Cisplatin

Chemistry and Biochemistry of a Leading Anticancer Drug

Cisplatin. Edited by Bernhard Lippert

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Bernhard Lippert (Ed.)

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Preface

The landmark discovery of the antitumor activity of *cis*-diammine-dichloroplatinum(II) (*cis*-$(NH_3)_2PtCl_2$, *cisplatin*, *cis*-DDP) by *Barnett Rosenberg*, first reported in 1969 in *Nature*, was an extremely fortunate one for a number of reasons.

First, and foremost, it was a discovery that gave and still gives patients suffering from various types of cancer hope for a prolongation and a better quality of life, and the chance of a cure. When clinical tests with *cisplatin* began in 1972, diagnosis of testicular cancer still meant a death sentence. That this is no longer the case is the undisputed achievement of *cisplatin*. Realization that this type of cancer can in fact be cured has led to high expectations about the possible effectiveness of *cisplatin* against other malignant diseases. It is ironic that at least three decades of research, aimed at a rational application of metal coordination complexes for the treatment of cancer, had proven largely unsuccessful. An experiment devised and carried out by a physicist, not at all aimed at finding a new antitumor agent, finally convinced coordination chemists and clinicians that it was indeed worthwhile pursuing investigations in this area. Today, a series of antitumor metal compounds are under investigation, and some look very promising.

Second, *Rosenberg'*s discovery that *cisplatin* was a powerful antitumor agent had an impact on inorganic and coordination chemistry that cannot be overestimated. The discovery occurred during a time that, in later years, would be called the 'renaissance of inorganic chemistry', and also during which, fully independently, the term 'biocoordination chemistry' was coined in Australia. Today, there is hardly a better example of the successful marriage of inorganic chemistry with other life science disciplines – medicine, pharmacology, biochemistry, molecular biology – than the success story of *cisplatin*, bioinorganic chemistry at its best! The awareness, established early on in *Rosenberg'*s laboratory, that *cisplatin* readily reacts with DNA, and that this reaction most likely is crucial to antitumor activity, focused a great deal of research activity on Pt-DNA studies. Much has been learned from these, from metal-binding patterns of nucleobases to the subtle effects of Pt coordination on base-pairing behavior. This knowledge is also useful today for other purposes, *e.g.*, a better understanding of heavy-metal toxicity and mutagenicity, the role of metal ions in ribozyme catalysis, and the understanding of chemical probes on metal basis for biomolecules, to give only a few examples. Today, 30 years after *Rosenberg'*s discovery, there is no other metal that is better understood in its reactivity toward DNA than platinum. And it was *cisplatin* that catalyzed many cutting-edge developments in metal-related molecular biology, *e.g.*, the role of metal ions in gene regulation.

Third, *Rosenberg's* discovery is a wonderful example of what basic research is capable of achieving and is a pledge for the support of basic science. It is important today to point out examples of this kind, now that it becomes increasingly difficult to obtain funding for scientific projects for which applications are not immediately foreseeable.

Cisplatin had its first appearance in chemistry in 1844, when synthesized by *Michel Peyrone*. It was not until 50 years later that *Alfred Werner*, in his theory of coordination chemistry, correctly assigned to this compound a *cis*-geometry, which was eventually unambiguously confirmed in an X-ray crystal-structure determination reported in 1966. It was around this time that *Rosenberg* had observed the curious effect of an electric field on the growth pattern of *E. coli* bacteria, which eventually led to the discovery of the antitumor activity of Pt coordination compounds. *Rosenberg* and co-workers published their results – 'Platinum Compounds: a New Class of Potent Antitumor Agents' – in *Nature* in 1969. Now, 30 years later, it is time to reminisce, to critically examine the usefulness of *cisplatin*, to value its impact on the development in the field of inorganic chemistry in general and to metal-DNA interactions in particular, and to look ahead for new directions and challenges. This is what this book is all about! It brings together various aspects of chemistry, biochemistry, biology, pharmacology, and medicine relevant to *cisplatin*, and also tells the story of how it all happened. The story of *cisplatin* is not finished yet. Too many questions remain unanswered, notably that of how *cisplatin* causes tumor cells to die, and why there is, after all, some selectivity between tumor and healthy cells.

The book contains 22 chapters and is divided into six *Parts*. The first chapter is by *Rosenberg* who, in a very personal manner, describes the time from the discovery of *cisplatin* to its acceptance as an established anticancer drug in the late seventies. The chapter in *Part 2*, written by *O'Dwyer* and colleagues, gives a topical account of the present clinical status of Pt antitumor agents.

Part 3 deals with the biochemistry of *cisplatin* as well as that of other Pt coordination compounds, and consists of four chapters. The first two chapters, by *Zamble* and *Lippard*, and by *Eastman*, provide detailed views of the picture we presently have of the mode of action of *cisplatin*. Both chapters make clear how the original perception of *cisplatin* causing a simple blockage of DNA functions has changed over the years to a picture of a complicated cascade of reactions triggered by a primary DNA adduct. The latter involves a number of key players in cell-cycle regulation, such as p53 and members of protein families controlled by p53, and can eventually lead to programmed cell death (apoptosis). *Villani*, *Tanguy Le Gac*, and *Hoffmann*, in their contribution, demonstrate that DNA replication is not automatically stopped by a *cisplatin* adduct. Rather, replication may proceed through the lesion ('DNA translesion synthesis') and lead to errors in the newly synthesized DNA, a mechanism causing mutations. Finally, *Malinge*

and *Leng* point out the potential significance of minor DNA adducts of *cisplatin* such as interstrand guanine-guanine cross-linking, and discuss the use of *trans*-diammineplatinum(II) entities in modifying oligonucleotides to give novel antisense and antigene agents.

In *Part 4*, chemistry relevant to interactions between Pt electrophiles and biomolecules (DNA, oligonucleotides, nucleotides and nucleosides, model nucleobases, amino acids, peptides) is described. The two introductory chapters by *Martin* and by *Arpalahti* address the hydrolytic activation of *cisplatin* and the reasons for the preference of Pt^{II} species for the N(7) sites of the purine bases, and they discuss several cases of unexpected migration processes of Pt^{II} at nucleobases. The nature of the actual *cisplatin* hydrolysis product that reacts with DNA is also the topic of the contribution by *Legendre* and *Chottard*, who, at the same time, provide detailed kinetic data on the individual binding steps of Pt^{II} to DNA. The power of NMR methods (^{1}H, ^{195}Pt, ^{15}N, ^{31}P), especially as applied in modern heteronuclear detection modes in Pt-DNA as well as Pt-protein binding studies, is described in two chapters by *Chen*, *Guo*, and *Sadler*, and by *Ano*, *Kuklenyik* and *Marzilli*. The importance of X-ray crystallography in understanding the base binding properties of *cisplatin* and the resulting DNA distortion, as deduced from simple model compounds up to the DNA-dodecamer level, is highlighted in the chapter by *Bau* and *Sabat*. *Part 4* is concluded by two chapters by *Reedijk* and *Teuben*, and by *Appleton* dealing with Pt-S as well as Pt-peptide interactions. Although, in the past, research has focused primarily on Pt-nucleic acid interactions, there is good reason to believe that reactions with other biomolecules (*e.g.*, peptides) as well as S-containing species may be important, *e.g.*, in the context of cytotoxicity of Pt drugs.

In examples compiled in *Part 5*, the impact of *cisplatin* on a specific field within inorganic chemistry – that of Pt compounds in unusual oxidation states – is described. A class of intensely colored Pt complexes derived from the hydrolysis products of *cisplatin* and the pyrimidine nucleobases uracil, thymine, cytosine, and related ligands ('platinum pyrimidine blues'), which was described by *Rosenberg* in 1973, excited interest in the re-investigation of mixed-valence Pt species such as 'Platinblau', a compound described at the beginning of the century. The startling antitumor activity of these 'blues' presented an additional challenge to unravel their nature and structure. The introductory chapter by *Lippert* surveys our present understanding of the 'blues'. Subsequently, *Randaccio* and *Zangrando* describe X-ray crystallographic work on pyrimidine-nucleobase complexes that model postulated structures of the 'blues'. Diplatinum(III) species belong to the class of compounds with unusual Pt-oxidation states as well, and have been studied in particular as oxidation products of the 'blues'. As pointed out in the chapter by *Natile*, *Intini*, and *Pacifico,* these compounds appear to have been overlooked for a long time, and may indeed prove to be

much more common than generally thought. *Matsumoto*, in the last chapter of this *Part*, describes the beginning of a fascinating organometallic chemistry with diplatinum(III), as well as related mixed-valence-state compounds.

The book concludes with *Part 6* dealing with several new developments in the field of antitumor Pt compounds. *Farrell et al.* present novel di- and trinuclear Pt^{II} compounds which display marked antitumor activity and, at the same time, have DNA-binding properties different from those of *cisplatin*. *Kelland* describes orally active Pt^{IV} drugs presently in Phase-I and Phase-II clinical trials. New and fast mechanism-based methods for screening Pt compounds for potential antitumor activity are the topic of the chapter by *Sandman* and *Lippard*. Finally, *Kozelka* critically examines the contribution that computational studies can make to the field of Pt-nucleic acid interactions. He ends with an optimistic outlook for using *ab initio* molecular-dynamics calculations in the near future.

It is the hope of the editor that this book reflects the tremendous progress that has been achieved over a period of 30 years in the understanding of the role of *cisplatin* as an antitumor agent. At the same time, it should be recognized that there is still much that we do not understand, and that, therefore, it makes sense to continue research in this field. The idea of producing new Pt or other metal-centered drugs with a spectrum of activity different from that of *cisplatin* and fewer adverse side effects, or finding new ways of administering these drugs, is an important goal that should continue to be the subject of intense investigation.

I whish to express my sincere appreciation for the contribution and help of many individuals, who eventually made this book possible; the authors, without whose willingness to write accounts of their work this endeavour would not have been successful; members of my group, especially *Jens Müller*, *Frank Glahé*, Dr. *Gabi Trötscher-Kaus*, *Markus Drumm*, and *Klaudia Passon*, for their help in putting the *Parts* together, redrawing figures, retyping, proof-reading *etc*.; and finally the production team. I owe special thanks to Dr. *Oliver Renn*, who, from the original idea to the final realization, has provided indispensable support and competence. I am also indebted to Dr. *M. Volkan Kisakürek*, *Verlag Helvetica Chimica Acta*, for accepting to publish this book. Special thanks to him and to *Pekka Jäckli* for his care with the preparation of the final version. Thanks also to Prof. *S. J. Lippard* (MIT) for providing the coordinates of the cover picture, and to *Fabian Lippert* for designing possible cover illustrations.

I dedicate this book to my dear friend *James H. Burness* and his family. *Jim* had been involved in the development of cisplatin at an early stage and is now fighting cancer. How I wish that all the knowledge and encouragement complied in this book can provide him with the strength to go on!

Dortmund, January 1999 Bernhard Lippert

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Part 1. The Start

Platinum Complexes for the Treatment of Cancer: Why the Search Goes On

Barnett Rosenberg

Cisplatin. Edited by Bernhard Lippert

Platinum Complexes for the Treatment of Cancer: Why the Search Goes On*

Barnett Rosenberg

Emeritus Professor of Chemistry, Michigan State University, President/Director of Research, Barros Research Institute, 2430 College Road, Holt, Michigan 48842, USA, Phone/Fax: +1 517 694 4788

Some platinum coordination complexes are active anticancer drugs in animals and man. This new class of chemotherapeutics was discovered during the course of investigation of the electric field effects on bacterial growth. The platinum electrodes electrolyzed during the experiment, releasing a platinum complex which caused complete cessation of cell division in the bacterial rods. With this filamentation assay system, we were able to identify the specific chemical as *cis*-dichlorodiammineplatinum(II), a complex known since 1845. The bacterial studies with many such complexes suggested to us the generalizations that charged platinum complexes were bacteriocidal, while the neutral platinum complexes induced filamentation and, in lysogenic bacteria, lysis. The neutral complexes have significant activity against transplantable, virally induced, and chemically induced cancers in animals. They are synergistic with almost every other anticancer drug in current use. In man, kidney toxicity is the dose-limiting side effect, but this is now completely ameliorated by simply hydrating the patient. The drug, in combination therapy, has proved to be curable for all forms of testicular cancer. Other cancers where the drug activity has begun to approach this are head and neck cancer, and ovarian cancer. Activity against the other major cancers is now being studied. The mechanism of action at a molecular level appears to depend upon a primary lesion formed on the cellular DNA by the platinum complex. This serendipitous discovery has led to a new class of anticancer agents, metal coordination complexes, which has now proved to be of significant value.

* *Editor's comment:* This article is taken in large part from a review article published more than twenty years ago in *Interdisciplinary Science Reviews*, Vol. 3, No. 2, pp 134–147 (1978). It tells the story of the discovery of cisplatin and reflects on its possible mode of action as an antitumor agent. While some of the ideas may have been revised or discarded today, the article represents a unique personal account of the discovery and at the same time is a beautiful example of science history. The chapter on the clinical results has been deleted, since the present status is covered in an up-to-date manner in this book's contribution by *O'Dwyer* and co-workers. The editor wishes to thank *John Wiley & Sons Limited* for permission to reproduce this work.

Cisplatin. Edited by Bernhard Lippert

Professor *Barnett Rosenberg*, Emeritus Professor of Chemistry (since January 1, 1997), Michigan State University; currently President and Director of Research, Barros Research Institute.

For reasons I cannot fathom, there is a new spirit of optimism in the field of cancer treatment. In the years since 1971, when the U. S. government instituted the 'War on Cancer', and the accumulated monies for research began to move into the tens of billions of dollars, the death rates from cancers have barely budged, despite a massive spate of clever new ideas and deeper understandings of the molecular biology, genetics and other basic sciences relevant to cancer. It should be, but is not, obvious to us by now, that cancer cures are difficult to find.

Researchers in the period of the 60's and 70's did produce a series of 'cytotoxic' agents with interesting antitumor activity. These included adriamycin (1970); cytoxan (1958); 5FU (1957); cisplatin (1971), and vinblastine (1960) among others. This period may, naively, be called the 'golden age' of cancer chemotherapy. It was the result of the beginning, in 1955, of the clinical trials program of the National Cancer Institute. As I recall, (and much of what I write here is from recollections and not documented) the program required testing, by the NCI, in mice, of all chemicals submitted by 'serious scientists' – no justification for testing was required. Eventually, about 50,000 compounds per year were tested, but only 5–10 compounds were able to pass on to clinical trials. So, useful anti-tumor agents were rare. But, in view of the large number tested, it was a good program for producing useful drugs. Using these compounds, usually in combination therapies, we now can achieve a high rate of cures for about 5 classes of cancers – particularly testicular cancers, *Hodgkin's* Disease and childhood leukemias.

By the mid 70's, the National Cancer Advisory Board, which was established to advise the U. S. President on these matters, concluded that we had a sufficiency of agents, but we did not know how to use them most effectively. Therefore, future research would be changed to emphasize improving the results obtained with the current agents, rather than seeking new ones. [This was told to me by *Charlie Heidelberger*, who was, at that time, a member of the Board.] The 'old rules', which did have some successes, 'went by the board'. Now, acceptance for testing by the NCI required a 'rational' reason for being tested; (the expected result of this was to skew the compound selection into the 'me too' category); the number of new compounds tested was reduced to about 15,000 per year; and the major emphasis was placed on finding *in vitro* tests to replace the 'mouse tumor tests' to select the actives.

The results of these changes were immediately obvious. The number of new drugs in the pipeline decreased precipitously. Papers appeared with titles like, 'why has the well run dry?' or 'who turned off the tap?' (well, hell, we did!). Then, the powers that be wrongly concluded that random screening was not the best way to identify new drugs. The research emphasis shifted dramatically to find the causes of cancers. Epidemiology, naturally, became the dominant approach. We then entered the period of the 'carcinogen of the week', and only succeeded in accusing many 'innocent' chemicals of being the major causes of cancers. However, when so many common chemicals tested positive in the well accepted *Ames* tests for mutagenicity (and presumably, therefore, carcinogenicity) even *Ames* balked at a too-easy acceptance of the relation. Many researchers, in recent years, have turned away from environmental carcinogens as the major causes of human cancers, and toward genetic faults, either inherited or induced by molecular changes of uncertain origin. Now the attack was at the most fundamental level. Many fresh new ideas emerged from this approach (and yet another new group of scientists entered the battle). Unhappily, again, after many years of effort, failure after failure plagued the field. At the present time, no significant new therapies have emerged, although there are still some that are, as yet, untested.

Oh yes – somewhere in this time period, viruses were suggested as causative agents of cancers (some few are), but we have neither been able to develop convincing evidence for a causal relation, nor have we developed successful agents for the killing of viruses in order to treat cancers (with the possible exception of *Kaposi's* Sarcoma).

In this dismal history, can we find some clue as to a possible cause and treatment? I believe so, since, as we have seen, we can already, in a small number of cancer types, achieve substantial cure rates. Testicular cancer, with a cure rate approaching 100%, is the prime example of this, and this is

mainly due to combination chemotherapy with a platinum drug, cisplatin or carboplatin. Newer evidence is now available that a number of other cancers respond well to such therapies, including ovarian, bone, and lung cancers. Since time of remission is the main criterion of cures, we must, therefore, wait yet a while.

Meanwhile, there is no reason to believe that we must restrict our research to platinum compounds – these were merely the first in the class of anti-cancer metal complexes to be discovered, and no one has come up with a good argument as to why they should be the best. They are likely not! Thus, the justification for this book.

It may be of some value to describe, briefly, the early history of the discovery of this class of anticancer drugs and the subsequent tortuous developments leading to its clinical use.

Early History

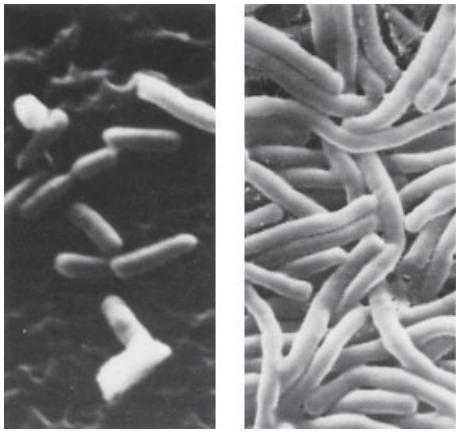
The story begins in 1961, when I left the Physics Department of New York University to help found the Biophysics Department at Michigan State University. With this change of departments there came an obligation to orient my research more toward biology. In my earlier reading I had been fascinated by the microphotographs of the mitotic figures in cells in process of division. They called to a physicist's mind nothing so much as the shape of an electric or magnetic dipole field, the kind one sees with iron filings over a bar magnet.

If such a dipole may be involved in cell division – as some had earlier speculated – then by tickling the dipole with electromagnetic radiation of a resonant frequency, or a subharmonic, to avoid the radiofrequency heating of cells, it may absorb some energy which may or may not be detrimental to the cell. Admittedly this is an overburden of 'mays', but I was intrigued by the idea of an experimental test. Having no competence in biology – few physicists have – *L. VanCamp* joined the laboratory to do the test. We set up a continuous-culture apparatus for the cells, but included in the growth chamber a set of platinum electrodes. Platinum of course, is known to be quite inert in a biologic environment.

The electrodes were powered by an audio amplifier whose input frequency was set by an audio oscillator. The impedance of the chamber, 6 Ω, was perfectly matched to the output impedance of the amplifier. To test the proper functioning of the apparatus before putting in mammalian cells, we used the common bacterium *Escherichia coli*. These, and prokaryotic cells generally, do not show mitotic figures in division. After the bacterial population reached a steady state, the electric field was turned on. The density

of bacteria started to decline, and we were in danger of having an aseptic chamber. When the field was turned off, the density returned to normal after a few hours.

A rather striking effect, but how striking we did not realize until we examined the bacterial cells in the effluent of the chamber. The bacterial rods normally look like the picture in *Fig. 1, a,* rods about 2–5 μm long, with a 1 μm diameter. After an exposure to the electric field they appeared as in *Fig. 1, b*; long filaments, up to 300 times the usual length. Now this required an explanation. The effect was not due to a direct action of the electric field on the bacterial cell but rather to electrolysis products from the platinum electrodes.



(a) (b)

Fig. 1. a) *Scanning electron microphotograph of normal* E. coli *(gram-negative rods).* b) *Scanning electron microphotograph of* E. coli *grown in medium containing a few parts per million of* cis*-dichlorodiammineplatinum(II).* Same magnification in both pictures. The platinum drug has inhibited cell division, but not growth, leading to long filaments. These pictures were taken by *D. Beck* of Bowling Green University.

We now brought our chemist, *T. Krigas*, in to isolate and identify these products. He clearly identified it as a platinum-containing compound, probably ammonium chloroplatinate $[NH_4]_2[PtCl_6]$. We were somewhat nonplussed, however, when addition of this compound at the detected concentration to bacterial cells in test-tube cultures led, not to filamentation, but to bacteriocidal activity. Many experiments later we found that a solution of this compound, after standing on our laboratory shelf for a few weeks, was able to produce a small amount of short filaments.

Some quick studies showed that light was the necessary agent for the change, and we were now deep into the photochemistry of platinum. In retrospect, this was not surprising. Platinum compounds antedate silver in pho-

tography. Ultraviolet light caused a series of chemical reactions in the solution, leading from the charged ions to a final neutral species $[Pt^{IV}(NH_3)_2Cl_4]$. Bacterial tests of the separated intermediates and final neutral product showed that the latter was the chemical causing filamentation, and was chemically identical to the electrolytically formed agent. *A. Thomson*, in our laboratory, synthesized the neutral species by known chemical techniques and tested it. It had no activity!

We had only one remaining possibility. The neutral compound exists in two isomeric modifications; the *trans*-$[Pt^{IV}(NH_3)_2Cl_4]$ and the *cis*-$[Pt^{IV}(NH_3)_2Cl_4]$. The former was the more thermodynamically stable and was the one we first prepared. We now synthesized the *cis*-configuration, and finally, achieved complete success.

Platinum has two dominant valence states, +2 and +4. The lower state forms square planar complexes, and the latter forms octahedral complexes. We now synthesized the 2+ complex, and it also was active in forming filaments. Thus the two active chemicals are *cis*-$[Pt^{II}(NH_3)_2Cl_2]$ and *cis*-$[Pt^{IV}(NH_3)_2Cl_4]$. These structures are shown in *Fig. 2*. The *trans*-structures have the two similar chemical groups (ligands) on opposite sides of the molecule, and both *trans*-species are inactive at low concentrations (parts per million in solution), but begin to suppress growth at higher concentrations.

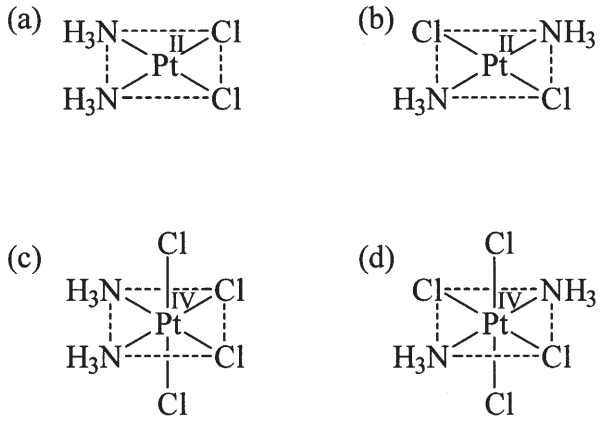


Fig. 2. *Molecular structures of anticancer active (*cis*-configurations) and nonactive (trans-configurations) platinum complexes*. a) *cis*-Dichlorodiammineplatinum(II); b) *trans*-dichlorodiammineplatinum(II); c) *cis*-tetrachlorodiammineplatinum(IV); d) *trans*-tetrachlorodiammineplatinum(IV).

Now we had done a strange thing, for by the circuitous route described, we had discovered a compound first synthesized in 1845 and known as *Peyrone's Chloride*. The molecular structural differences between the *cis*-

and *trans*-complexes had been solved by *Werner* in 1890, who, in so doing, established the basis of modern coordination chemistry. What little value we added by this whole exercise was the use of a biologic test for identification of the complex, thus establishing a clear and interesting biologic activity of some coordination complexes of platinum.

The Effects of Platinum Complexes on Bacterial Cells

Clinical use of metal complexes, particularly of arsenic, antimony and mercury, in the treatment of bacterial infections has a long history. The noblest scion was probably Salvarsan, developed by *P. Ehrlich* about the turn of the century as a specific for syphilis. It was also almost the last of the line. For, in the first half of this century, rapid progress in organic chemistry and biochemistry produced a proliferation of antibacterial drugs, culminating in the enormously successful sulphonamides and finally, the antibiotics. This success fixed the attitude of the next generations of scientists, and metal complexes were largely ignored thereafter.

The antibacterial activity of some platinum group metal complexes was first studied by *F. P. Dwyer* and his co-workers in 1953. They found the relatively inert chelated complexes of ruthenium with phenanthroline to be quite good bacteriostatic and bacteriocidal agents against Gram-positive microorganisms. Unfortunately, these charged complexes also produced a severe neuromuscular toxicity, 'curare-like' behavior, which limited their use to topical (skin) administration. Limited clinical trials did establish a usefulness for these complexes in the treatment of some skin infections such as dermatosis, dermatomycosis and others, but little further work was done to bring these complexes into general use.

Our laboratory first called attention to the bacterial effects of the simpler complexes in 1965. Over the next few years, in cooperative studies with microbiologists, a number of papers were published describing a multiplicity of effects on microorganisms caused by various complexes of platinum group metals; platinum, palladium, ruthenium, rhodium, osmium, and iridium.

Consider first the filamentation effect. Trials of many complexes established that mainly those complexes which were neutral and had no electrically charged ions in solution, markedly inhibited cell division in bacteria. The *cis*-configuration was active, the *trans* was not. They did not inhibit growth unless the concentrations were greatly increased. They were associated in the cell primarily with nucleic acids (RNA and DNA) and with some soluble proteins.

Gram-negative rods were the most sensitive to this effect, Gram-positive rods much less so, and spherical bacilli (cocci) not at all. Forming a fil-

ament was not a terminal event for the bacterium. If the platinum complex was removed from the solution, or the filaments transferred to a normal medium suitable for growth, the filaments begin to divide into normal bacteria, looking much like a string of sausages in the process, growing into quite normal colonies. The division occurs all along the length of the filament and not just at the ends. This was quite a different pattern from the filamentation caused by chemicals such as the nitrogen mustards, where filamentation is a terminal event.

The difference may reside in the fact that the nitrogen mustards block DNA synthesis and each such filament contains only a small number of copies of the genetic information (genome) whereas the platinum complex does not stop new DNA synthesis in bacteria at the concentration causing filaments to appear, and the DNA exists in multiple genome copies as continuous strands or large clumps throughout the filament. This, by the way, is quite different from the effects of these complexes on mammalian cells as discussed below. It is also one of the major differences in the biological effects of the nitrogen mustards, bifunctional alkylating agents and potent anticancer agents, and the platinum complexes.

The complexes which form ions in solution such as $[NH_4]_2[PtCl_4]$, which ionizes to $PtCl_4^{2-}$ and $2NH_4^+$ are quite poisonous to the bacteria, causing a large cell kill at low concentrations, and few or no filaments. These ions react with proteins in the cytoplasm of the cell almost exclusively, compared to the strong nucleic-acid association of the neutral complexes.

Measurements of these various reactions required a sensitive technique for detecting the minute amounts of platinum incorporated by the cells. This necessitated the use of a radioactive isotope of platinum as a tracer. *E. Renshaw* and *A. Thomson* produced the isotope ^{191}Pt by irradiating an iridium foil in the proton beam of the Michigan State University cyclotron, chemically separating the platinum isotope from the other metals present and synthesizing the charged and the neutral complexes for the bacterial tests. In recent years, the radioactive isotope ^{195m}Pt has been generously prepared for us at Oak Ridge Laboratories by *K. Poggenberg*. This also is a γ-ray emitting isotope with a three day half-life. This means a bout of hectic activity in our laboratory with each delivery to accomplish all our experiments before the level of radioactivity diminishes below our detection threshold.

R. Gillard and his co-workers at Kent University have extended these studies, and shown that organic complexes of rhodium produce similar effects. *G. Gale* and his associates at the Medical University of South Carolina developed a parallel story of the photochemical transformations and filamentation of bacteria by the *cis*-isomer of the neutral complex with iridium instead of platinum. Thus, the experience now accumulated suggests a

generality of the bacterial phenomena with the complexes of the other platinum group metals.

Certainly the development of new bacteriocidal agents, particularly those which are active against Gram-negative bacteria, is a very desirable goal. However, the report of anticancer activity of these complexes shifted the weight of research to this more urgent problem. And, just as the electric-field experiment was bypassed – temporarily I hope – so too the bacteriocidal utility was put in limbo by more exciting developments. Before moving on to these developments a third bacterial effect needs discussion since it provides some insight into the possible mechanism of how cancers are affected by these complexes.

S. Vasilukova, née *Reslova*, a young Czechoslovakian microbiologist, and an ex-student of *J. Drobnik* who contributed much to our microbial experiments, worked with strains of *E. coli* bacteria that had been previously infected with a bacterial virus (λ-bacteriophage). In these lysogenic bacteria, the genetic information of the virus has been incorporated into the cell, but it is repressed so that it is not normally detectable. It replicates during cell division along with the bacterial DNA and so is not lost or diluted out after many divisions.

This is the bacterial equivalent to slow, or latent virus infections in mammals and man. These bacterial strains are called lysogenic, since a number of physical agents such as X-rays or UV light, and some chemicals, such as the nitrogen mustards and carcinogens, can derepress the viral genome causing an active viral infection leading to the dissolution – the lysis – of the cell. These effects are easily measured when the bacteria are grown in test tube cultures. The platinum complexes, for example, are added as a few parts per million concentration in the growth medium. The bacteria grow, forming filaments for about three hours, then rather quickly, the milky opacity of the culture diminishes, and in a few hours the culture is water clear; the cells have all lysed. The *cis*-$[Pt^{II}(NH_3)_2Cl_2]$ complex is extremely efficient in inducing such lysis – less than 0.1 ppm in the culture produces a detectable effect.

It should be pointed out here that all the platinum complexes which are active anticancer agents are also efficient inducers. Those complexes which are not active, do not cause lysis. So far, there is a complete isomorphism between the set of active anticancer complexes and the set of efficient inducers. Earlier, we had believed that a good correlation existed between anticancer active complexes and the filament-forming complexes. After a while, however, exceptions in both classes occurred which decreased our faith in this correlation. The correlation of lytic induction and anticancer activity has held up well. In fact, when *R. Adamson* at the National Cancer Institute reported the anticancer activity of gallium salts, we tested these and, indeed, they did induce lysis in lysogenic bacteria.

We then tested salts of other group IIIa elements, aluminium and indium, and these too proved to be inducers. It was only after we had predicted, but not published, the activity of aluminium and indium in these tests that *Adamson* reported them active as anticancer agents. The verification of the prediction tended to reinforce our belief in the correlation and, more importantly, in the possibility of a similar mechanism of action in the two apparently dissimilar effects.

In 1953, *A. Lwoff*, had reviewed evidence showing that water soluble mutagens, carcinogens and anticancer drugs were potent inducers of lysogenic bacteria, a strong hint that underlying these four different effects there was a common mechanism, and that it involved an interaction of the causative agent with cellular DNA. The importance of the agent-caused lesion in the DNA in these processes was further enhanced when *Vasilukova* returned to her native land and performed the experiment called 'indirect induction'. In this case a strain of nonlysogenic bacteria with the sexual transduction factor, F^+, was treated with the platinum complex. These cells were allowed sexually to conjugate with a lysogenic strain, F^-, which had not been treated with the platinum complex. In this process, only a portion of the DNA of the cell is transferred. Yet the recipient cells were induced to lyse. Later on I will speculate on the sequence of events arising from this correlation in order to account for the anticancer action of the platinum drugs. But first I must carry the story forward to the discovery of their utility as cancer drugs.

The Anticancer Activity of Platinum Complexes

By 1968 we had achieved a certain degree of understanding of the bacteriologic effects of the platinum complexes, and we had synthesized and repeatedly tested the *cis*-dichlorodiammineplatinum(II) which we now took as a model for the active neutral complexes. We were primed to try the chemical against a cancer. The logic was somewhat naïve: the complex stopped cell division in bacteria at concentrations without marked toxicity, perhaps then it would stop cell division in tumors which grow rapidly, without unacceptable toxicity to the host animal. *J. Toth-Allen* first determined the safe dose levels which could be injected into the peritoneal cavity of mice. The dose which killed 50% of the animals (LD_{50}) was about 13 mg of the drug per kilogram of animal body weight. A dose of 8 mg kg^{-1} was nonlethal.

VanCamp then implanted in these mice a standard transplantable animal tumor, the solid Sarcoma-180. This was administered as a 10 mg piece of tumor tissue inoculated beneath the skin under one armpit. The tumor fragment increased its mass about 100 times over the next ten days. The tu-

mor could be cut out, since it remained localized, – non metastatic – and weighed. The standard protocols of the National Cancer Institute called for implantation of the tumor on day 0; injection of the drug on day 1; and sacrifice of the animals on day 8. The average tumor size of the treated group is divided by the average tumor size of the untreated group, the negative controls. For a drug to be considered effective against the tumor, the treated-to-control (T/C) tumor-size ratio should be less than 0.5. Our first test values were well below this. We repeated this test more than half a dozen times to be sure it was not a peculiarity due to our inexperience. It was not; each new test reconfirmed the activity.

We also tested a number of other neutral platinum complexes and in this new biological effect, we again saw the stereospecificity that had occurred in the bacterial tests; the *cis*-configurations were active, the corresponding *trans*-configurations were not. The implications of this are significant: it shows that the platinum complexes retain their geometry in the biologic environment, they were not degraded to heavy metal ions which could be nonspecific poisons. The specific chemical reaction leading to the biological effect was sensitive to molecular geometry, and was most likely to involve a macromolecule such as a protein or a nucleic acid. We also were presented with a simple test to determine the significant chemical reaction. Both *cis*- and *trans*- complexes undergo roughly similar, multiple reactions in the cell, but obviously only those reactions which the *cis*-configuration can undergo, but the *trans*-configuration cannot, are likely to be significant.

After confirming these results I contacted *G. Zubrod*, head of the chemotherapy branch of the National Cancer Institute and apprized him of the results. I was invited to discuss this with his associates at Bethesda, Maryland. After my short lecture, which was received with perceptible, but understandable coolness, I left samples of the four complexes to be tested in their tumor screen, the L1210 leukemia in mice. A few months later I was informed that the complexes were also active in their system, and it was suggested to me that a grant proposal to pursue this research would not be unfavorably received. A proposal was duly submitted, and approved, and NCI support has continued ever since.

But meanwhile, we had tried a variant of the protocols and this gave us the first hint of the true potency of these complexes. Instead of injecting the complexes on day 1, we waited until the tumor was about a gram in weight (in a 20 g mouse!) and then injected the drugs (on day 8). All the tumors regressed and all the animals were cured. A time sequence photograph of two mice is shown in *Fig. 3*. This was an unusual result since we were not aware of any other anticancer drug capable of regressing large Sarcoma-180 tumors. As would be expected, the surviving animals showed strong immunologic rejections of reimplants of the same tumor up to the longest time

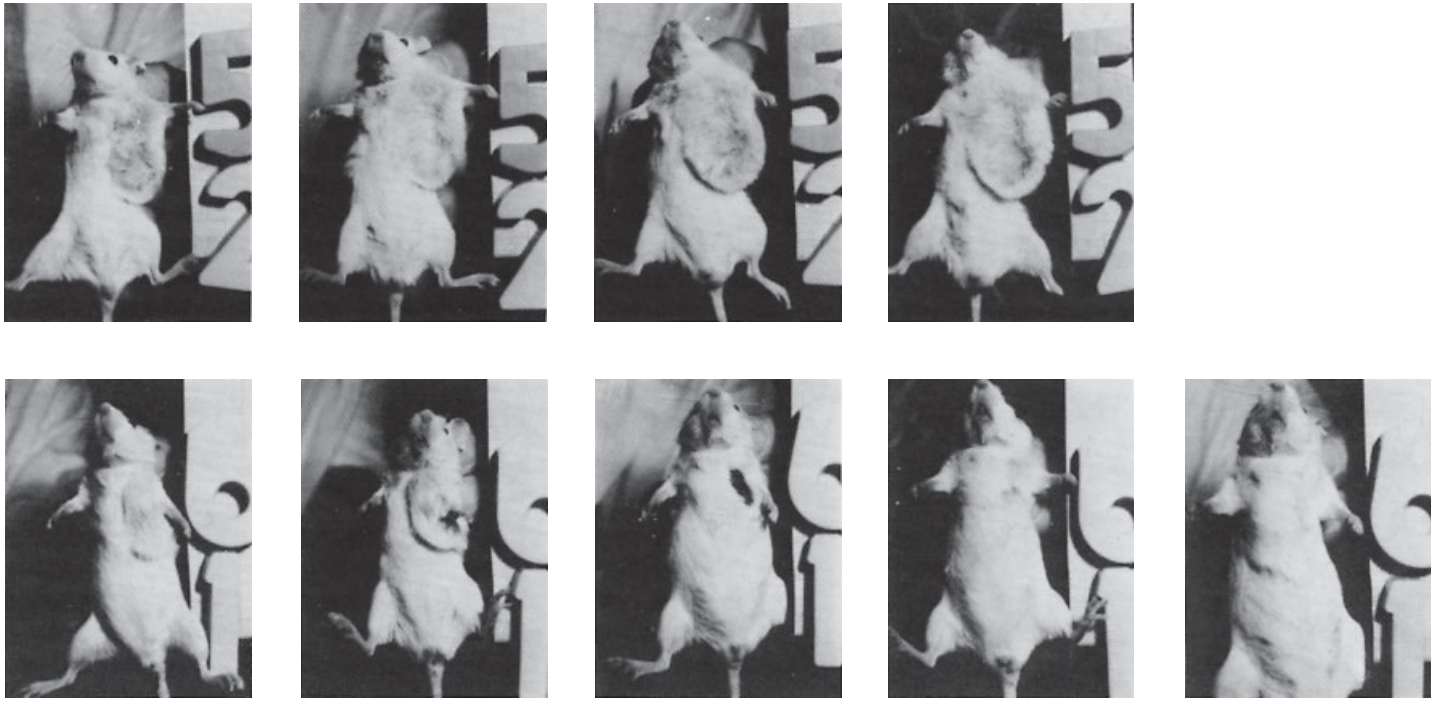


Fig. 3. *Time sequence photographs of two mice with solid Sarcoma-180 tumors.* The mouse at the top was an untreated negative control. She died on day 21 when the tumor weighed about 3 g. The bottom mouse was in the group treated on day 8 with an intraperitoneal injection of *cis*-dichlorodiammineplatinum(II). Her tumor was completely regressed six days after treatment, and she died of age-related causes almost 3 years later.

tested, 11 months. The animals lived out their full life expectancy, about 30 months, and died of normal, age-related, causes.

With confirmed activity against two different animal tumors we were ready to publish the preliminary results, which we did in a short paper in *Nature* in 1969 [1]. An American journal of almost equal distinction had turned the manuscript down because a referee had commented that it was not noteworthy since so many new drugs with activity were being found. Indeed, there was a strong possibility that the *Nature* paper would be lost in a crowd of similar reports of new anticancer chemicals that were flooding the literature at the time. It was rescued from potential oblivion by the interest and good graces of Professor *Sir Alexander Haddow*, then head of the Chester Beatty Institute in London.

Curiously, he had an intuitive feeling that platinum complexes might be effective anticancer agents, and had already tested some earlier, without success. On hearing of our results he had these new complexes synthesized and tested against a different tumor system, a myeloma tumor (ADJ/PC6) in mice, and again, confirmed the activity. He wrote to me of the results obtained at the Chester Beatty Institute and extended an invitation to visit with him and some of his colleagues, which I accepted with alacrity. This began a strong cooperative group in Britain which included *T. A. Connors* and *J. J. Roberts* of the Chester Beatty Institute, *R. J. P. Williams* of Oxford University, and *M. Tobe* at University College, London, all of whom have contributed much to the advance of this new research field.

[*Personal comment*: We later learned that *R. Mason*, who had helped in our earlier bacterial studies, had sent some *cis*-dichlorodiammineplatinum(II) to a friend to test for anticancer activity in 1966. His friend overdosed the animals, they all died, and he reported back that the drug was too toxic! There must surely be a lesson somewhere in this story.]

In recent years, *cis*-dichlorodiammineplatinum(II) has been tested against a wide variety of animal tumors [2]. While this drug is by no means the most active of the platinum complexes, it was the first chosen by the National Cancer Institute to be slated for clinical trials. This fact made studies with this drug more imperative, and most further research making up the bulk of the, by now, over 600 papers in the field, are concerned with it. A tabulation of the best animal test results is shown in the *Table*.

The best results are indicated here solely to convey a qualitative impression of the activity. Most tests were performed with small numbers of animals, making statistical analyses meaningless. Besides, the perversity of animal responses, which indicates a lack of appreciation or knowledge of all the important variables necessary to control, makes either the average or best results of dubious numerical value. Nevertheless, we have compiled a list of 16 tumor types, including transplantable tumors, chemically derived

Table. *Best Results of the Antitumor Activity of* cis-*Dichlorodiammineplatinum(II) in Animal Systems*

Tumor	Host	Best Results
Sarcoma-180 solid	Swiss white mice	T/C = 2–10 %[a])
Sarcoma-180 solid (advanced)	Swiss white mice	100 % cures
Sarcoma-180 ascites	Swiss white mice	100 % cures
Leukemia L1210	BDF_1 mice	% ILS = 379 %; 4/10 cures[b])
Primary *Lewis* lung carcinoma	BDF_1 mice	100 % inhibition
Ehrlich ascites	BALB/c mice	% ILS = 300 %
Walker 256 carcino-sarcoma (advanced)	*Fisher* 344 rats	100 % cures; T. I. > 50 [c])
Dunning leukemia (advanced)	*Fisher* 344 rats	100 % cures
P388 lymphocytic leukemia	BDF_1 mice	% ILS = 533 % [b]); 6/10 cures
Reticulum cell sarcoma	C+ mice	% ILS = 141 % [b])
B-16 melano-carcinoma	BDF_1 mice	% ILS = 279 % [b]); 8/10 cures
ADJ/PC6	BALB/c mice	100 % cures; T. I. = 8 [c])
AK leukemia (lymphoma)	AKR/LW mice	% ILS = 225 % [b]); 3/10 cures
Ependymoblastoma	C57BL/6 mice	% ILS = 141 % [b]); 1/6 cures
Rous sarcoma (advanced)	15-l chickens	65 % cures
DMBA-induced mammary carcinoma	*Sprague Dawley* rats	77 % total regressions 3/9 free of all tumors
ICI 42, 464-induced myeloid and lymphatic leukemias	*Alderly Park* rats	% ILS = 400 % [b])

[a]) $\text{T/C} = \frac{\text{Tumor mass in treated animals}}{\text{Tumor mass in control animals}} \times 100.$

[b]) % ILS = % increase in lifespan of treated over control animals.

[c]) TI = Therapeutic index (LD_{50}/ED_{90}), ED_{90} = effective dose to inhibit tumors by 90 %.

tumors (from carcinogens) and virally derived tumors (from oncogenic viruses). The drug is active against all types. The conclusions that may be drawn from these tests are that the drug:

1. exhibits marked, rather than marginal antitumor activity;
2. has a broad spectrum of activity against drug-resistant as well as drug-sensitive tumors;
3. is active against slowly growing as well as rapidly growing tumors;
4. is active against tumors normally insensitive to 'S' phase (DNA replicative stage) inhibitors;
5. regresses transplantable as well as chemically and virally induced tumors;
6. has shown no animal specificity since it works in mice and rats, either inbred or random bred, and in chickens;
7. is useful for disseminated (*e.g.*, leukemias) as well as solid (*e.g.*, sarcoma) tumors;
8. is potent, in that it can rescue animals when injected a few days before death from certain types of tumors.

Thus the credentials of the drug, and by implication, others in the class of platinum group complexes, as an active anticancer agent in animals is well established.

We were then faced with a series of questions, the answers to which were urgently needed, and which required for these answers expert competence in coordination chemistry, biochemistry, biophysics, molecular biology, physiology, pathology, pharmacology, electron microscopy, immunology, and finally, clinical medicine. In short, the entire panoply of disciplines in chemistry and biology was needed. We alone could not do it, nor could any small group of laboratories. A worldwide network of cooperating laboratories was called for, and established. They were supported by public funds, cancer societies, and to a generous degree, the platinum industry.

[*Personal comment:* I recall two strict admonitions from my major professor when I informed him of my growing interest in biophysics. These were, not to work with medical doctors untrained in research and to avoid cancer research, since many had tarnished their reputations from a malignant neglect of scientific objectivity in their desire to do something useful. I have broken both injunctions, but I cannot say that I am sorry. With a very few exceptions, all connected with the network impressed me as dedicated, selfless, humane scientists. The expected ego clashes and political infighting that characterize so much of science seems to have been muted by the urgency of the problem at hand.]

Molecular Structure Determines the Anticancer Activity

Of the myriad questions arising from this discovery of the anticancer activity in mice of some platinum complexes, one which we did feel competent to attack, particularly since we had the advice of some very able inorganic chemists, *R. Mason* of Sussex University, *R. J. P. Williams* of Oxford University and *M. Tobe* of University College, London, was the so-called structure-activity relation. Without requiring detailed knowledge of the molecular interactions of the chemicals in the biologic system, we simply induced structural changes in the molecules by known synthetic techniques and tested them against a standard mouse-tumor. If many variations are tried, then a catalogue of these chemicals, with a simple numerical measure of their activity, should exhibit some regularities. This allows them to be grouped in subclasses, and each subclass can be analyzed for common chemical properties. The more chemicals that are tested, the sharper will be the subclassification. However, a reasonable limit had to be set for these syntheses.

As an example, one starts with one metal of the platinum groups, platinum itself. It has the two major valence states +2 and +4. Take the latter.

It can associate in an octahedral complex with six ligands, atomic groups bound to the metal. The individual ligands may be chosen from a large group, but let us restrict it to just ten. Therefore, for this one metal valence state we have about one million potential variations. Obviously the required manpower to synthesize, purify and characterize such numbers of chemicals is beyond the world's capacity even if all laboratories were recruited for this sole purpose; to say nothing of the 30 million mice required.

A drastic compromise was called for, and here the intuition of the experts in coordination chemistry was essential. Actually, only about 1000

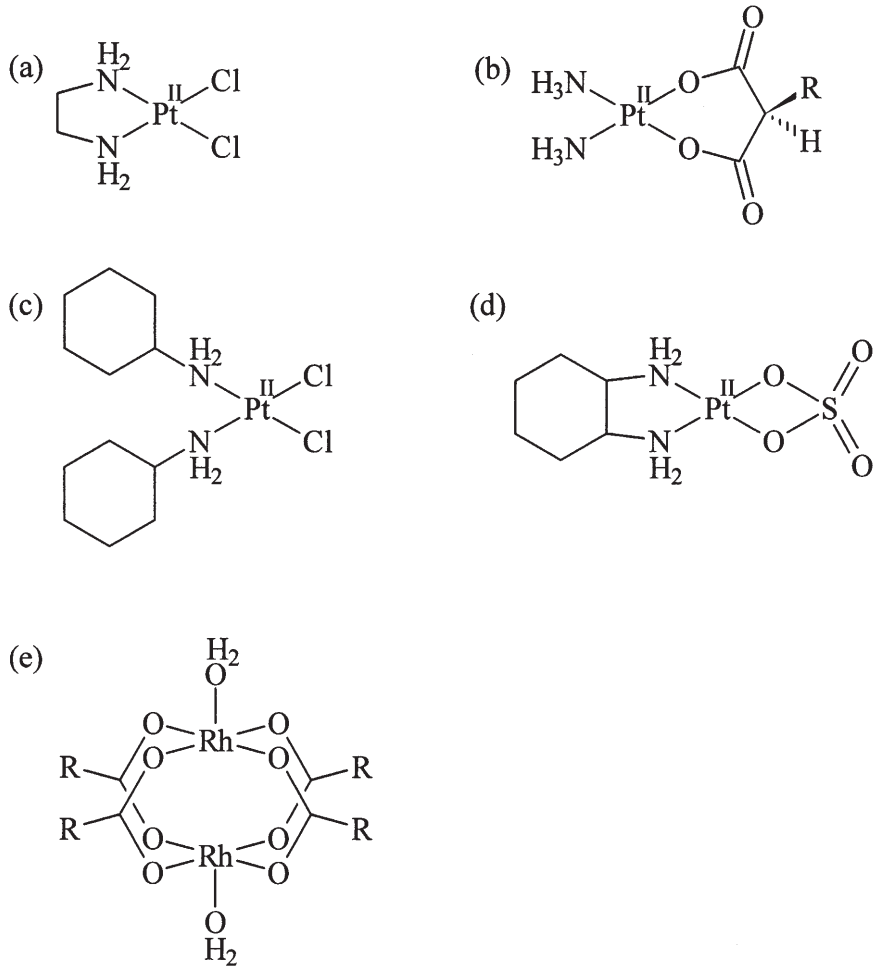


Fig. 4. *Molecular structures of new platinum group metal complexes with high activity against animal cancers*. a) Dichloroethylenediamineplatinum(II); b) substituted (R) malonatodiammineplatinum(II); c) *cis*-dichlorobis(cyclohexylamine)platinum(II); d) sulfato-1,2 diaminocyclohexaneplatinum(II); e) rhodium(II) carboxylate.

complexes have been studied in various laboratories. Of these, about 10–20% are active. The molecular structures of some of the most active complexes are shown in *Fig. 4*. The number of 'actives' is this large simply because most have started with a known active complex and produced small variations upon it. In a large morass of chemicals, we have found, through luck or cleverness, a few small islands of success, and we stray far from these only at some peril. It is not a very satisfying situation when endangered grant renewal is the penalty for boldness.

Nevertheless, in the areas explored, some common features have emerged which link structure to activity. We embody these here in a set of 'rules of thumb', since they can hardly lay claim to general validity [3]. These are:

1. the complexes exchange only some of their ligands quickly in reactions with biological molecules;
2. the complexes should be electrically neutral, although the active form may be charged after undergoing ligand exchanges in the animal;
3. the geometry of the complexes are either square planar or octahedral;
4. two *cis*-monodentate or one bidentate leaving group (exchangeable ligands) are required; the corresponding *trans*-isomers of the monodentate leaving groups are generally inactive;
5. the rates of exchange of these groups should fall into a restricted region, since too high a reactivity will mean that the chemical reacts immediately with blood constituents and never gets to the tumor cells, while too low a reactivity would allow it to get to the cells, but they would do nothing once there;
6. the leaving groups should be approximately 3.4 Å apart on the molecule (an interesting number, since the spacing between the steps of the *Watson-Crick* DNA ladder is also 3.4 Å);
7. the groups across the molecule from the leaving groups should be strongly bonded, relatively inert amine type systems.

We certainly do not intend these rules to restrict future research, but only to encompass a large amount of past experience with platinum(II) complexes. Obviously exceptions will, and have already, occurred. For example, the high activity of bidentate leaving groups such as oxalate and malonate (see structures of *Fig. 4*) first synthesized by *M. Cleare* and *J. Hoeschele* in this laboratory are not encompassed; nor is the effect of cyclic amines, developed by *Tobe*, which decrease the solubility of the complexes, but markedly enhance the antitumor activity. Here, studies of the rela-

tive solubilities in oil and water, the partition coefficient, may be significant in determining activity.

We can now present a broad outline of the fate of the drug, *cis*-dichlorodiammineplatinum(II), after injection into the peritoneal cavity of the mouse. Within minutes the drug leaves the cavity through the blood and lymph circulation. The high chloride concentration of these extracellular fluids prevents the chlorides from leaving the molecule, thus maintaining the structural integrity. The intact drug is rapidly excreted in the urine, with a half-life in the body of about one hour. The excreted drug is 95 % the unchanged molecule but about 5 % is attached to proteins. The drug is passively transported across the cellular membrane - no active transport (carrier) is necessary. Once inside the cell, the lower chloride content of the cytoplasm (1/30 of that outside the cell) allows the chloride to exchange with water according to the following scheme:

$$Pt^{II}(NH_3)_2Cl_2 + H_2O \rightleftharpoons [Pt^{II}(NH_3)_2(H_2O)Cl]^+ + Cl^-$$
$$[Pt^{II}(NH_3)_2(H_2O)Cl]^+ + H_2O \rightleftharpoons [Pt^{II}(NH_3)_2(H_2O)_2]^{2+} + Cl^-$$

Depending on the hydrogen-ion concentration, the H_2O may be changed to $(OH)^-$. This aquated species reacts primarily with the nitrogens of the DNA bases leading to the primary lesion responsible for the anticancer effect. While the formula for the diaquo species implies a simple, single structure, we have recently discovered that it is slightly more complicated than that. In fact, isolation of crystal species of the diaquo complex under slightly different conditions have yielded one monomer, one hydroxy-bridged dimer, two hydroxy-bridged trimers, a tetramer and two other not yet resolved crystal forms. This emerged from a cooperative study between *B. Lippert* of this laboratory and *C. J. L. Lock* of McMaster University. It is not yet clear what role, if any, these various structures have in the anticancer activity or toxicity of the parent drug.

I have been purposely nebulous so far on where the DNA is, and what sites of the DNA are involved. In order to be more concrete we require a short description of the molecular biology studies.

What Do the Platinum Complexes Do to Mammalian Cells?

Two options are available generally to study the effects of drugs on cells; first, inject the drug in animals, excise the desired cells and examine these for changes, the *in vivo* system; second, use purified cells growing in tissue culture, the *in vitro* system. The former is more relevant, but the latter is scientifically ‘cleaner’. Both should be done, and in the case of the

platinum drugs, were simultaneously and independently performed in two laboratories. Both the techniques were used by *Gale* and his associates at the Medical University of South Carolina, while only the second was done in our laboratory. There is general agreement on the results obtained by the two methods [4].

By the use of radioactively labelled precursor chemicals, the cell's ability to synthesize macromolecules such as DNA, RNA, and proteins, after treatment with the drug, can be measured. A typical result is shown in *Fig. 5*, for exposure of the cells to the equivalent level of drug found in tumor tissue of a treated animal. The synthesis of new total DNA is selectively and persistently inhibited. Total RNA and protein syntheses are not markedly affected until much higher drug-dose levels, which are frankly toxic to

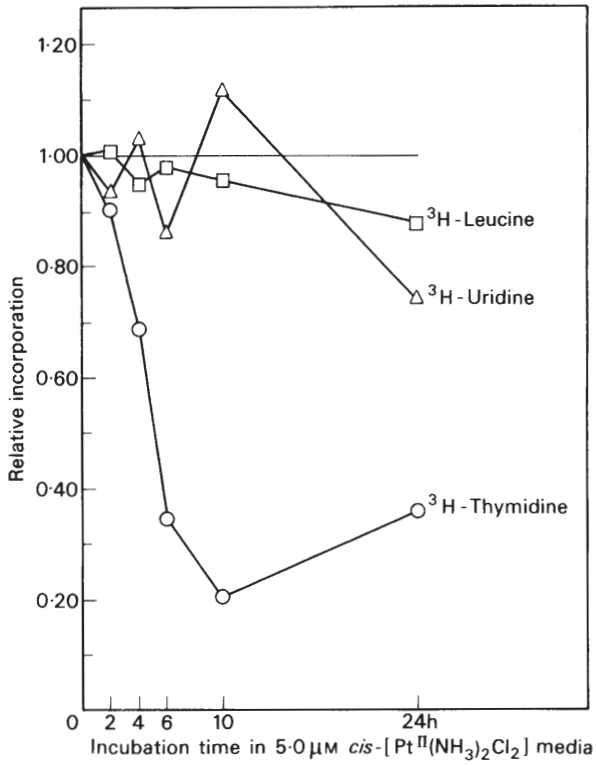


Fig. 5. *The effects of* cis*-dichlorodiammineplatinum(II) on macromolecular syntheses in human amniotic cells in tissue culture at a concentration* (5 μM ≈ 1 ppm*) similar to that found in tumor cells in animals treated with a therapeutic dose of the drug*. DNA Synthesis is measured by the incorporation of radioactively labeled thymidine and is severely and persistently inhibited. The synthesis of RNA, measured by radioactive uridine, and protein, measured by radioactive leucine is not significantly different from control (nontreated) cells represented by the horizontal bar at 1.00.

the cells, are used. The level of inhibition of DNA synthesis is dose-dependent. Its onset is slow, taking about 4–6 h after drug exposure to reach a nadir. It was surprising that there was not a large cell kill at the therapeutic dose level. The cells first grew into giant cells which, after a few days, showed the appearance of many nuclei and eventually divided into a number of single cells. I will return to this important result later.

H. Harder, then in our laboratory but now at George Washington University, was responsible for these studies. He also checked that the synthesis of precursor molecules for DNA, and the transport of these across membranes was not responsible for the inhibition. He has more recently shown that the ability of the DNA to act as a template for new synthesis is strongly inhibited by the platinum drug. These results can be most reasonably explained by the hypothesis that the anticancer activity of the platinum drugs arises from a primary attack on DNA. The battle to discover the molecular mechanism of action was, therefore, joined on the field of metal complex interactions with DNA, and numerous other laboratories entered the fray. The booty has been rich, embarrasingly so.

We now know that *cis*-dichlorodiammineplatinum(II) can crosslink the two strands of the double helix of DNA, an exciting discovery since this type of linkage had already been invoked to account for the anticancer activity of the bifunctional alkylating agents such as the nitrogen mustards. It was made almost simultaneously in three laboratories, but most elegantly by *J. J. Roberts* and his co-workers at the Chester Beatty Institute. It can also, apparently, crosslink two neighboring bases stacked on a single strand, which significantly, the complex in the *trans*-configuration should not do. The platinum drug does not react with the sugar-phosphate backbone of the strands, but only with the bases. Nor does it appear to intercalate between the bases. It reacts most strongly with the G-C rich regions of DNA, and can, through the technique of gradient centrifugation, be used to characterize the relative G-C/A-T content of DNA.

The platinum complex-DNA reaction is very slowly reversible *in vitro*, but it may be removed more rapidly within the cell by the actions of DNA repair enzymes. The two available exchangeable groups of the platinum can react at two sites on a given base (primarily the purines, guanine and adenine), or with single sites on two different bases, or finally, a single ligand of platinum exchanges at only one site of a base. It will take a considerable period to sort out the multiplicity of such reactions and to identify finally one or more as the necessary lesion for anticancer activity. In the meantime, it is clear to many of us that metal complex interactions with nucleic acids are too poorly understood, and too important to remain so.

It must surely be nagging the reader by now, as it has us for some years, that in all of the above work no clear distinction has emerged between ef-

fects on tumor cells and on normal cells. The same effects qualitatively, and very likely, quantitatively appear in many cell types, and yet we have suggested that these effects are the primary lesion leading to anticancer activity. Chemical studies of DNA are no doubt important, but since we cannot say in what way DNA differs in cancer cells from normal cells, we cannot answer the question of why the cancers are killed and not the animals. The question is by no means trivial. It cuts to the heart of cancer chemotherapy.

Justifiably, some people are unhappy that we have not yet discovered drugs with higher curative power against cancer. However, many researchers, myself included, are surprised that we have discovered so many that are so good, because we do not know why. All cancer drugs are cellular poisons, but not all cellular poisons are cancer drugs. If the drugs were not eventually more poisonous to cancer cells than normal cells, we would not be injecting them into patients. With the possible exception of L-asparaginase, we have not yet been able to seize upon a unique, exploitable characteristic of cancer cells to produce specific, or even selective tumor-cell kill. Yet in the host animal this can, and does, occur with presently useable drugs. This, admittedly simplistic, logic leads naturally to the invocation of the host-tumor interaction rather than the drug-tumor interaction as the source of specificity in the anticancer activity of drugs. Such specificity is usually associated with the host's immune response.

How Does Selective Cancer Destruction Occur?

Here I should like to touch on a more speculative side of the research. We had to face up to the strong evidence at the molecular level that the platinum drug produced a lesion on the DNA of cells, which did not necessarily lead to cell death, and in any case, was not restricted to cancer cells alone, and the final clinical observation that the cancers disappeared in the animal, without unacceptable side effects. There is a wide gap between molecular biology and clinical results. Could we bridge some or all of it with testable hypotheses?

The specificity of the cure is a good clue, since as we noted, specificity is usually associated with the host's immunologic responses. Is there any evidence for such responses of the host? There is, but this evidence consists mainly of an accumulation of weak arguments which cannot be summed to make a strong argument. Briefly, these are: our earliest screening studies of coordination complexes brought to light the peculiar result that some complexes increased the rate of tumor growth by about 200% compared to untreated controls. This is consistent with the already established suggestions that the host animal exerts some constraint on the growing tumor through

immunologic reactions, and if these constraints are inhibited (by immunosuppressive agents) without these agents simultaneously exerting antitumor activity, then increased tumor growth rates are expected.

The second involvement of the immune system occurred when we were able to cure large solid Sarcoma-180 tumors in ICR mice. The cured animals rejected any new attempt to reimplant this tumor up to 11 months later. They have obviously developed a heightened immunologic reactivity for this tumor. Interestingly enough, the cure of small tumors did not produce such an immunologic rejection reaction.

This experiment also produced a third unexpected result. It has been accepted, since the classic work of *H. E. Skipper* and co-workers at the Southern Research Institute, that at least for leukemia, a given dose of a drug kills a constant fraction of the tumor cells present, in fact a first order kinetic process. Yet, we are able to cure small tumors and large tumors, with a given optimal dose of the platinum drug, but not intermediate-sized tumors. This is not sensible if one considers direct cell kill only. Similarly, if the optimal dose cures the large tumors, then a much smaller dose should cure the smaller tumors. This, too, is contrary to our experiments. Something other than direct chemical cell kill must be operating to achieve cures.

Dead Sarcoma-180 cells injected into mice do not cause tumors, neither do they induce an immune reaction to reimplanted live tumor cells. Here one must be cautious since only small numbers of live implanted cells (~ 40) can eventually lead to large tumors and death. But cells treated with the platinum drug at low concentrations, 100 times less than the concentration required to produce extensive cell kill, implanted in the mice do not produce tumors, but do induce an immunologic rejection of pristine tumor cells implanted two weeks later. This experiment is difficult to interpret without invoking the immune system of the host in causing tumor cell death.

If the immune system is involved, then one could anticipate that modulating the host's immune competence should modulate the anticancer activity of the drug. Preliminary experiments by *P. Conran* in this laboratory, and now at the University of Connecticut, suggest that this is true. Decreasing the immunocompetence of mice by hydrocortisone injections decreases the cure rate of the platinum drug against Sarcoma-180 in ICR mice, while, conversely, the nonspecific immune stimulant, zymosan, increases the cure rate against the Sarcoma-180 in BALB/c mice. Unfortunately these systems are not as 'immunologically clean' as one would like, so the experiments are now being repeated using acceptable systems both in our and *Conran's* laboratories.

One of *Tobe's* complexes, *c*is-dichlorobis(cyclohexylamine)platinum(II) was tested by *T. A. Connors* against the ADJ/PC6 myeloma tumor in mice. It cured the tumors completely at a dose 1/500 of the LD_{50}. Such specificity, especially in the absence of any evidence of selective tumor up-

take of the drug, is utterly inconsistent with the direct cell-toxicity hypothesis. A host response must again be invoked, one with high specificity. And again, the immune system alone has that characteristic.

Finally, we can count the number of tumor cells in the animal as a function of time after a platinum drug treatment known to produce large-percent cures. The Ascites Sarcoma-180 in ICR mice is ideal for this purpose. We inject four million cells into the peritoneal cavity on day 0. They multiply rapidly to two thousand million cells 15 days later, remaining localized in the cavity and killing 100% of the animals. Now we inject the platinum drug on day 1, and sacrifice small numbers of the animals every day. We wash, clean, and count the tumor cells. The results are shown in *Fig. 6*. The cells divide about 2–4 times, increasing in number up to 40 million cells by days 4–5, before a turnaround occurs and the cell number drops to 0 on day 9–10. If direct tumor-cell kill by the drug were operative, we should expect a fast decline from about eight million cells to 0 on day 2, with no further cell di-

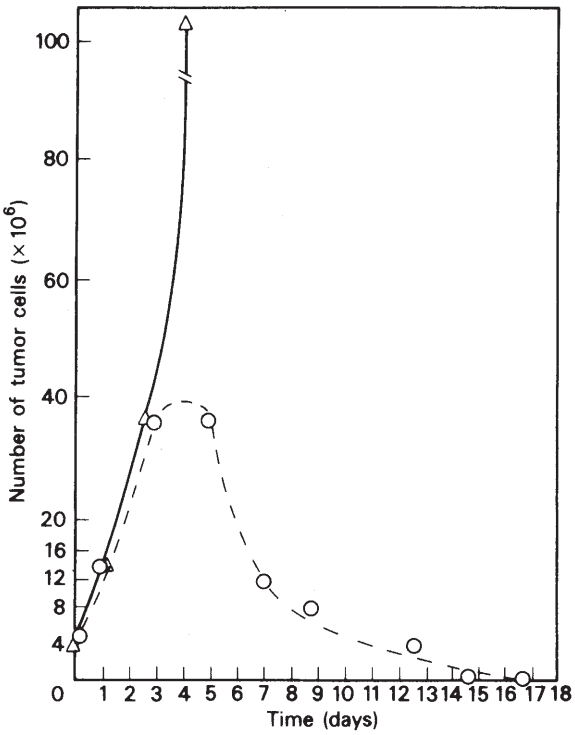


Fig. 6. *The growth of Ascites Sarcoma-180 tumor cells in untreated ICR mice* (solid line) *and in treated mice* (dashed line). The treatment consisted of five injections of 1.5 mg kg^{-1} given on day 1 after inoculation of 4 000 000 tumor cells on day 0. The number of cells continues to increase by repeated cell division up to day 4 and then slowly decreases to zero cells (cured animals).

visions and no continued growth. This is contrary to the experimental results and again suggests a host mechanism for tumor-cell destruction.

We propose that these arguments are consistent with, but do not corroborate, the hypothesis that the platinum drug enhances the antigenic character of the tumor cells, tipping the balance in favor of the host's immunologic intervention to destroy the cancer. The question then becomes, 'how does the platinum drug accomplish this?'. Now we return to the derepression story based on *Reslova's* work. In brief, *Fig. 7* outlines one potential sequence of molecular events in a mammalian cell which could produce the desired result.

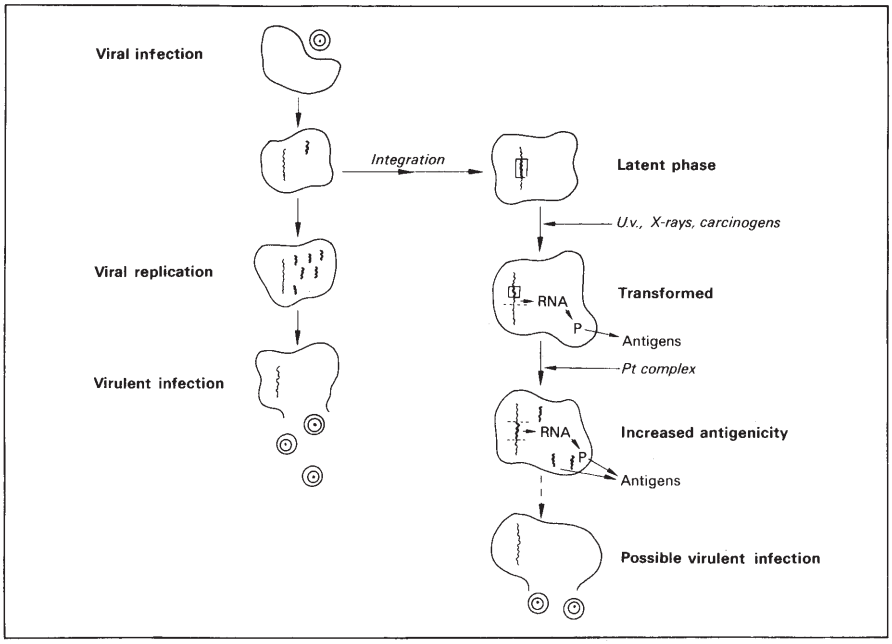


Fig. 7. *A schematic diagram of one possible hypothesis of the molecular action of* cis-*dichlorodiammineplatinum(II) leading to an enhanced antigenicity of tumor cells by the derepression of virally coded information latent in the cell.*

Without making a definite commitment, let us assume the hypothesis that expression of viral DNA is the causative factor in the cell transformation to a cancer state. There is certainly a significant body of experiments indicating that this is true in many mammals, but solid evidence in humans still eludes us. The viral genome, incorporated in the cellular genome, is completely repressed for long periods compared to most cells' division times. As in the case of lysogenic bacteria, a wide variety of chemical and physi-

cal agents are able to cause a depression of a small part of this latent viral genome, enough say to code the production of one or two proteins. These proteins transform the cell. The existence of temperature-sensitive mutants for cell transformation tells us that the production of as little as one virally coded protein is a necessary (but not sufficient) cause of cell transformation. This small number of proteins is also the cause of the antigenicity of the tumor cell. The chemical and physical agents causing derepression are, therefore, carcinogens. If now we add the platinum drug to the cell, it effectively derepresses a larger fraction or all of the viral genomic information. This inevitably leads to the production of a larger number and variety of proteins, and this enhances the antigenicity of the cell.

This scheme, incidentally, provides a simple explanation of '*Haddow*'s paradox', that is, certain classes of chemicals and agents that cause cancer, can also cure cancer. The difference between cause and cure indicated here is simply a quantitative one. It is the amount of derepression of the viral genome.

Some experimental information consistent with our hypothesis exists. *V. Vonka* and co-workers at the Institute of Sera and Vaccines in Prague, were able to induce up to 300% increase in the number of *Epstein Barr* virus (an oncogenic *Herpes* type virus) positive cells in a culture of *Burkitt* lymphoma cells (EB3) by treatment with *cis*-dichlorodiammineplatinum(II). The induction of the new, virus-associated antigens was monitored both by an indirect immunofluorescence test for the coat proteins of the virus appearing at the cell surface, and by the visualization of virus-like particles in the treated cells by electron microscopy.

Thus the platinum drug, at least in this case, causes the hypothesized derepression in a cancer cell line, and has enhanced the antigenicity of the cells. While the enhanced antigenicity hypothesis is consistent with a large body of information, and does bridge the gap between molecular events involving the platinum drug interaction with cellular DNA and the host immunologic intervention, it still leaves unexplained the detailed mechanisms of derepression of latent viral genomes, its role in cell transformation and the nature of the immune response.

In the chapters that follow, the latest research results and the newest ideas, are masterfully presented.

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Part 2. Cisplatin – How Good is it?

Clinical Status of Cisplatin, Carboplatin, and Other Platinum-Based Antitumor Drugs

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Cisplatin. Edited by Bernhard Lippert

Clinical Status of Cisplatin, Carboplatin, and Other Platinum-Based Antitumor Drugs

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The platinum drugs represent a unique and important class of antitumor agents. The initial discovery of the antitumor properties of cisplatin by Dr. *Barnett Rosenberg* was quickly followed by clinical trials demonstrating its efficacy in a variety of solid tumors. It was soon realized, however, that nephrotoxicity and the emergence of drug-resistant tumor cells limited the overall efficacy of cisplatin. The search for new platinum analogues that could circumvent the deleterious aspects of cisplatin therapy soon followed. Carboplatin is a cisplatin analogue that is more easily administered and is less toxic at standard doses. This is due to a different pharmacokinetic profile resulting from the substitution of a more stable leaving group. Carboplatin and cisplatin form similar DNA adducts, which may explain, in part, the similar efficacies observed with the drugs in most solid tumors. The search for platinum analogues that do not exhibit cross-resistance with cisplatin and carboplatin has led to the synthesis of the DACH platinum compounds. The DACH platinum drug, oxaliplatin, has been shown to be active in combination with 5-fluorouracil and leucovorin for the treatment of colorectal cancer, a disease in which cisplatin and carboplatin show little activity. It appears that the clinical use of cisplatin and its analogues will continue to evolve, guided by pharmacologic principles, and these drugs will remain indispensable to combination chemotherapeutic regimens for many years to come.

Introduction

The development of cisplatin marked a watershed in the treatment of cancer. The three major classes of anticancer drugs then available – antimetabolites, alkylating agents, and anthracyclines – shared a common origin in the treatment of leukemia. The exception – 5-fluorouracil – was developed as a thymidine analogue, but surprisingly was relatively inactive in the more rapidly replicating acute leukemias. Cisplatin was remarkable for its lack of myelosuppression and so its investigation was targeted to solid tumors. Now over 25 years after the description of its anticancer activity, the

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continuing central role of cisplatin (and of its close congener carboplatin) in the initial management of several major solid tumors attests to its therapeutic importance.

In this chapter we will provide a detailed accounting of the impact that cisplatin and carboplatin have had in the treatment of cancer. We will address the major issues that constitute opportunities to enhance this impact: the emergence of drug resistance, and the approaches to platinum-refractory cancers. Finally we will describe the clinical pharmacology of these agents, through an understanding of which improved treatments are expected.

Developmental Perspective

Early Clinical Trials

As *Rosenberg* has indicated in *Part 1* of this volume, the early studies of cisplatin revealed it to be clinically challenging for patients and physicians alike. The initial human studies were characterized by toxicity of a degree hitherto unprecedented. Severe nausea and vomiting and nephrotoxicity in the form of renal failure almost led to studies being discontinued [1]. The demonstration by *Cvitkovic* and co-workers, first in an animal model, then in a clinical trial, that aggressive diuresis could prevent the severe renal damage permitted the further investigation of the drug [2][3]. These methods are still in standard use today. The nausea and vomiting were ameliorated largely as a result of the investigation of intensive antiemetic regimens in a series by *Gralla et al.* ([4] and references therein). Ultimately the discovery of 5-HT_3-receptor blockers (*Zofran*® and *Kytril*®) rendered this uncomfortable toxicity tolerable. The observation that patients with refractory tumors were deriving substantial benefit from treatment propelled continued clinical development.

Development of Carboplatin

The side effects associated with cisplatin therapy (including neurotoxicity and fatigue) prompted a parallel synthesis effort to design more effective and less toxic platinum analogues [5]. It was hypothesized that modification of cisplatin to contain less labile leaving groups could alter toxicity. The search for a less toxic agent was pursued at the Institute for Cancer Research in the U. K., which led to the development of carboplatin (reviewed in [6]). Using a murine screen for nephrotoxicity, it was found that substi-

tution of more stable ligands for the chloride leaving groups did indeed diminish renal effects, while antitumor activity was retained [7]. Carboplatin (*Fig.*), in which the leaving group is a cyclobutanedicarboxylate ligand, was found to have bone-marrow suppression as its predominant toxicity [8]. This differed from cisplatin, with which marrow suppression is neither common

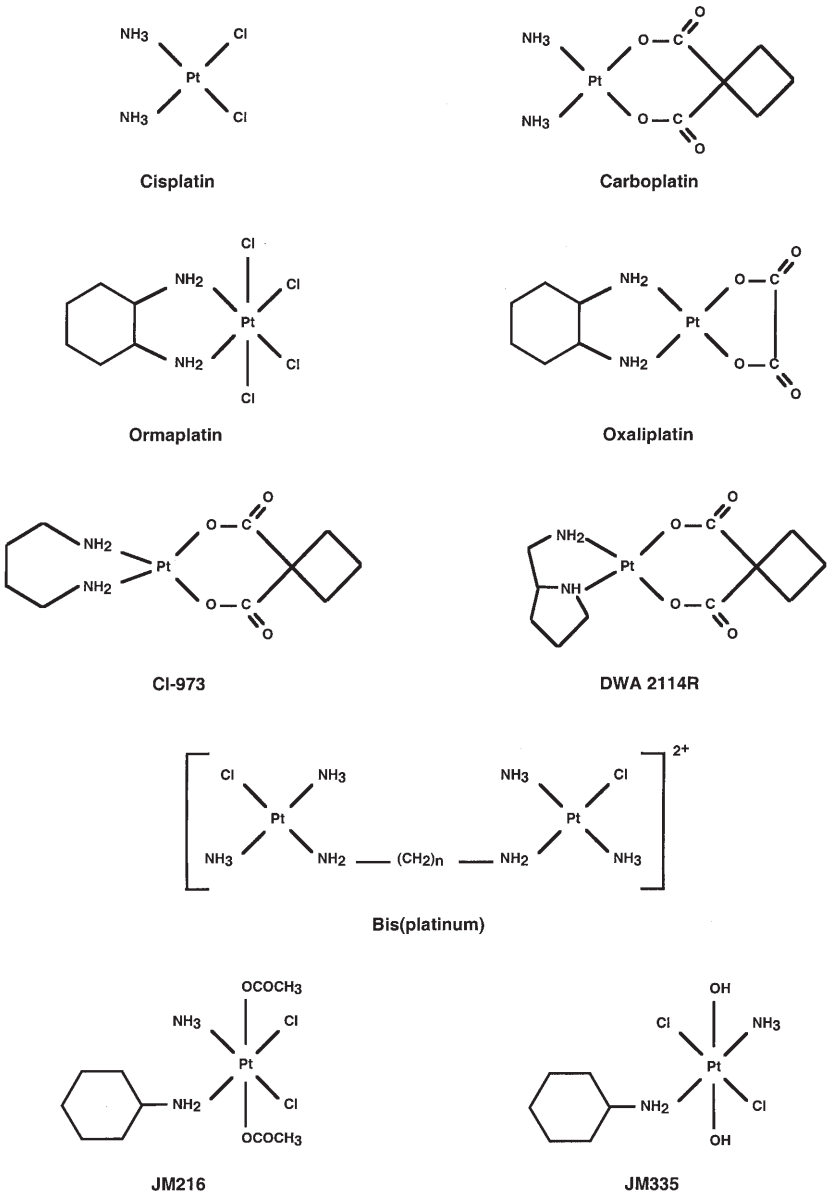

Figure. *Structures of platinum complexes*

nor severe. Also, carboplatin did not require pretreatment with a rigorous hydration regimen. At effective doses, carboplatin produced substantially less nausea, vomiting, and neurotoxicity than cisplatin. Phase III trials demonstrated the equivalence of carboplatin and cisplatin in the treatment of ovarian cancer [9], but in testicular, and head and neck cancers, cisplatin appears to be superior. Therefore, on the basis of superior therapeutic index, greater ease of administration, and more predictable individualized dosing (as will be described), carboplatin has largely replaced cisplatin in the treatment of many but not all platinum-sensitive tumors.

Development of New Analogues

Altering the structure of the leaving group appears to influence tissue and intracellular distribution of the platinum coordination complexes. Upon interacting with DNA, the stable (carrier) amine group determines the structure of the adduct. Thus, the adducts produced by cisplatin and carboplatin are identical. This seems to explain their very similar patterns of tumor sensitivity. While one agent or the other may have slightly better efficacy in certain tumors, there are no tumors that are resistant to one while highly sensitive to the other. Therefore, it was hypothesized that modification of the parent drug to obtain analogues that produced different DNA lesions might result in compounds with a broader spectrum of anticancer activity. Examples of analogues recently or imminently to be in clinical development are shown in the *Figure*.

1,2-Diaminocyclohexane (DACH) Derivatives and their Analogues. The initial screening for platinum analogues with broader antitumor activity was conducted in murine leukemias with acquired resistance to cisplatin (reviewed by *Harrap* [6]). Compounds containing the DACH ligand as a stable carrier group were first synthesized by *Connors et al*. [5], and *Burchenal et al.* first demonstrated their activity in murine models [10]. Based on these studies (reviewed in [11]), a number of compounds were developed for potential clinical use: DACH-(malonato)platinum(II) was insufficiently soluble [12], DACH-(4-carboxyphthalato)platinum(II) was not active in limited Phase-II testing [13], and tetraplatin (ormaplatin) caused severe and cumulative neurotoxicity in Phase-I trials [14].

More recently, however, a DACH compound oxaliplatin [DACH-(oxalato)platinum(II)] ([SP-4-2-(1*R-trans*)]-(1,2-cyclohexanediamine-*N*,*N′*)-[ethanedioato(2-)-*O*,*O′*]platinum(II)) has been successfully developed in France (*Fig.*) [15–17]. The spectrum of DNA adducts formed by oxaliplatin are the same as that observed with cisplatin and carboplatin [18]. The

major adducts formed are d(GpG)Pt and d(ApG)Pt intrastrand crosslinks. *Saris et al*. [19] showed that at equimolar concentrations in cultured cells, oxaliplatin forms fewer intrastrand crosslinks than cisplatin. Oxaliplatin is active in several cisplatin-resistant tumor cell lines [20]. Moreover, comparative analysis of the results from the NCI human tumor screen suggests that oxaliplatin and other DACH-ligand-containing platinum drugs form a distinct family of agents with a pattern of tumor sensitivity that differs from that of cisplatin [21][22]. Evidence for a lack of cross-resistance has also been obtained *in vivo* [23]. Phase-II and -III trials indicate that oxaliplatin has activity in colorectal cancer that has developed resistance to 5-fluorouracil [24][25]. Its potential role in the initial treatment of colorectal cancer is currently being investigated.

This important result lends substantial support to the hypothesis that structural modifications of the carrier ligand may greatly alter the spectrum of antitumor activity, and so overcome resistance. The major difference between the diammine-based cisplatin and carboplatin, and DACH- and other ligand-containing derivatives is likely to be in the manner in which cellular proteins recognize and process platinum-DNA adducts. *Chaney* and co-workers have shown that some cisplatin-resistant human ovarian cancer cells have the capacity to replicate DNA past cisplatin-induced DNA adducts, but not past DACH-platinum adducts [26]. The local effects of adducts formed with various carrier ligands upon DNA structure may differ, and may result in the recruitment of more than one type of DNA damage recognition protein, possibly initiating various repair and/or tolerance pathways. Thus, the results of the treatment of cisplatin- and carboplatin-resistant human tumors with oxaliplatin are awaited with interest.

Alternative Alicyclic Carrier Ligands. Additional carrier ligands with aliphatic cyclic components have also been tested. Enloplatin (*Fig.*) was nephrotoxic in Phase-I trials and has been abandoned [27]. Two compounds continue in Phase-II development. Lobaplatin (D-10466) has a cyclobutane-derived carrier group, and lactate as a leaving group. Like cisplatin and oxaliplatin, lobaplatin also forms predominantly d(GpG)Pt and d(ApG)Pt intrastrand crosslinks in DNA [19]. In Phase-I trials, thrombocytopenia was dose-limiting, and responses have been observed in patients with ovarian cancer previously treated with cisplatin and/or carboplatin [28][29]. DWA 2114R has a 2-(aminomethyl)pyrrolidine carrier group: in Phase-I trials the dose-limiting toxicity was neutropenia [30]. In Phase-II trials a 44% response rate was observed in patients with relapsed ovarian cancer [31]. These data are interesting in that they suggest that the structural modification may have yielded a drug with the capacity to overcome cisplatin resistance.

Aliphatic non-cyclic carrier ligand substitutions have also been tested. CI-973 or NK-121 has a 1,2-diamine-methylbutane carrier ligand, combined with a cyclobutanodicarboxylate leaving group. This compound exhibited myelosuppression as its dose-limiting toxicity in Phase-I trials, but was inactive in limited Phase-II testing [32].

Platinum (IV) Structures. The oxidation state of the platinum atom in platinum coordination compounds determines the steric configuration of the molecule: platinum(II) structures are planar molecules, while platinum(IV) derivatives assume an octahedral shape. Though it was hoped that these differences could be used to circumvent platinum resistance, the two compounds developed in the clinic, iproplatin and ormaplatin, have not proven useful. In the case of the former, testing in Phase-II trials failed to reveal activity. In the case of ormaplatin, the platinum(IV) configuration is not maintained under biological conditions: conversion to a platinum(II) metabolite occurs within minutes [14]. A series of novel platinum(IV) and mixed ammine/amine derivatives being developed at the Institute for Cancer Research are described in this volume by *Kelland*.

Bis-platinum Derivatives. Based on the recognition that cisplatin cytotoxicity is predicated upon the formation of bifunctional interastrand and interstrand crosslinks, *Farrell* and colleagues have synthesized a series of DNA-binding drugs based on a binuclear platinum structure. These drugs form DNA complexes that differ markedly in structure, sequence specificity, and formation kinetics from those of cisplatin [33]. The incorporation of more than one platinum molecule, each capable of adduct formation, together with a variable linker region, results in novel, structurally distinct interstrand crosslinks [34]. Unlike the case with cisplatin, interstrand crosslinks are more common than intrastrand lesions with the bis-platinum derivatives. Furthermore, these lesions have more profound effects upon DNA replication and gene transcription, and there is evidence that because of the conformational changes they exert on DNA, they are detected less efficiently by DNA damage-recognition proteins. As a result, repair of the lesions may be less readily accomplished. Some of these complexes themselves may bind to and inactivate repair proteins [33]. The preclinical evaluation of bis-platinum drugs demonstrates that they have *in vivo* activity in a variety of cisplatin-resistant models, including murine leukemias and human ovarian cancers. Clinical trials with these drugs have been initiated.

For all of these analogues, it may be expected that varying characteristics of cellular uptake, interaction with cytoplasmic nucleophiles, DNA-adduct formation, DNA topologic alterations, and interaction with DNA-repair systems will determine their cellular pharmacology. Understanding

these characteristics will determine their optimal clinical use and will permit the broader application of platinum drugs to refractory tumors.

Clinical Applications in Oncology

Cisplatin first provided the opportunity to cure over 80% of patients with testicular cancer, a disease that afflicts young men in their prime. It greatly improved survival in patients with advanced ovarian cancer, often a disease of young and middle-aged women. Its use provided the first cures in patients with small-cell lung cancer, and cisplatin is the cornerstone of regimens that showed that chemotherapy could improve outcome in advanced non-small-cell lung cancer. In patients who present with earlier stages of lung cancer, the same regimens enhance cure rates. In head and neck, and bladder cancers, substantially improved outcomes result from treatment with platinum-containing combinations.

In this section we will detail some of the studies that have established for cisplatin a central role in the treatment of cancer. In most cases it will be observed that the drug is used in combination. The development of combinations has been guided by both preclinical and clinical studies, by mechanism-based hypotheses and by empiricism. All have contributed to the investigation of treatment strategies that are now standard throughout the world.

Testicular Cancer

Testicular cancer is the most common tumor in men between the ages of 15 and 35 years, with an incidence of about 3/100,000. There is evidence that the incidence of this tumor has doubled over the past 50 years, both in Europe and in the United States. Until the 1970's, testicular cancer was usually a fatal diagnosis, and fewer than 10% of patients enjoyed long-term survival. With the advent of aggressive chemotherapy along with surgery and radiation therapy, over 90% of patients are now expected to be cured. There is general agreement that the most important contribution to this success has been the development of cisplatin and its incorporation into combination regimens for the treatment of this disease.

Prior to the discovery of cisplatin, testicular cancer was known to be somewhat sensitive to chemotherapy: the most active agent was dactinomycin, which produced responses in some 50% of patients, 10% of which were complete [35]. Only 5% of patients were cured. As a result, patients were available to enter trials of new agents, and in the earliest Phase-I trials

of cisplatin, responses were found in patients with testicular tumors [36]. From this beginning, two groups of investigators were instrumental in the rapid development of platinum-containing combinations in this disease. At Memorial Sloan Kettering, cisplatin was added to a pre-existing VAB (vinblastine, dactinomycin, and bleomycin) regimen to produce VAB-II [37]. The addition of cisplatin increased complete response rates from 14 to 50%, and appreciable proportions of long-term survivors (24%) were observed. It is important to mention that the very rapid growth of testicular cancer results in the detection of relapse early (within months), so that long-term survival equates to cure. Various modifications of the VAB regimen were made subsequently until the development of VAB-VI, which featured the addition of cyclophosphamide, and dose intensification of cisplatin to 120 mg/m^2 on the fourth day of treatment [38]. In a study of this regimen from 1979 to 1982, a complete remission rate of 78% was observed among 166 patients, and the long-term survival rate was 74%.

Einhorn and *Donohue* at Indiana University derived a simpler but aggressive regimen that was administered over 12 weeks [39]. Bearing the acronym PVB, this regimen consisted of cisplatin administered in five daily doses of 20 mg/m^2, vinblastine in two daily doses, and bleomycin weekly. The regimen was repeated every three weeks for four cycles. A maintenance regimen of vinblastine treatment for a total for two years was included. In the initial trial of this regimen, 47 patients were treated, of whom 33 (70%) achieved complete remission [39]. Five additional patients were rendered free of disease by surgical resection of residual masses. Of the entire group, updated later, 64% survived five years and 60% ten years. Subsequent randomized studies show that vinblastine doses could be reduced without sacrificing efficacy, and that maintenance treatment was unnecessary [40–42]. In all of these trials, over 80% long-term survivors were obtained.

Why was there a progressive increase in effectiveness of therapy with essentially the same regimen over about 10 years? Factors that may be important include the rapid and early referral of suitable patients for treatment, reflecting the growing appreciation that testicular cancer was now a potentially curable disease. Treated earlier, with lower tumor burdens, and with less general debilitation as a consequence of advanced cancer, patients could expect greater success of therapy. However, these data also reflect the more general learning curve associated with the broad use of cisplatin in the community. The revelation of its curative potential encouraged physicians both to adhere rigidly to doses and schedules of administration, and aggressively seek to ameliorate side-effects so that patients would not refuse treatment. As a result, a pattern developed for the future use of cisplatin in the treatment of other tumor types. Progress in the discovery of anti-emetics, in strict adherence to hydration regimens, and in anticipating and treating electro-

lyte abnormalities facilitated the investigation of high-dose and regional approaches, especially in ovarian and lung cancers.

In the 1970's and 1980's while this research was in progress, two additional developments contributed to progress in the treatment of testicular cancer. The identification of tumor markers in serum provided a sensitive indicator of tumor progression or regression. Alpha-fetoprotein, a fetal glycoprotein normally barely detectable in adult serum is elevated in up to 60% of patients, and implies an embryonal cell component to the tumor. Beta-human chorionic gonadotrophin may be elevated in both seminomatous and non-seminomatous testicular tumors, also in up to 60% of patients. The population of patients with elevated markers increases with increasing disease stage. The availability of these markers facilitated the diagnosis of testicular cancer at earlier stages. They defined which patients might need more than the four cycles of chemotherapy to eradicate all vestiges of disease. In the new era of cisplatin-based chemotherapy, they subsequently became central to the identification of good-risk and poor-risk patients at diagnosis.

The second important technological development was the widespread availability of computerized tomographic (CT) scanning. For testicular cancer this was critical to the initial quantitation of the extent of disease in a tumor that commonly involves the retroperitoneum and mediastinum, both difficult to assess by other means. Scanning was invaluable to define the presence of residual masses following chemotherapy: surgical resection of these masses has been shown to maximize cure rates.

With these tools progress in the treatment of testicular cancer could be reliably documented. The striking cure rates from platinum-containing chemotherapy led to a classification of patients based on their likelihood of cure. Testicular cancer of the nonseminomatous type was classified into good, intermediate, or poor-risk, based on the degree of serum marker elevation, site of primary, and involvement of organs other than testis, lymph nodes, and lung. The seminomatous type, for which prognosis is generally better, was classified into good and intermediate risk.

The focus in good-risk patients was to decrease the toxicity of treatment. Substitution of cisplatin by carboplatin was attempted in two randomized trials [43][44]. In both, outcomes were worse in patients who received carboplatin. Thus, in testicular cancer standard dose carboplatin is not therapeutically equivalent to cisplatin.

Three studies have addressed the need for bleomycin in patients with good risk disease: in one the number of cycles was reduced from four to three [45], and the regimen without bleomycin was inferior; in the other two (both four cycles) results were equivalent with and without bleomycin [46][47].

A new agent for this disease was provided by the observation that etoposide was active in patients with resistant disease [48]. Its incorporation with

bleomycin and cisplatin in patients with poor-risk disease resulted in activity greater than that observed with PVB [49]. As a result, the BEP regimen is now standard in these patients. An attempt to enhance results by intensifying the dose of cisplatin to 200 mg/m^2 per course did not, however, improve results [50]. This was important in that it limited enthusiasm for dose-intensification trials using cisplatin. Carboplatin, limited mainly by myelosuppression, can be dose-escalated to a much greater extent than cisplatin, and research is currently directed to determining if high-dose carboplatin can improve outcome in patients with very poor-risk disease.

Ovarian Cancer

Compared to other solid tumors, ovarian cancer is relatively responsive to chemotherapy, but unlike testicular cancer, cure is not common for patients with advanced disease. Prior to the incorporation of cisplatin or carboplatin into treatment regimens, chemotherapy for advanced-stage ovarian cancer consisted of combinations of alkylating agents and doxorubicin. Response rates from such regimens were of the order of 33–65%, and fewer than 10% of patients survived 5 years [51].

In an initial Phase-II clinical trial in 1974, cisplatin treatment resulted in 7 of 25 (28%) responses in patients with ovarian cancer [52]. In two subsequent trials, response rates of 27% and 29% were reported in drug-refractory ovarian cancer patients treated with single-agent cisplatin [53][54]. These promising results led to studies investigating cisplatin alone or in combination with other drugs as first line treatment for advanced-stage ovarian cancer. As a single-agent, cisplatin yields response rates of approximately 50% in previously untreated patients. In combination with drugs such as cyclophosphamide, doxorubicin, and hexamethylmelamine, response rates of 55 to 96% may be achieved [2]. Three randomized trials comparing combinations with or without cisplatin have been performed [55–57]. The largest, by the Gynecologic Oncology Group (GOG), randomized 227 patients to receive either cyclophosphamide/doxorubicin or cyclophosphamide/doxorubicin/cisplatin. The response rates (26% *vs*. 51%), median response duration (8.8 *vs*. 14.6 months), and survival (9.7 *vs*. 15.7 months) all favored the cisplatin-containing regimen [57]. Similar findings were obtained in the other studies. These three trials established the role of cisplatin in ovarian cancer.

Prior to the mid 1990's, the standard treatment for advanced-stage ovarian cancer had become either cisplatin or carboplatin in combination with cyclophosphamide, although some clinicians still favored single-agent carboplatin. The basis lay in comparative studies of cisplatin- *vs*. carboplatin-

containing combinations. Two North American trials of cisplatin-cyclophosphamide *vs*. carboplatin-cyclophosphamide showed no differences in response rates or survival [58][59]. A European study by *ten Bokkel Huinink* and co-workers showed no significant difference in response rates, but a suggestion of a survival advantage favoring cisplatin in patients with small-volume disease, but not in those with bulky disease [60][61]. Single-agent randomized studies (cisplatin *vs*. carboplatin) have also been conducted in Europe: no differences in outcome have emerged between the two analogues [62–65]. These data taken together support the equivalence of cisplatin and carboplatin in advanced ovarian cancer.

Despite the broad use of combination chemotherapy, the superiority of this approach over the use of single-agents is disputed by some. Two meta-analyses pooled the results of several clinical trials to compare the survival of ovarian cancer patients treated with a single platinum drug *vs.* a platinum drug combination [62]. In one study, the survival curves suggested a difference in favor of platinum-based combination chemotherapy at two years, however, the two curves converged by year eight [62]. In contrast, the other study reported that the survival curves were similar up to two years after which time they diverged, with increased survival occurring in patients treated with platinum combinations [66]. At year eight, 23% of patients receiving platinum combinations had survived, whereas only 14% of the patients receiving single-agent cisplatin or carboplatin were alive. These two studies also indicated that the survival curves of patients treated with cisplatin or cisplatin-based regimens was similar to that of patients treated with carboplatin or carboplatin-based regimens.

In 1994, it was shown that paclitaxel demonstrated significant activity in previously untreated and platinum-drug refractory ovarian cancer [67]. In order to compare the efficacy of paclitaxel against standard chemotherapy, a clinical trial was performed by the Gynecologic Oncology Group (GOG) to compare the combination of cisplatin and paclitaxel *vs.* cisplatin and cyclophosphamide. The results indicated that 73% of the patients receiving cisplatin/paclitaxel responded to treatment, whereas 60% of the patients receiving cisplatin/cyclophosphamide responded [68]. Median survival was also significantly higher in the cisplatin/paclitaxel arm (38 month *vs*. 24 months). A confirmatory trial of cisplatin/paclitaxel has been performed by European and Canadian investigators [69]. At the present time, the combination of cisplatin or carboplatin with paclitaxel is the standard regimen for the treatment of advanced-stage disease in the United States. Many issues remain to be examined with regard to platinum/paclitaxel chemotherapy, such as optimizing scheduling, duration of treatment, incorporation of new agents, and the role of high-dose carboplatin [70].

Lung Cancer

Platinum-based combination chemotherapy has become the cornerstone of therapy for both non-small-cell (NSCLC) and small-cell (SCLC) lung cancers. Systematic evaluation in large randomized trials in the cooperative group setting following single-institution Phase-II studies has provided substantial data for the practicing oncologist, yet no clear consensus regarding the superiority of a single regimen. What is clear is that platinum combinations can improve the survival and quality of life of patients with advanced lung cancers.

Non-Small-Cell Lung Cancer. During the 1970's, small studies of single-agent cisplatin therapy in patients with metastatic NSCLC demonstrated a low level of activity, with response rates approximating 10% [71]. However, the success of combination chemotherapy regimens in other tumor types led investigators to pursue combination chemotherapy. Phase II combination studies of cisplatin paired with vindesine (VP) [72] and etoposide (EP) [73], or incorporated into triplets with mitomycin C and vinblastine (MVP) [74], and cyclophosphamide and doxorubicin (CAP) [75] revealed increments in response rates (30–53%). Results of larger randomized studies conducted by the ECOG investigating these and other combinations underscored the shortcomings of smaller single-institution studies: MVP was associated with the highest response rate (31%), yet no combination conferred a clear survival advantage [76][77]. Therefore, the choice of a regimen in practice became a rather subjective one for the oncologist, and the EP and VP regimens became popular in the 1980's and early 1990's, based upon their schedules and more favorable toxicity profiles. While no single combination proved superior, the results of a meta-analysis performed by the NSCLC collaborative group [78], a randomized National Cancer Institute (NCI) of Canada study [79], and a prospective review of SWOG NSCLC trials [80] confirmed that platinum-based chemotherapy provided a small but significant survival benefit in metastatic NSCLC over supportive care alone or non-platinum-containing therapy.

Differences in response rates were substantial in small studies, and especially between single-institution and large cooperative group trials. The major reason for these discrepancies is attributed to selection of patients for study: those entered in single-institution studies tended to have better performance status and perhaps to have less extensive disease. These conclusions were reinforced by a comparison of the NCI Canada study that demonstrated a survival advantage for chemotherapy over best supportive care with two other randomized trials that failed to show such a difference. The negative trials included patients with performance status of 2, while the NCI

Canada trial was limited to those with performance status of 0 or 1. Thus, the benefit of cisplatin-containing chemotherapy in patients with advanced symptomatic disease remains to be definitively established.

The optimal dose of cisplatin for application in lung cancer has also been the subject of some controversy: preclinical data suggested that cisplatin-induced cytotoxicity and DNA-adduct formation were concentration dependent [81]. *Gralla et al.* stimulated interest in high-dose regimens after reporting improved duration of response and survival in responding patients treated with 120 mg/m^2 *vs*. 60 mg/m^2 of cisplatin combined with vindesine in a single-institution randomized study [72]. Survival for all patients treated was not reported, however, leaving their conclusion open to question. Subsequent randomized studies performed by SWOG and by *Klastersky et al.* in Belgium failed to demonstrate a survival benefit for doses of cisplatin > 60 mg/m^2, while the high-dose regimens induced significantly more Grade III/IV toxicities [82][83]. These data do not support the use of high-dose cisplatin in advanced NSCLC, although as shall be described below, doses > 75 mg/m^2 continue to be routinely studied in combination therapy.

Early trials of carboplatin as a single-agent in NSCLC produced somewhat surprising results: a randomized ECOG trial showed that carboplatin therapy was associated with a survival advantage over combination regimens, but it produced an objective response rate of only 9% [84]. CALGB compared carboplatin and iproplatin in a randomized fashion in patients with metastatic NSCLC and observed a higher response rate and a 5 week median survival advantage in the carboplatin arm [85]. These results, in light of carboplatin's modest toxicity profile, generated interest in carboplatin-based combination regimens. The Belgian group compared cisplatin/VP-16 and carboplatin/VP-16 in a large Phase-III study and found that, while there were more responses in the cisplatin arm (27% *vs*. 16%), there was no difference in overall survival between the two combinations [86]. These results underscore the drawbacks inherent in the use of response rate as a primary endpoint in NSCLC trials: patient survival is better correlated with host factors such as performance status and gender (females do better than males), and the prognostic implications of tumor biology continue to be explored.

The development of several new agents with broad antitumor activity in the early 1990's has had a major impact on the treatment of NSCLC. The single-agent response rates observed with paclitaxel (24%), vinorelbine (27%), docetaxel (38%), gemcitabine (20%), and irinotecan (32%) in single-institution studies has led to a 'second generation' of combination regimens in NSCLC, as all of these agents can effectively be incorporated into platinum-based combinations [87–91]. The results of randomized trials involving these agents are presented in *Table 1*. The addition of paclitaxel,

Table 1. *Recent Randomized Trials of Platinum Regimens in Non-Small-Cell Lung Cancer*[a])

Study	Regimen	N	RR [%]	MS	Comment
ECOG 5592 [96]	CDDP (75 mg/m^2) VP-16 (100 mg/m^2 × 3)	194	12	7.6 months	p = 0.048 for survival when both paclitaxel-CDDP arms combined
	CDDP (75 mg/m^2) Paclitaxel (135 mg/m^2 over 24 h)	189	26.5	9.5 months	
	CDDP (75 mg/m^2) Paclitaxel (250 mg/m^2 over 3 h) with G-CSF support	191	32.1	10 months	
EORTC 08925 [97]	CDDP (80 mg/m^2) Teniposide (100 mg/m^2 × 3)	162	28	9.9 months	More grade III/IV heme toxicity in teniposide arm (p = 0.002). Quality-of-life scores favored paclitaxel arm
	CDDP (80 mg/m^2) Paclitaxel (175 mg/m^2 over 3h)	155	41	9.7 months	
Belani et al. [98]	CDDP (75 mg/m^2) VP-16 (100 mg/m^2 × 3)	179	14	NR	p = 0.059 for RR. Median survival 8.25 months for entire patient population.
	CBDCA (AUC 6) Paclitaxel (225 mg/m^2 over 3 h)	190	21.6	NR	
Le Chevalier et al. [99]	CDDP (120 mg/m^2) Vindesine (3 mg/m^2 weekly)	200	19	32 weeks	Survival and response rates superior (p = 0.04 and 0.02) for vinorelbine-CDDP over vindesine-CDDP.
	CDDP (120 mg/m^2) Vinorelbine (30 mg/m^2 weekly)	206	30	40 weeks	
	Vinorelbine (30 mg/m^2)	206	14	31 weeks	
Wozniak et al. [93]	CDDP (100 mg/m^2)	218	12	6 months	Significant survival advantage for vinorelbine-CDDP (p = 0.0018)
	CDDP (100 mg/m^2) Vinorelbine (25 mg/m^2 weekly)	214	26	8 months	
Sandler et al. [94]	CDDP (100 mg/m^2)	Interim analysis	9	7.6 months	p = 0.078 for survival. 30% of patients censored at time of report.
	CDDP (100 mg/m^2) Gemcitabine (1000 mg/m^2 × 3 weeks)	of 309 patients	31	8.7 months	

Table 1. (continued)

Study	Regimen	N	RR [%]	MS	Comment
Gatzemeier et al. [92]	CDDP (100 mg/m^2)	206	17	8.6 months	Quality-of-life scores superior in paclitaxel-CDDP group.
	CDDP (80 mg/m^2) Paclitaxel (175 mg/m^2 over 3 h)	202	26	8.1 months	
Von Pawel et al. [95]	CDDP (75 mg/m^2)	219	14	27.7 weeks	Significant survival advantage for tirapazamine-CDDP (p = 0.0047).
	CDDP (75 mg/m^2) Tirapazamine (390 mg/m^2 × 3 weeks)	218	28	34.6 weeks	

[a]) RR = response rate; MS = median survival; NR= not reported, CDDP = Cisplatin; CBDCA = low-dose carboplatin.

vinorelbine, gemcitabine, and the novel bioreductive agent tirapazamine to cisplatin has produced increments in response rate and survival over cisplatin alone in studies to date [92–95]. However, the superiority of these combinations over the previous 'standard' platinum-containing combinations in terms of survival has not yet been convincingly demonstrated. The ECOG found that the addition of paclitaxel at moderate dose (135 mg/m^2 over 24 h) and high dose (250 mg/m^2 with G-CSF support) to cisplatin resulted in a 2-month increment in median survival over patients treated with standard cisplatin and VP-16 [96]. Two randomized studies, one of the cisplatin/paclitaxel combination *vs.* standard cisplatin/teniposide and another comparing carboplatin/paclitaxel with cisplatin/VP-16 failed to demonstrate a survival advantage for the platinum/paclitaxel arms [97] [98]. However, the toxicity of cisplatin/paclitaxel was modest in comparison and was associated with superior quality-of-life indices in the EORTC study [97].

The large study reported by *Le Chevalier et al.* (512 patients) did demonstrate a significant improvement in survival with high-dose cisplatin and weekly vinorelbine when compared to cisplatin/vindesine or vinorelbine alone [99]. Because of their favorable toxicity profiles and at least comparable activity, these newer doublets have become the *de facto* standard of care for patients with advanced NSCLC in North America and Europe. The relevant question to be answered presently is whether one of these second generation combinations is superior: two large Phase-III studies in the ECOG (ongoing) and SWOG (recently completed accrual) will provide important data in this regard (*Table 2*).

The recognition that distant metastases were the most common type of recurrence in patients with early-stage NSCLC following resection prompted investigation of adjuvant chemotherapy. Early trials in the 1960's and 1970's with non-platinum-containing regimens were negative, while studies with platinum-based therapies produced mixed results. The Collaborative Group meta-analysis detected a small (5% at 5 years) overall survival benefit with the use of adjuvant platinum-based regimens that bordered on statistical significance ($p = 0.08$) [78]. No clear consensus regarding adjuvant therapy has emerged.

The superior response rate observed when cytotoxic agents are administered pre-operatively in a variety of tumors prompted studies of induction regimens in locally advanced (Stage IIIA/B) NSCLC. A landmark CALGB study reported a four month increase in median survival and a doubling in the number of three-year survivors with two cycles of cisplatin and vinblastine administered prior to radiotherapy *vs.* radiotherapy alone in patients with stage III disease [100]. Selection criteria caused some to question the applicability of these results to the majority with Stage III NSCLC, however, the benefits of platinum-based chemotherapy followed by radiotherapy in patients with locoregional disease were confirmed in subsequent studies by the RTOG/ECOG and *Le Chevalier*, as well as the meta-analysis [78][101][102].

Small randomized studies have also demonstrated a survival benefit to induction therapy with cisplatin-based chemotherapy followed by surgical

Table 2. *Ongoing Randomized Trials of Platinum-Based Regimens in Non-Small-Cell Lung Cancer*[a])

Study	Therapy
ECOG 1594	CDDP (75 mg/m^2 on day 2) Paclitaxel (135 mg/m^2 over 24 h on day 1)
	CDDP (100 mg/m^2 on day 1) Gemcitabine (1000 mg/ m^2 weekly × 3)
	CDDP (75 mg/m^2) Docetaxel (75 mg/m^2 × 3 weeks)
	CBDCA (AUC 6) Paclitaxel (225 mg/m^2 over 3 h)
SWOG 9509[b])	CDDP (100 mg/m^2 on day 1) Vinorelbine (25 mg/m^2 weekly)
	CBDCA (AUC 6) Paclitaxel (225 mg/m^2 over 3 h)

[a]) CDDP = Cisplatin; CBDCA = low-dose carboplatin.
[b]) Completed accrual in January, 1998.

resection [103–105]. The discovery that cisplatin and carboplatin can act as radiosensitizers has prompted investigation of concurrent chemoradiotherapy regimens, and the results of two recently reported studies by *Choy et al.* [106] and *Belani et al.* [107] of concurrent weekly carboplatin/paclitaxel and radiotherapy in locally advanced NSCLC are encouraging. The rather compelling evidence from trials to date has led to the routine use of platinum-based chemotherapy regimens as initial therapy in patients with Stage III NSCLC. The question remains as to the need for surgery following chemotherapy and radiation and this is being investigated by the RTOG and the EORTC in ongoing randomized trials.

Small-Cell Lung Cancer. The value of platinum agents in combination regimens is strikingly evident in SCLC, which displays chemosensitivity in both limited and extensive stages. While cisplatin was only modestly effective as a single-agent in SCLC trials from the late 1970's, it proved to be significantly active when combined with VP-16: reponse rates of 60-80% were reported in several Phase-II studies of untreated patients with extensive stage disease, and median survival approached 10 months [108–110]. Randomized studies from Japan and the SECSG failed to demonstrate superiority of cisplatin/VP-16 over standard CAV (cyclophosphamide, doxorubicin, vincristine) [111][112]. However the activity of EP in patients refractory to CAV and its applicability to combined-modality approaches in patients with limited stage disease led this to become the most favored regimen for SCLC in the late 1980's and early 1990's.

Carboplatin, in contrast to cisplatin, did demonstrate significant single-agent activity in SCLC, with response rates approaching 60% [113]. Phase II studies of the carboplatin/VP-16 combination showed response rates of 60–85% in extensive-disease patients, which approximated those previously reported with cisplatin/VP-16 [114–116]. The Hellenic Cooperative Oncology Group observed similar response rates and median survival with both combinations in their randomized study [114]. These results have led the carboplatin/VP-16 regimen to be considered the standard of care in SCLC for most oncologists. Other investigators have studied carboplatin/VP-16-based triplets, with the addition of ifosfamide and paclitaxel, and have reported favorable results at the expense of greater toxicity. There are no randomized data yet available for these combinations, and they, therefore, should be considered investigational [117][118]. The incorporation of newer agents with significant activity in SCLC such as irinotecan, docetaxel, gemcitabine, and topotecan into platinum-based combination regimens will likely be the focus of clinical studies in the near future.

Cancer of the Head and Neck

Unlike lung cancers, in which control of distant metastases with platinum-based chemotherapy has resulted in measurable improvements in survival, the therapeutic focus in head and neck carcinomas has been locoregional disease and its attendant morbidity.

Phase-II studies of single-agent cisplatin in recurrent and metastatic disease yielded response rates averaging 28% with no definitive impact on survival [119]. Trials with high-dose regimens suggested improvement in efficacy, however, *Veronesi et al.* found no benefit to high-dose (120 mg/m^2) over moderate-dose (60 mg/m^2) cisplatin in their randomized study [120]. Carboplatin also proved to have significant activity as a single-agent in recurrent head and neck cancers, yielding response rates of up to 30% with a favorable toxicity profile in comparison to cisplatin [121]. The incorporation of cisplatin into combination therapies generated excitement, as substantial numbers of complete responses could be demonstrated. *Kish et al.* [122] at Wayne State reported a 72% response rate (22% CRs) when cisplatin was administered at a dose of 100 mg/m^2 with 5-day continuous infusion 5-FU (PF). Randomized trials from the 1980's comparing the PF regimen to other combinations and single-agents generally indicated higher response rates with PF but no clear survival benefit [123–127].

The inability of chemotherapy to effect significant improvements in survival in a malignancy, where death as a result of distant disease is the exception rather than the rule, led to its application as primary therapy prior to definitive local treatment. The Head and Neck Contracts Program provided the first randomized test of platinum-based induction chemotherapy in their study, which began in the late 1970's [128]. Although this study showed no benefit to one cycle of induction treatment with cisplatin/bleomycin, it paved the way for well-designed studies of the PF regimen in the 1980's, when it became evident that organ preservation in laryngeal carcinoma was an important therapeutic objective. The Veterans Affairs Laryngeal Cancer Study Group reported a 62% organ-preservation rate in patients with stage III/IV disease following two to three cycles of PF. A significant reduction in distant metastases was noted in the chemotherapy group (11% *vs*. 17%), but there was no difference in overall survival noted between the two arms (68% at two years) [129]. Subsequent randomized studies confirmed these results [130–132], and Phase-II data suggested that three cycles of induction therapy induced more complete responses that one or two [133]. Thus, while substantial survival improvements were not observed, induction chemotherapy with PF followed by surgery or radiotherapy has become standard therapy for patients with Stage III/IV carcinomas of the larynx and hypopharynx.

Investigators have also attempted to exploit the synergistic interaction between platinum agents and radiotherapy through concurrent chemoradiotherapy protocols. Initial intergroup studies of single-agent cisplatin revealed significant improvements in response rate (73% *vs*. 59%) over radiotherapy alone but no survival benefit [134]. The suboptimal dose of cisplatin used in these studies may have precluded the detection of a survival benefit: the RTOG observed an improvement in survival (34% alive at four years) with concurrent high-dose cisplatin (100 mg/m^2) when compared to historical controls who received radiation alone [135].

Combination chemotherapy administered concurrent with radiation has produced the most promising results in advanced, unresectable disease. The important study of *Merlano et al.* randomized 157 patients to conventional radiotherapy *vs.* cisplatin/5-FU given concurrent with radiation in alternating weekly fashion. They reported a 3-year survival rate of 41% with concurrent therapy *vs*. 23% with radiation alone ($p < 0.05$) and 5-year survival rate of 24% *vs*. 10% ($p < 0.02$) [136]. *Taylor et al.* reported significantly improved disease-free survival rates in patients treated with concomitant cisplatin/5-FU and radiation over sequential therapy (17 months *vs*. 13 months, $p = 0.003$) in their study of 214 patients with unresectable disease [137].

Investigators in France reported their randomized results at the 1998 ASCO meeting using carboplatin-based chemoradiotherapy: 226 patients received radiation alone versus carboplatin (70 mg/m^2 daily × 4) and 96-hour 5-FU infusion every 21 days with radiation. Three-year survival was significantly prolonged in the chemoradiotherapy arm (51% *vs*. 31%, $p = 0.002$) [138]. A meta-analysis of 63 randomized studies of over 10,000 patients by *Bourhis*, *Pignon et al.* confirmed an absolute survival benefit of 8% for chemoradiotherapy in locally advanced head and neck cancer [139]. Concurrent platinum-based chemoradiotherapy has also become the standard of care for patients with nasopharyngeal carcinoma based on intergroup results first reported at ASCO in 1996 and updated in 1998. Patients who received concomitant cisplatin/radiation prior to resection had a surprising 83% 3-year survival compared to 45% with radiation alone [140]. Recent investigations have focused on the use of hyperfractionated radiation given concomitantly with chemotherapy, and *Wendt et al.* [141] in Munich recently reported a significant survival benefit when cisplatin and 5FU were administered with hyperfractionated radiotherapy in a randomized trial.

Newer platinum-based combinations have proven effective in Phase-II studies of patients with advanced disease. Carboplatin/paclitaxel (response rate 23%) and cisplatin/gemcitabine (response rate 24%) have figured prominently [142][143]. The carboplatin/paclitaxel combination appears remarkably active in the induction setting, as *Dang et al.* reported response rates of 95% [144], and also when given weekly with concurrent radiotherapy

(55% complete response rate) [145] in recently reported single-institution studies. Randomized results are awaited. Cisplatin also lends itself to combination with novel therapies, which is being studied in head and neck cancer. These include monoclonal antibodies directed to the epidermal growth factor receptor and ONYX-015, an E1B-attenuated adenovirus that is selectively toxic to cells containing mutated p53 [146–148].

Urothelial Cancer

Cisplatin has an integral role in the treatment of advanced transitional carcinomas of the bladder, and is included in all of the most active combination regimens that have been identified to date. The present standard of care is the M-VAC regimen (methotrexate, vinblastine, doxorubicin, and cisplatin), developed at Memorial in the 1980's [149]. A response rate of 69% (37% CR) and three-year survival rate of 55% was reported by *Sternberg et al.* in their initial experience with 83 patients with advanced disease. Randomized comparisons of M-VAC to single-agent cisplatin and CISCA (cisplatin, cyclophosphamide, and doxorubicin) showed M-VAC to be superior in terms of response rate and median survival [150][151]. Remarkably, identical median survivals of 12 months were observed with M-VAC therapy in these two studies. The CMV regimen (cisplatin, methotrexate, and vinblastine), developed in the early 1980's, is also commonly employed. *Harker et al.* [152] reported a 56% response rate (28% CR) and median survival of eight months from their single-institution trial. There has been no randomized comparison of M-VAC and CMV. The choice of regimen is left to the treating oncologist. The Memorial group recently analyzed their experience with M-VAC and found that the presence of visceral metastases and a *Karnofsky* performance status < 80% were independently associated with poorer survival [153]. Dose intensification has not proven beneficial [154].

The routine use of cisplatin-based combinations in the adjuvant setting following cystectomy for patients with muscle-invasive disease is the subject of some controversy. *Skinner et al.* at USC reported a significant improvement in time to progression with adjuvant CISCA *vs*. no therapy and median survival favored the CISCA arm (4.3 years *vs*. 2.4 years, $p = 0.0062$) in a small study of 91 patients [155]. *Stockle et al.* [156] in Germany did observe a survival benefit with adjuvant M-VAC (or M-VEC, with epirubicin), however, their conclusion has been questioned based on their trial design and lack of intent-to-treat analysis. A third positive trial was reported from Stanford, where *Freiha et al.* found that four cycles of adjuvant CMV significantly improved freedom from progression (37 months *vs*.

12 months, p = 0.01) [157]. Survival was almost doubled in the treatment group, yet the difference was not significant. The fact that patients in the observation arm received CMV at relapse was felt to account for this lack of significance. Viewed in a different way, however, this result suggests that systemic therapy at the time of progression may be a reasonable and equivalent option. These underpowered studies allow no definitive conclusion to the hypothesis that adjuvant chemotherapy benefits patients with muscle-invasive bladder cancer.

Neoadjuvant therapy with platinum-based regimens prior to cystectomy or bladder-conserving surgery in node-negative disease can produce 30% complete pathologic responses, yet no randomized study to date has demonstrated a survival advantage. Neoadjuvant and adjuvant combined-modality approaches have proven of value in highly selected patients who are candidates for bladder preservation. Five-year survival rates of 40% have been reported [158-160]. The import of molecular markers such as *p53* and *Rb* gene mutations are being explored to refine the selection process of patients who may be better served by conservative approaches [161][162].

Carboplatin in combination with paclitaxel has shown activity in recently reported phase II trials. *Vaughn et al.* [163] at the University of Pennsylvania reported a 50% response rate in previously untreated patients with advanced disease, while *Droz et al.* [164] observed responses in 14 of 38 patients (2 CRs). Randomized comparisons of this combination with M-VAC are expected. *Kaufman et al.* [165] reported promising results with the cisplatin/gemcitabine combination at ASCO in 1998: a 66% response rate (13 CRs) was observed in their Phase-II study of 47 patients.

Other Cancers

Upper Gastrointestinal-Tract Cancer. Cisplatin is a mainstay of therapy for squamous-cell carcinomas and adenocarcinomas of the esophagus. Response rates of up to 40% were reported with cisplatin alone in patients with advanced disease, and the EORTC noted a doubling of the response rate (35% *vs*. 19%) when cisplatin was administered with 5-FU by continuous infusion *vs*. cisplatin alone [166–168]. Although similar survival rates were noted in the randomized EORTC study, the improvement in response rate with combination therapy suggested that an impact might be made in earlier stages of disease. Phase-II studies of preoperative cisplatin/5-FU in patients with squamous-cell histology showed responses in 42–66% with complete response rates of up to 10% [169][170].

Esophageal adenocarcinomas do not appear to be as responsive [171]. *Ajani et al.* [172] have studies neoadjuvant EAP (VP-16, doxorubicin, and cisplatin) for patients with adenocarcinomas of the distal esophagus and gastroesophageal junction and observed a 42% response rate: 78% of treated patients underwent curative resection and overall median survival was 23 months.

Randomized studies comparing primary chemotherapy followed by surgery to surgery alone have failed to demonstrate a survival advantage with neoadjuvant therapy [173][174]. Combined-modality treatment with platinum-based regimens such as cisplatin/5-FU administered concurrently with radiation was superior when compared to radiation alone in an important study conducted by the GI intergroup. Median survival was over 4 months longer in the combined-modality arm [175][176]. It has yet to be proven that surgical resection following chemotherapy and radiation is of added benefit. Median survival rates from Phase-II studies of chemoradiation followed by resection are not clearly superior. Randomized data are awaited.

The impressive activity of paclitaxel has led to its incorporation into combination regimens with cisplatin, cisplatin/5-FU, and carboplatin/5-FU in combined modality protocols. *Safran* and colleagues observed a response rate of 71% (26% CRs) with weekly cisplatin/paclitaxel given concurrent with radiation, followed by surgical resection [177]. The group at the Sarah Cannon Cancer Center in Nashville administered carboplatin, paclitaxel, and continuous infusion 5-FU with radiation and observed responses in 80% of patients with an impressive 50% pathologic complete response rate and 62% 1-year survival [178]. Whether carboplatin is comparable to cisplatin in combined modality approaches remains to be seen, although early data suggest that this may be so.

Cisplatin is also commonly employed in combination therapy for gastric cancer. Cisplatin-based combinations including cisplatin/5-FU, FAP (5-FU, doxorubicin, cisplatin), ECF (epirubicin, cisplatin, 5-FU), and EAP (VP-16, doxorubicin, cisplatin) have been thoroughly investigated. Response rates up to 60% with complete responses in 15–20% have been reported in studies involving patients with advanced disease [179–182]. Randomized trials comparing the various regimens have produced no consensus, although the recent study reported by *Webb at al.* from the Royal Marsden suggests that the ECF regimen might be considered the standard of care. ECF was associated with a significant survival advantage when compared to FAM-TX (8.9 months vs. 5.7 months, $p = 0.0009$) [183].

Neoadjuvant platinum-based approaches have shown promise in several Phase-II studies. *Ajani et al.* [184] found that 77% of patients who received three cycles of preoperative EAP underwent potentially curative resections, with a median survival of 16 months. *Leichman et al.* at USC re-

ported a 76% resection rate and median survival over 17 months with two cycles of neoadjuvant 5-FU, leucovorin, and cisplatin followed by intraperitoneal FUdR and cisplatin [185]. The results of several neoadjuvant studies suggest that primary chemotherapy does not add to operative morbidity. Data from randomized trials and further studies of adjuvant intraperitoneal chemotherapy should help define optimal approaches to the peri-operative management of patients with potentially resectable gastric cancer.

Cervical and Endometrial Cancer. Cisplatin is one of the most active agents available for the treatment of squamous-cell cancers of the cervix. Single-agent trials have indicated response rates of up to 31% in patients with advanced disease, with nearly one-third being complete responses [186]. The GOG reported the results of a large (394 patients) randomized trial in 1989 comparing carboplatin and iproplatin monotherapy and noted a 15% response rate with carboplatin, suggesting that its activity may not be comparable to cisplatin in cervical cancer [187].

Marked increments in response were observed with the incorporation of cisplatin into combination regimens; response rates greater that 50% were reported in Phase-III studies of cisplatin/ifosfamide, cisplatin/ifosfamide/bleomycin, and cisplatin/5-FU [188–190]. Randomized trials of these combinations administered prior to radiotherapy in locally advanced disease have not shown a survival advantage; however, a recent GOG trial of concurrent cisplatin or cisplatin/5-FU/hydroxyurea and radiation was associated with significantly improved progression-free survival versus concurrent hydroxyurea and radiation in patients with Stage IIB–IVA cervical cancers [191]. Combined cisplatin and paclitaxel produced responses in 9 of 11 patients in a recent GOG study [192] and will be the focus of larger trials in the future.

Phase-II trials have suggested that cisplatin and carboplatin have comparable activity as single-agents in the treatment of advanced endometrial carcinoma, and produce responses in up to 30% in previously untreated patients [193][194]. Platinum-based combinations such as AP (doxorubicin, cisplatin), PAC (cisplatin, doxorubicin, cyclophosphamide), APV (doxorubicin, cisplatin, vinblastine), and M-VAC have been associated with improved response rates, yet there is presently no randomized data comparing these various regimens to single-agent therapy [195-198]. The GOG is presently comparing circadian administration of AP to standard dosing. The addition of paclitaxel to combination regimens will likely have some impact. *Lissoni et al.* [199] observed responses in 11 of 13 patients treated with cisplatin, epirubicin, and paclitaxel in a study reported at ASCO in 1998.

Osteogenic Sarcoma. Cisplatin figures prominently in the treatment of primary bone sarcomas. Single-agent response rates approximate 30% in

advanced osteosacomas [200]. Adjuvant studies of cisplatin-containing combinations have suggested that cure rates of up to 60% are possible following resection, however, randomized data are lacking to support this approach [201][202]. Neoadjuvant therapy came to the fore as limb-sparing surgical approaches were developed in the 1970's. Early experiences at Memorial with the T10 regimen suggested that tumor response at the time of resection following primary chemotherapy could be used to determine the choice of agents post-operatively [203]. Responding patients with good prognostic features continued with the same therapy as was administered pre-operatively, while poor responders received doxorubicin and high-dose cisplatin (120 mg/m^2) as adjuvant treatment. Initial results reported by *Rosen* and co-workers [204] were promising, however long-term follow-up did not support the concept of tailoring therapy based on response, as disease-free survival rates in poor-risk patients was not improved.

What has become clear is the fact that neoadjuvant chemotherapy applied to patients with extremity osteosarcomas can allow limb-sparing surgery to be performed on patients who may otherwise have undergone amputation. The groups at the Rizzoli Institute and MD Anderson employed the use of intra-arterial administration of cisplatin to the tumor as a way of maximizing drug delivery [205][206]. While more favorable responses were observed with intra-arterial dosing, disease-free and overall survival and limb-salvage rates were similar to those reported in studies of intravenous administration [207][208]. Thus, intra-arterial cisplatin therapy remains investigational in osteosarcomas, and has fallen out of favor to some degree.

The ever-increasing use of bone marrow and peripheral blood-stem cell rescue following dose-intense therapy has allowed investigators to study high-dose regimens in osteosarcoma patients with advanced disease. *Patel et al.* [209] at MD Anderson reported that 13 of 15 patients treated with cisplatin (120 mg/m^2), ifosfamide (10 gm/m^2), and doxorubicin (75 mg/m^2) every 28 days for three cycles with peripheral blood-stem-cell support were able to undergo resection with an attempt at cure. Ten of 13 had over 75% tumor necrosis at surgery indicating a favorable response to high-dose therapy: further results are awaited. *Weigel et al.* added carboplatin (400 mg/m^2) to ifosfamide and etoposide and observed complete responses in 3 of 5 patients with relapsed disease as well as one partial response in a small study that produced interesting results [210].

Clinical Pharmacology

Differences in toxicity and in specificity for particular tumor types are influenced by the cellular pharmacology and by the pharmacokinetic char-

acteristics of the various platinum drugs. These variables determine the clinical role of the drugs for the treatment of various diseases. In turn, more than for any other drug class, an understanding of the pharmacology of these agents has influenced the clinical use of platinum compounds.

Clinical Administration

Cisplatin is administered in a chloride-containing solution IV over 0.5 to 2 h. To minimize the risk of nephrotoxicity, patients are prehydrated with at least 500 ml of salt-containing fluid. Immediately before cisplatin administration, mannitol is given parenterally to maximize urine flow, along with parenteral anti-emetics. A minimum of 1 liter of post-hydration fluid is usually given [1]. The intensity of hydration varies somewhat with the dose of cisplatin. High-dose cisplatin (up to 200 mg/m^2/course) may be administered in a formulation containing 3% sodium chloride, but the indications for this therapy are not well-established [211]. This onerous method of treatment over 3–6 hours is burdensome for clinical resources and tiring for cancer patients. Previously given as in-hospital treatment, it is now usually administered in the outpatient setting. The exigencies of the modern healthcare environment have contributed to the expanding use of carboplatin as an alternative to cisplatin except in circumstances where cisplatin is clearly the superior agent.

Cisplatin may also be administered regionally to increase local drug exposure and diminish side effects. Its intraperitoneal use was defined by *Ozols et al.* and by *Howell et al.* [212][213]. Measured drug exposure in the peritoneal cavity is some 50-fold higher as compared to levels achieved with i.v. administration [213]. At standard doses in ovarian cancer patients with low-volume disease, a randomized intergroup trial suggests that intraperitoneal administration is superior to intravenous cisplatin in combination with IV cyclophosphamide [214]. Used at doses of 100 or 200 mg/m^2, the regimen for hydration and premedication is identical to that described above for intravenous therapy. The development of combinations of carboplatin with paclitaxel has, however, superseded this technique in ovarian cancer, and the intraperitoneal route is now infrequently used. Regional use also includes intraarterial delivery (as for hepatic tumors, melanoma and glioblastoma), but none has been adopted as a standard method of treatment.

Carboplatin is substantially simpler to administer. Extensive hydration is not required because of the lack of nephrotoxicity at standard doses [215]. Carboplatin is reconstituted in chloride-free solutions (chloride can displace the leaving groups), and administered over 30 minutes as a rapid intravenous infusion. Carboplatin has been incorporated in high-dose chemo-

therapy regimens at doses over 3-fold higher than those of the standard regimens [216]. In some regimens, continuous infusion has been substituted for a rapid intravenous infusion. It is doubtful that there is an advantage for this approach: carboplatin doses up to 20 mg · min/ml may be safely administered in 200 ml of D5W over 2 h [217].

Oxaliplatin is also uncomplicated in its clinical administration. For bolus administration of oxaliplatin, the required dose is administered in 500 ml of D5W as a short infusion. A five-daily dose regimen is most commonly used. In studies in colorectal cancer, oxaliplatin has been administered as a 5-day continuous infusion, during which the dosage rate has been modified to observe principles of chronopharmacologic administration [218]. Using programmable pumps, a sinusoidal-shaped infusion-rate curve was used to maximize the infusion rate at 4 p.m. The evidence to favor schedule dependence of oxaliplatin is limited, and additional studies will be required to determine if the chronopharmacologic approach is a necessary component of drug activity.

Side-Effects/Scheduling

A substantial body of literature documents the side effects of platinum compounds. The nephrotoxicity of the parent compound cisplatin almost led to its abandonment, until *Cvitkovic et al.* introduced aggressive hydration, which prevented the development of acute renal failure [2][3]. As noted above, the toxicity of cisplatin was a driving force both in the search for less toxic analogues and for more effective treatments for its side effects, especially nausea and vomiting.

Cisplatin. The side effects associated with cisplatin (at single doses ≥ 50 mg/m^2) include nausea and vomiting, nephrotoxicity, ototoxicity, neuropathy, and myelosuppression. Rare effects include visual impairment, seizures, arrhythmias, acute ischemic vascular events, glucose intolerance, and pancreatitis [1]. The nausea and vomiting stimulated a search for new anti-emetics. It is currently best managed with 5-HT$_3$ antagonists usually given with a glucocorticoid, though other combinations of agents are still widely used. In the weeks after treatment, continuous anti-emetic therapy may be required. Nephrotoxicity is ameliorated but not completely prevented by hydration. The renal damage to both glomeruli and tubules is cumulative, and after cisplatin treatment, the serum creatinine is no longer a reliable guide to the glomerular filtration rate. Acute elevation of serum creatinine may follow a cisplatin dose, but this index returns to normal with time. Tubule damage may be reflected in a salt-losing syndrome that resolves with time.

Ototoxicity is also a cumulative and irreversible side effect of cisplatin treatment that results from damage to the inner ear. Therefore, audiograms are recommended every 2 to 3 cycles [1]. The initial audiographic manifestation is loss of high-frequency acuity (4000 to 8000 Hz). When acuity is affected in the range of speech, cisplatin should be discontinued under most circumstances and carboplatin substituted where appropriate. Peripheral neuropathy is also cumulative, though less common than with agents such as vinca alkaloids. This neuropathy is usually reversible, though recovery is often slow. A number of agents with the potential for protection from neuropathy have been developed, but none is yet used widely [219].

Carboplatin. Myelosuppression, which is not usually severe with cisplatin, is the dose-limiting toxicity of carboplatin [215]. The drug is most toxic to the platelet precursors, but neutropenia and anemia are frequently observed. The lowest platelet counts following a single-dose of carboplatin are observed 17 to 21 days later, and recovery usually occurs by day 28. The effect is dose-dependent, but individuals vary widely in their susceptibility. As shown by *Egorin et al.* [220] and *Calvert et al.* [221], the severity of platelet toxicity is best accounted for by a measure of the drug exposure in an individual, the area-under-the-concentration-time curve (AUC). Both groups derived pharmacologically-based formulas to predict toxicity and guide carboplatin dosing. That of *Calvert et al.* targets a particular exposure to carboplatin:

$$\text{Dose [mg]} = \text{Target AUC [mg} \cdot \text{min/ml]} \times (\text{GFR [ml/min]} + 25)$$

This formula has been widely used to individualize carboplatin dosing and permits targeting an acceptable level of toxicity. Patients who are elderly or have a poor performance status or a history of extensive pretreatment have a higher risk of toxicity even when the dose is calculated based on these methods [220][221], but the safety of drug administration has been enhanced. In the combination of carboplatin and paclitaxel, AUC-based dosing has helped to maximize the dose intensity of carboplatin [222]. Doses some 30% higher than a dosing strategy based solely on body surface area may safely be used. Determination of whether this approach to dosing improves outcome requires a randomized trial, which is in progress.

The other toxicities of carboplatin are generally milder and better tolerated than those of cisplatin. Nausea and vomiting, though frequent, is less severe, shorter in duration, and more easily controlled with standard antiemetics (for example compazine, dexamethasone, lorazepam) than that following cisplatin treatment. Renal impairment is infrequent, though alopecia is common, especially with the paclitaxel-containing combinations. Neu-

rotoxicity is also less common than with cisplatin, though with the increasing use of high-dose regimens, it is observed more frequently. Ototoxicity is also less common. As might be anticipated, the incidence of neurologic findings appears to be cumulative, and patients receiving higher doses should be followed carefully.

Oxaliplatin. Oxaliplatin also has less nephrotoxicity than cisplatin, presumably related to its more slowly hydrolyzed leaving group. The dose-limiting toxicity of oxaliplatin is sensory neuropathy, a characteristic of all DACH-containing platinum derivatives. The severity of the toxicity is dramatically less than that observed with another DACH-containing analogue, ormaplatin. This side effect takes two forms. First, a tingling of the extremities, that may even involve the perioral region, which occurs early and usually resolves within a few days. With repeated dosing, symptoms may last longer between cycles, but do not appear to be of long duration or cumulative. A second neuropathy, more typical of that seen with cisplatin, affects the extremities and increases to affect about 10% of patients with repeated doses. This effect resolves over 3–4 months in the majority of patients. Ototoxicity is not observed with oxaliplatin. Nausea and vomiting do occur and generally respond to $5HT_3$ antagonists. Myelosuppression is uncommon and is not severe with oxaliplatin as a single-agent, but it is a feature of combinations including this drug.

Pharmacokinetics

The major therapeutic target of platinum-coordination compounds is DNA, but all bind to a greater or lesser extent to many macromolecules. Plasma protein binding is extensive, and its degree is influenced by the composition of the leaving group. In addition to being reversibly bound by electrostatic forces, platinum drugs become covalently bound to proteins and a proportion of an administered dose is eliminated only as the macromolecules themselves turn over. Thus, for most of the analogues, it is important to measure bioavailable drug in plasma ultrafiltrate.

Interpretation of pharmacokinetic data is also complicated by biotransformation processes. Cisplatin is metabolized to various aquated species and in the low-chloride intracellular environment these predominate. Platinum(IV) compounds are converted rapidly to platinum(II) derivatives in plasma, and multiple distinct circulating molecular species may be produced [223]. While some HPLC assays may distinguish among metabolites, sensitivity often limits resolution of the various molecular species. Pharmacokinetic data should be interpreted accordingly. Recently it has been possible

using LC-MS to simultaneously quantitate metabolites of JM216 *in vivo* [224] and this technology should be availed of where possible with new platinum agents. As with toxicity, the pharmacokinetic behavior of analogues appears to be determined in large part by the structure of the leaving group. Agents with less easily displaced groups (*e.g.* cyclobutanedicarboxylates) have lower plasma protein binding, longer plasma half-lives, and greater renal excretion. These agents must be dosed carefully in patients with compromised renal function.

Pharmacodynamics

Pharmacodynamics relates pharmacokinetic indices of drug exposure to biological measures of drug effect, usually toxicity to normal tissues or tumor cell kill. Toxicity to normal tissues can be quantitated as a continuous variable when the drug is myelosuppresive. Thus, the early studies of carboplatin demonstrated a close relationship of changes in platelet counts to the area under the concentration-time curve in the individual. The AUC was itself closely related to renal function determined as creatinine clearance. Based on these observations, *Egorin et al.* [220] and *Calvert et al.* [221] derived formulas based on creatinine clearance to predict either the percent change in platelet count, or a target AUC. Application of *Calvert*'s formula has been widely adopted as a means of avoiding overdosage (by producing acceptable nadir platelet counts) and of maximizing dose-intensity in the individual. *Egorin et al.* have quantitated the effects of combinations of drugs on carboplatin pharmacodynamics. They have shown that interactions are complex and require extensive simultaneous pharmacokinetic measures. These studies are continuing to pursue the goal of using pharmacodynamic measures to optimize treatment. A key question, however, is whether maximizing carboplatin exposure in each individual can measurably increase the probability of tumor shrinkage or of survival.

There is much support in the literature that dose-intensity is a powerful determinant of treatment outcome. In many analyses higher doses result in higher response rates. This hypothesis underlies high-dose chemotherapy approaches to some patients with metastatic disease. In an indirect approach to this issue, *Egorin et al.* [225] analyzed a trial of cyclophosphamide and carboplatin in a cohort of over 200 patients with ovarian cancer. While toxicity was clearly related to the delivered calculated AUC of carboplatin, response rates were not clearly related to this index. In this study, patients were administered a relatively small range of AUC's. For this and a number of other reasons a relationship may have been obscured. A more rigorous study will compare standard dosing of carboplatin to AUC-based dosing.

A concern with AUC-targeting based on renal function surrounds the measurement of creatinine clearance. The formulas of *Calvert et al.* were developed using EDTA clearance, measurement of which is not widely available. They have shown that neither standard measured creatinine clearance, nor the calculation of this index are as accurate or as reproducible. To circumvent this difficulty an alternative dosing strategy has been developed by *Chatelut*, *Canal* and co-workers [226]. This dosing approach is being tested in clinical trials.

Cellular Pharmacodynamics

The pharmacodynamic understanding of platinum-drug action may also be approached by investigating the cellular pharmacology of these agents [223]. Platinum compounds form numerous DNA adducts as discussed extensively elsewhere in this volume. The formation and repair of these adducts in human cells are not easily measured. One approach is to measure specific adducts (using antibody-based assays), another is to measure total platinum bound to DNA. The formation and repair of DNA-platinum adducts has been studied in white blood cells obtained from various groups of patients [226][227]. In a cohort of patients with ovarian cancer treated with cisplatin-containing chemotherapy, responders had higher peak platinum-DNA adduct levels than non-responders, though substantial overlap existed among the groups [228][229]. *Schellens* and co-workers [230] have re-evaluated the pharmacokinetic-pharmacodynamic interactions of cisplatin as a single agent in a series of recent studies. They found that white blood cell platinum-DNA adducts could be readily quantitated in the 24 h following the administration of 70–80 mg/m^2 of cisplatin. Peak adduct levels were found at the end of the infusion, and declined over the subsequent 18 h. In a series of patients with head and neck cancer, they found that cisplatin exposure (measured as the AUC) was closely correlated with both the peak DNA-adduct content ($r = 0.073$) and the area under the DNA-adduct time curve ($r = 0.78$) [230]. These three measures were important predictors of response, both individually and in logistic regression analysis. This group has now embarked upon an adaptive dosing study in which the dose of cisplatin will be escalated to tolerance in patients with low AUC or DNA-adduct levels [231].

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Part 3. How Does it Possibly Work? – Biochemistry

The Response of Cellular Proteins to Cisplatin-Damaged DNA

Deborah B. Zamble and *Stephen J. Lippard*

The Mechanism of Action of Cisplatin: From Adducts to Apoptosis

Alan Eastman

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Cisplatin. Edited by Bernhard Lippert

The Response of Cellular Proteins to Cisplatin-Damaged DNA

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The anticancer drug cisplatin forms a variety of covalent DNA adducts. The consequences of this DNA damage are mediated by proteins which either bind to the cisplatin-DNA cross-links or influence cellular pathways in response to the genotoxic stress. In either case, these proteins can regulate the processing of the cisplatin lesions and thereby affect cellular sensitivity to the drug. Identification of these proteins and exploration of their cellular functions has implicated multiple systems including several classes of DNA repair, transcription, cell cycle and cell death responses. Complete knowledge of how cisplatin-DNA adducts affect the components of these pathways will provide a basis for understanding the cisplatin mechanism of action.

Introduction

The chemotherapeutic agent *cis*-diamminedichloroplatinum(II), *cis*-DDP, or cisplatin, can form covalent adducts with many cellular macromolecules, but there is convincing evidence that its cytotoxic properties are a consequence of bifunctional-DNA adduct formation [1][2]. Platinum binds to the N(7) position of purine nucleotides, resulting predominantly in 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links, but also in 1,3-d(GpNpG) intrastrand, interstrand and protein-DNA cross-links [3][4]. The 1,2-intrastrand cross-links, which comprise 90% of the DNA adducts, are not formed by the clinically inactive *trans*-DDP because of geometric constraints, and attention has therefore focused on these adducts as the active lesions in the anticancer activity of the drug.

Numerous studies, carried out to determine how ligation of platinum affects the conformation and stability of normal B-DNA, revealed significant destabilization of the double helix (reviewed in [5]; see also [6]). More detailed information has been provided by recent structural analyses of the

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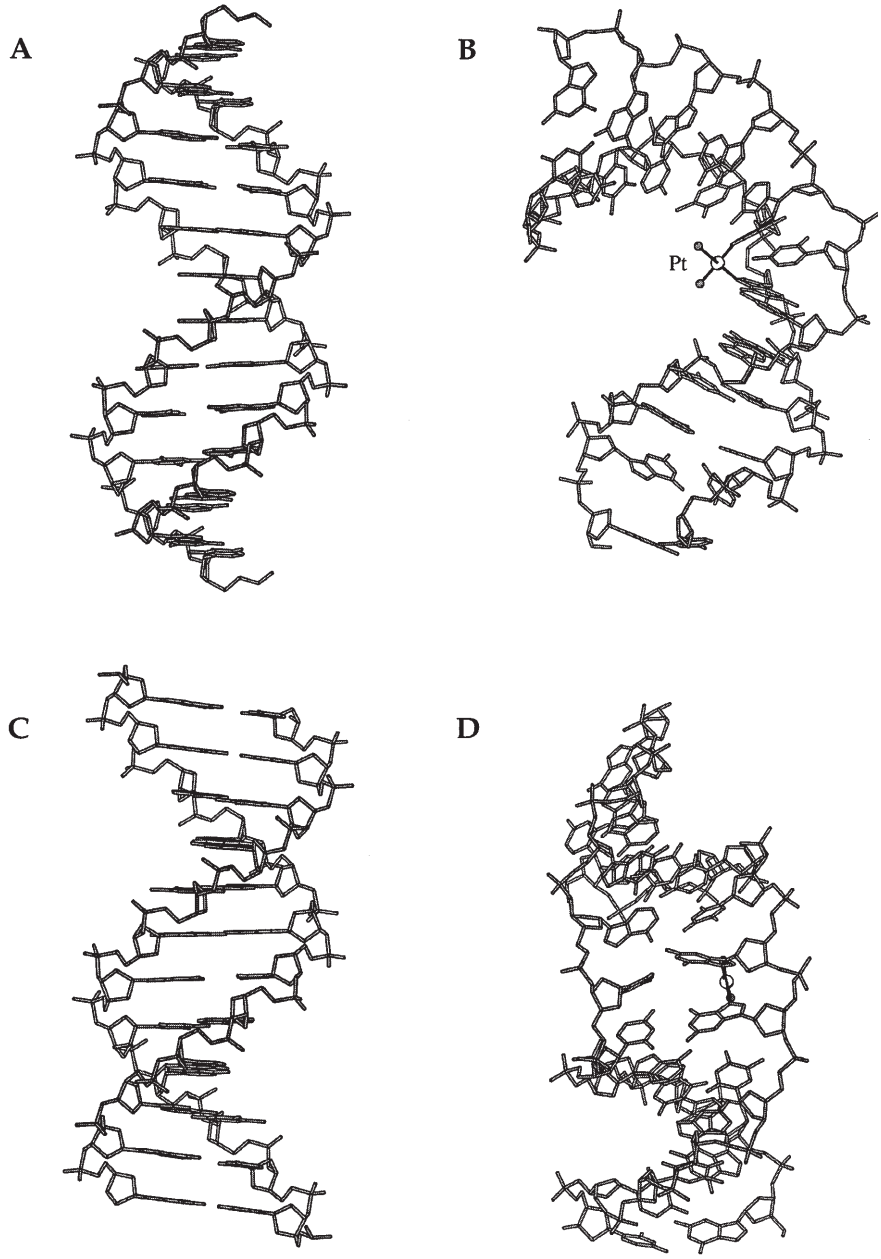

Fig. 1. *DNA Distortions caused by a 1,2-d(GpG)-cisplatin intrastrand adduct in a double-stranded deoxyoligonucleotide with the sequence d(CCTCTG*G*TCTCC).* A) Major groove of normal B-DNA. B) DNA bend caused by the cisplatin adduct. C) Minor groove of normal B-DNA. D) Widening of the minor groove by the cisplatin adduct.

1,2-d(GpG) intrastrand cross-link in duplex DNA (*Fig. 1*), performed both by X-ray crystallography [7][8] and NMR spectroscopy [9–11]. Although these structures differ in some details, they all reveal that the platinum induces a roll of 26°–50° between the guanine bases involved in the cross-link, displacement of platinum from the planes of the guanine rings, and a global bend of the helix axis towards the major groove. In addition, hydrogen-bonding within the 5′-coordinated GC base pair is severely perturbed, resulting in enhanced solvent accessibility. The cisplatin cross-link also unwinds the duplex and induces a widening and flattening of the minor groove in the vicinity of the adduct. These structural features strikingly resemble those observed in some DNA-protein complexes [12], the consequences of which will be discussed below.

NMR solution structures of two cisplatin-DNA decamers containing interstrand cross-links demonstrated that this minor adduct also has characteristic structural features [13][14]. In particular, the platinum atom lies in the minor groove, the complementary cytosines are extrahelical, and there is a switch in the double helix to a left-handed form localized at the site of the adduct. These distortions bend the helix axis towards the minor groove by approximately 20°–40° and unwind the duplex by 76°–80°. More detailed descriptions of Pt-DNA complexes may be found in other chapters in this volume.

The unusual conformations at the sites of cisplatin-DNA adducts suggest that they might serve as recognition elements for proteins that bind to and process damaged DNA. Studies with cell-free extracts, designed to test this hypothesis, demonstrated the presence of factors that bind DNA modified with cisplatin but not *trans*-DDP [15–17]. Subsequent gel mobility shift assays revealed the binding activity of one of these proteins to be specific for the 1,2-intrastrand cross-links [15]. The possibility that these cisplatin-damage recognition proteins play a role in the cytotoxic mechanism of the drug was supported by studies revealing that some proteins in this class are overexpressed in cell lines having acquired cisplatin resistance [16][18–20]. In addition, several damage recognition proteins could be induced in cultured cells exposed to cisplatin [19][21].

The purpose of this chapter is to review the types of proteins that interact with or respond to cisplatin-damaged DNA, and to discuss how these proteins can modulate cellular sensitivity to the drug through their participation in various biochemical pathways (*Fig. 2*). Included are proteins dedicated to dealing with genotoxic stress, such as components of the DNA repair and p53-regulated pathways, but also proteins affected by the DNA structural distortions induced by cisplatin adduct formation, such as transcription factors and architectural proteins. The manner by which such proteins affect the processing of cisplatin-DNA adducts can determine wheth-

er a cell attempts to repair the damage or activates an irreversible cell death program. These proteins most likely contribute to the anticancer activity of this drug. Moreover, differential protein expression and activity may explain why certain types of tumors, such as testicular, ovarian, head, and neck, are successfully treated by cisplatin whereas others are resistant [22][23]. Because many factors are involved in the mechanisms of action and resistance, we make no attempt to cover this field comprehensively. Topics beyond the scope of this review include oncogene activation, replication bypass and mutagenesis, reactions with sulfur-containing molecules, and mechanisms of drug uptake and export. For additional information, the reader is directed to other review articles (Chapters by *Eastman*, *Villiani et al.*, and *Reedijk* and *Teuben* in this book as well as [24–26]).

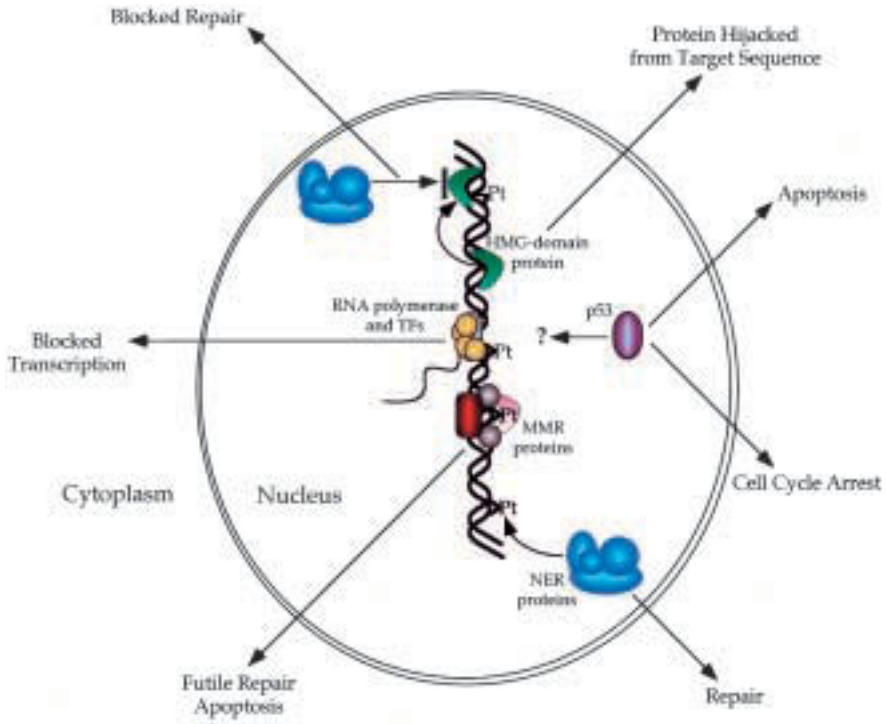

Fig. 2. *Effects of cisplatin-DNA adducts on some of the proteins in the nucleus that interact with the lesions*

DNA Repair Pathways

Nucleotide Excision Repair

There are numerous sources of DNA damage in the environment, including both naturally and artificially introduced substances. To limit genetic mutations and prevent the ensuing malignant transformation that might arise from exposure to such agents, there are a variety of cellular defense mechanisms which remove lesions from DNA and correct any unwanted changes. One of the most versatile systems is nucleotide excision repair (NER). In this pathway, many types of DNA lesions are excised as a small, single-stranded oligonucleotide fragment, and new DNA is synthesized to fill the resulting gap (*Fig. 3*; for reviews see [27][28]). The study of NER has been facilitated by several genetic diseases associated with DNA repair deficiencies including *xeroderma pigmentosum* (XP), an inherited disorder characterized by unusual sensitivity to sunlight and a predisposition to neurological abnormalities and cancer [29]. Most of the essential mammalian excision repair factors have been cloned by complementation studies with such human XP (XPA-XPG) or rodent (ERCC1-ERCC11) mutant cell lines. Experiments with purified proteins demonstrated that 14–16 polypeptides are necessary to reconstitute fully the dual incision activity of the excinuclease (*Table 1*) [30–32].

Recognition of DNA Damage by NER Proteins

If cisplatin-DNA adducts are substrates for NER, they should be recognized specifically by proteins involved in the first, and possibly rate-limiting, damage recognition step [27]. The zinc-finger protein XPA plays a central role in sensing genetic damage and recruiting the excinuclease to the site of the lesion [33]. Gel mobility shift and nitrocellulose binding assays demonstrated that XPA binds to cisplatin-modified DNA, although the affinity for the damaged over undamaged DNA was fairly modest, only a factor of 5 or less [34–36]. Another essential component of the excision nuclease, the human single-stranded binding protein RPA, was detected in a complex with cisplatin-modified DNA isolated from cell extracts [37][38]. XPA binds tightly to RPA *in vitro* [27], so it is likely that together they recognize DNA damage with an increased specificity of binding. Recent work with purified repair factors, however, did not detect specific binding of the two proteins to a damaged substrate [39]. Rather, it was suggested that XPA/RPA may bind DNA lesions weakly, and that recruitment of TFIIH and XPC, as well as ATP-dependent DNA-unwinding, are all required to form the first stable complex in the NER pathway [39][40].

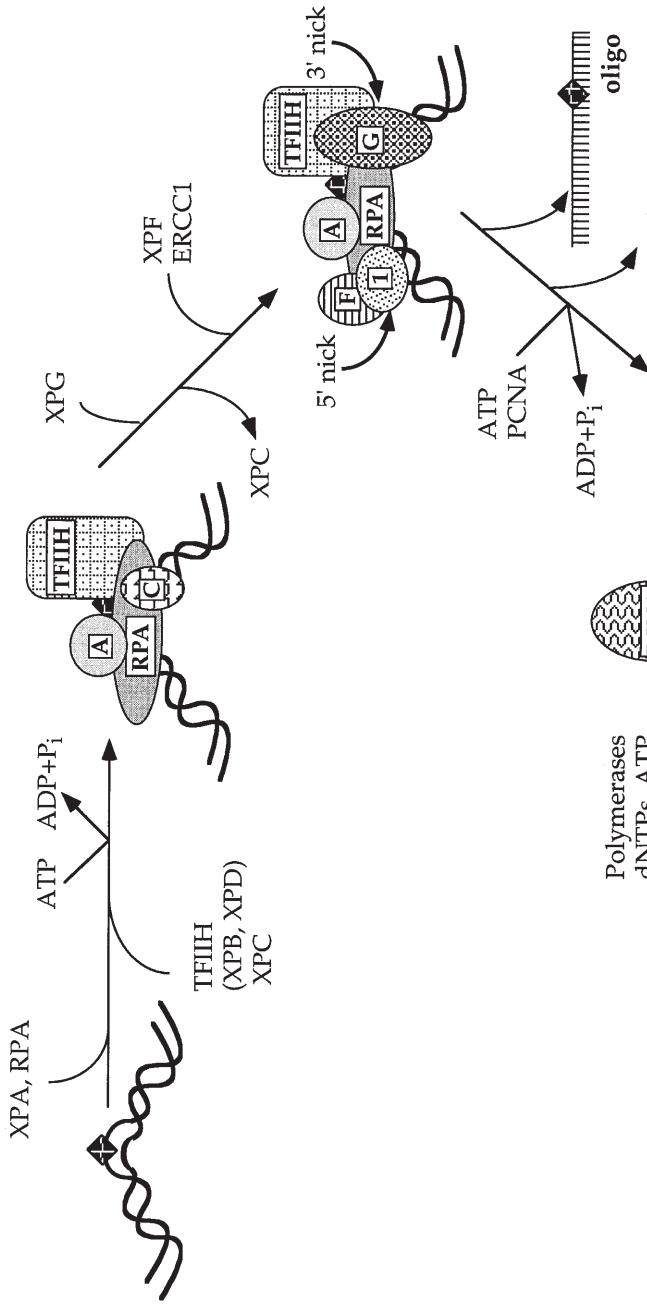

Fig. 3. *Nucleotide excision repair pathway*. XPA and RPA are thought to be involved in damage recognition, but the first stable complex is formed after ATP hydrolysis and the recruitment of TFIIH and XPC. This complex unwinds the DNA helix around the lesion. The addition of XPG and the departure of XPC results in the next detectable preincision complex, and dual incision occurs after the final addition of XPF/ERCC1. The damaged oligonucleotide is excised and then most of the excinuclease dissociates and is replaced by PCNA in an ATP-dependent process, followed by repair synthesis by DNA polymerases δ or ε and DNA ligases.

Another DNA-damage binding activity was found to be deficient in some XPE cells [41]. This activity is expressed at higher levels in some human tumor cells selected for cisplatin resistance, accompanied by enhanced levels of repair [18]. In another study, the small subunit of the putative XPE protein was induced by cisplatin and its induction was greater in resistant cell lines. The diminished cisplatin sensitivity was attributed to replicative bypass, however, not to excision repair [42][43]. Further investigation of the XPE phenotype, which presents with only mild XP symptoms and partial reduction in repair capacity, demonstrated that the damaged-DNA binding activity is absent in cells from only a few XPE patients (reviewed in [27]). Moreover, the purified protein does not complement the repair activity of XPE extracts and it is not required to reconstitute the excinuclease completely [30][31]. It is therefore impossible at present to delineate what role this protein might play in excision repair or the cytotoxic activity of cisplatin.

Table 1. *Components of the Excinuclease* [a])

Mammalian protein		Yeast homolog[b])	Role in repair
XPA		Rad14	Damage recognition
RPA/HSSB		Rpa	Damage recognition, also involved in repair synthesis
TFIIH			
includes:	XPB/ERCC3	Rad25/Ssl2	Contains helicases and DNA-dependent ATPase activity, required for formation of preincision complex and transcription-repair coupling
	XPD/ERCC2	Rad3	
	p62	Tfb1	
	p52/Cdk7	Tfb2	
	p44	Ssl1	
	p34	Tfb4	
	p38/CycH	Ccl1	
XPC		Rad4	Stabilization and protection of preincision complex, not required for repair of some lesions
hHR23B		Rad23	Binds XPC
XPG/ERCC5		Rad2	3′-endonuclease
XPF/ERCC4		Rad1	Subunit of 5′-endonuclease
ERCC1		Rad10	Subunit of 5′-endonuclease

[a]) Proteins required for repair synthesis include RFC, PCNA, RPA, DNA polymerase ε or δ and DNA ligase.
[b]) *S. cerevisae*.

Repair of Cisplatin-DNA Adducts

It is now certain that cisplatin-DNA cross-links are removed by the nucleotide excision repair pathway [1]. In fact, one of the original pieces of evidence indicating that cisplatin-damaged DNA is cytotoxic was the enhanced sensitivity of bacterial mutants deficient in components of the UvrABC excision nuclease [44][45]. Parallel studies in mammalian cells revealed the abnormal cisplatin sensitivity of human XPA and XPF cell lines [46–48] and of a Chinese hamster ovary cell line lacking ERCC1 [49]. The sensitivity of these cells was attributed to defective adduct removal from genomic DNA. This conclusion was supported by work with an *in vitro* repair synthesis assay which measures the formation of new DNA patches following removal of the platinum adducts by the excinuclease. The signal was diminished in extracts from XPA cells when compared to extracts from normal lymphoid cells [50][51].

Experiments were also performed with an alternative assay which makes use of a linear DNA substrate containing a site-specific lesion positioned close to a radioactive phosphodiester group. Incubation with cell free extracts and resolution of the DNA by denaturing gel electrophoresis afforded small oligonucleotides containing the adduct and the label [52]. By using this excision assay, it was revealed that both cisplatin 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links are substrates for the mammalian excinuclease [53][54]. The minor 1,3-d(GpTpG) cross-link is removed much more efficiently, however (*Table 2*). The relative rates of repair of the various cisplatin-DNA adducts by the mammalian excinuclease (GTG ≫ GG) agree with prokaryotic excinuclease recognition of DNA adducts formed by a diaminocyclohexane-platinum compound ([55], see, however, [56]) and reflects the degree of unwinding caused by the different adducts [57]. The correlation between helix-destabilization and relative rates of repair is not surprising, given that components of the damage-recognition complex (XPA and RPA) preferentially bind single-stranded over double-stranded DNA [33].

Table 2. *Site-Specific Cisplatin-DNA Adducts Repaired by Mammalian NER*

Adduct	Repair detected	Reference
GG	+	[53]
AG	+	[54]
GTG	+++	[53] [238]
GC interstrand cross-link	–	[54]
GG plus mismatch	++	[62] [63]

Although cisplatin interstrand cross-links also significantly distort DNA (*see above*), it is unclear whether they are repaired by NER. Study of a psoralen interstrand cross-link suggested that the excinuclease recognizes this type of damage, but with an unusual outcome [58]. Two cleavage sites were identified, both 5′ to the damage, such that the adduct was not removed from the DNA. It was suggested that the ensuing gap might serve as a recombinogenic signal to activate subsequent removal of the cross-link through a different repair pathway. When a cisplatin interstrand adduct was incubated with mammalian cell free extracts, however, no such reaction products were detected [54]. Cells from *Fanconi's* anemia patients (FA) are very sensitive to cross-linking agents but proficient in NER [59]. The unusual sensitivity of this cell type to cisplatin is associated with an inability to remove interstrand adducts from the DNA [46][47], indicating that the adducts are repaired by an alternative pathway. There is some evidence to suggest that mammalian repair of interstrand cross-links requires a few proteins which are also components of excision repair [60]. The repair synthesis signal arising from a cisplatin-modified plasmid enriched for the interstrand cross-link was larger than that detected for randomly modified DNA [61], and this activity was absent in extracts made from XPA cells. Although these experiments provide some information about the repair of cisplatin interstrand cross-links, the detailed mechanism still remains to be elucidated.

Cisplatin is also a carcinogen [2], at least in part, because mutations across from its DNA adducts are generated during replicative bypass [4]. Studies of a cisplatin 1,2-d(GpG)-containing substrate revealed that a thymine across from the adduct enhanced the excinuclease activity when compared with the correct complementary bases [62][63]. This effect probably reflects the superimposition of structural distortions. The action of an excinuclease on the Pt-damaged strand of such a compound lesion would permanently fix the mutation in the genome. A compound lesion would also be recognized by the mismatch repair proteins, the consequences of which are discussed below.

The Role of NER in the Anticancer Activity of Cisplatin

Over the years, it has become apparent that the antitumor activity of cisplatin is a consequence of several different cellular phenomena [24][25]. DNA repair has been investigated as one factor that could influence cisplatin sensitivity, since failure to remove DNA lesions would allow them to persist and interfere with essential cellular systems. Several early studies provided evidence that *trans*-DDP was ineffective because its DNA adducts were repaired more efficiently than those of the *cis*-isomer [4]. This result

might be a consequence of differential recognition of the platinum adducts by the repair enzymes or by some other factor that modulates repair. In addition, in contrast to previous reports (reviewed in [3]), recent evidence suggests the *trans*-DDP forms predominantly monofunctional and interstrand DNA cross-links [64], which might be processed in a different fashion than intrastrand adducts.

Sublines with reduced sensitivity to cisplatin have been produced *in vitro* by first exposing cells to low levels of the drug and then increasing the concentration in a stepwise fashion as the cells adapt to its presence. Enhanced DNA repair has been implicated as a mechanism of drug tolerance in a variety of model systems, including murine leukemia [65–67], rat colon adenocarcinoma [68], human ovarian carcinoma [69], and human testicular tumor cell lines [70][71]. In some cases, it was evident that other factors were involved since the increase in DNA repair was not proportional to the level of resistance. In addition, the resistance phenotype may be caused by mutations that reverse a DNA repair deficiency in the parental cells. For example, the murine leukemia L1210/0 cell line has been used in many studies of acquired resistance to cisplatin. A recent investigation revealed the parental cell line to be deficient in nucleotide excision repair, due to an XPG genetic defect. In the course of selection for drug resistance, the XPG function was restored, probably by a platinum-induced mutation [72]. Whatever the explanation, the possibility that increased DNA repair could confer resistance to cisplatin led to studies of agents that inhibit repair [5][26]. Although the results seem to depend on cell type, many of the compounds identified in this manner produced a synergistic response with cisplatin *in vitro*, and provided promising leads for clinical evaluation.

In *E. coli*, RNA polymerases stalled at sites of DNA damage are recognized by the coupling factor TCRF, which recruits repair factors [27]. In mammalian cells, the coupling factor has not been clearly identified, but mutations in the two genes responsible for *Cockayne's* syndrome, another repair-related disease, result in defective transcription-coupled repair. Various types of DNA damage including cisplatin adducts are removed more rapidly from actively transcribed genes [73], and intrastrand adducts in particular are preferentially removed from the transcribed strand [74]. Transcription-coupled repair of the major cisplatin-DNA adducts has not been linked to drug sensitivity. In contrast, enhanced gene-specific repair of the cisplatin interstrand cross-link was detected in several cell lines with acquired cisplatin resistance [75][76], but its repair mechanism has not been delineated.

A reduced capacity to repair cisplatin-DNA adducts may be responsible for the clinical effectiveness of the drug in the treatment of certain types of cancer. Cell lines derived from human testicular tumors are hypersensitive

to a variety of DNA-damaging agents, including cisplatin [77]. In comparison to relatively resistant bladder cancer cell lines, testis tumor cells have lower levels of platinum-adduct removal from the whole genome [78]. This result was reproduced when the repair of both the actively transcribed N-ras gene and the inactive CD3δ gene was analyzed. In particular, immunochemical analysis of the different cisplatin-DNA adducts demonstrated a deficiency in the repair of the 1,2-intrastrand cross-links [79][80]. The biological basis for this effect has not yet been established. Possible explanations include a defect in the nucleotide excision repair pathway, lower expression levels of the repair proteins, the absence of some excinuclease-activating signal, or the presence of a repair-inhibiting factor. Further work is required to ascertain whether DNA repair is the major determinant of cisplatin sensitivity in testicular cancer.

In vitro experiments with cultured cell lines have provided a wealth of information about the biological mechanism of cisplatin. Although there is some correlation between the sensitivity of these cells and related tumors [81], it is likely that they provide only a limited representation. An extreme case is a murine mammary tumor that acquired cisplatin resistance *in vivo* but seemed to lose drug tolerance when cultured in a monolayer *in vitro* [82]. Reimplantation *in vivo* restored cisplatin resistance, demonstrating the importance of cellular context when examining drug sensitivity. For this reason, the mechanism of action of cisplatin should be examined in human tissue when possible.

By using leukocytes from chemotherapy patients with squamous-cell carcinoma of the head and neck region, it was demonstrated that damage removal from DNA was related to cisplatin resistance [83]. This type of study assumes the profile of adduct formation and repair to be the same in peripheral and tumor tissue. The hypothesis was supported by several early studies which employed either atomic absorption spectroscopy or immunochemical techniques to demonstrate a relationship between DNA adduct formation in blood cells and disease response [84–89]. Subsequent work revealed, however, that cisplatin-DNA adduct levels do not always correlate with survival [90] and can vary substantially between individuals [91].

DNA repair components have been analyzed in a few tumor samples. Elevated levels of RNA for ERCC1 and XPA were detected in the tumors of ovarian cancer patients who were clinically resistant to cisplatin chemotherapy when compared to the responders [92][93]. In these studies, the tissue was harvested before drug treatment, the higher levels of expression of the NER components providing a biochemical basis for *inherent* cisplatin resistance in some of the patients. The mechanism of *acquired* resistance was investigated in a malignant oligodendroglioma by examining tissue both before and after failed cisplatin therapy [94]. The level of repair of cispla-

tin-DNA adducts, as determined by a host cell reactivation assay, was higher in the tumor procured after therapy and associated with increases in DNA polymerase β and ligase activity.

Clearly, the NER proteins play a major role in the biological processing of cisplatin-DNA adducts. As a consequence, it is likely that they can modulate cellular sensitivity to cisplatin. So far, however, there is only limited information about the relative repair capacities of malignant and normal tissue. To determine whether DNA repair contributes to tumor responses during cisplatin chemotherapy, it is necessary to quantitate directly the DNA repair activity from the tissue. Recent studies of human tissue demonstrated a significant variability in the repair capacity of tumors from different patients [95]. When the levels of repair in extracts from different rat organs were compared, no correlation with cisplatin toxicity was observed [96]. Such experiments are difficult to evaluate, however, since current methods involve the preparation of crude extracts, which must be normalized to one another, and the use of *in vitro* repair assays which are complicated by inherent variability [97]. Improved technical protocols, along with a better understanding of the intricacies of neoplastic transformation, should make it easier to study the excinuclease activity in the context of the complex cellular environment.

Mismatch Repair

Normal DNA processing activities are inherently error-prone, and the existence of several mechanisms to remove genetic misinformation indicates how disastrous the propagation of mutations can be. The consequences of such negligence was made evident by the discovery that almost all cases of hereditary nonpolyposis colon cancer as well as a variety of sporadic tumors arise from defects in the mismatch repair pathway [98]. In addition to the maintenance of genetic integrity (for reviews on mismatch repair, see [98–100]), however, it is becoming apparent that mismatch repair may also mediate the cytotoxicity of a variety of clinically effective drugs, including cisplatin [101][102]. A connection between cisplatin sensitivity and mismatch repair was made with the discovery that repair-deficient cell lines were resistant to cisplatin and carboplatin, but not to all DNA-damaging agents [103][104]. Furthermore, some ovarian cancer cell lines selected for cisplatin resistance lose the ability to express hMLH1 [105–107], one of the essential components of the mismatch repair machinery [99][100]. In a model proposed to explain drug tolerance associated with defects in mismatch repair, it is assumed that replication bypass of cisplatin-modified bases produces mutations (*Fig. 4, A*). During mismatch repair, the strand to be cor-

rected is nicked, an oligonucleotide containing the mismatch is excised, and new DNA is synthesized. The mismatch repair proteins always replace the incorrect sequence in the daughter strand through some as yet undetermined signal, which would leave the exogenous platinum damage unrepaired. This activity initiates a futile cycle. During DNA synthesis to replace the excised oligonucleotide, the polymerases would again incorporate mutations, which would be followed by attempts to fix them. The repeated breaks in DNA formed at each ineffective cycle of repair could trigger a cell death response.

A more direct association was made by experiments showing that cisplatin-modified DNA is recognized by mismatch repair proteins. The MutSα heterodimer, a putative mismatch recognition factor, binds to a 32-bp

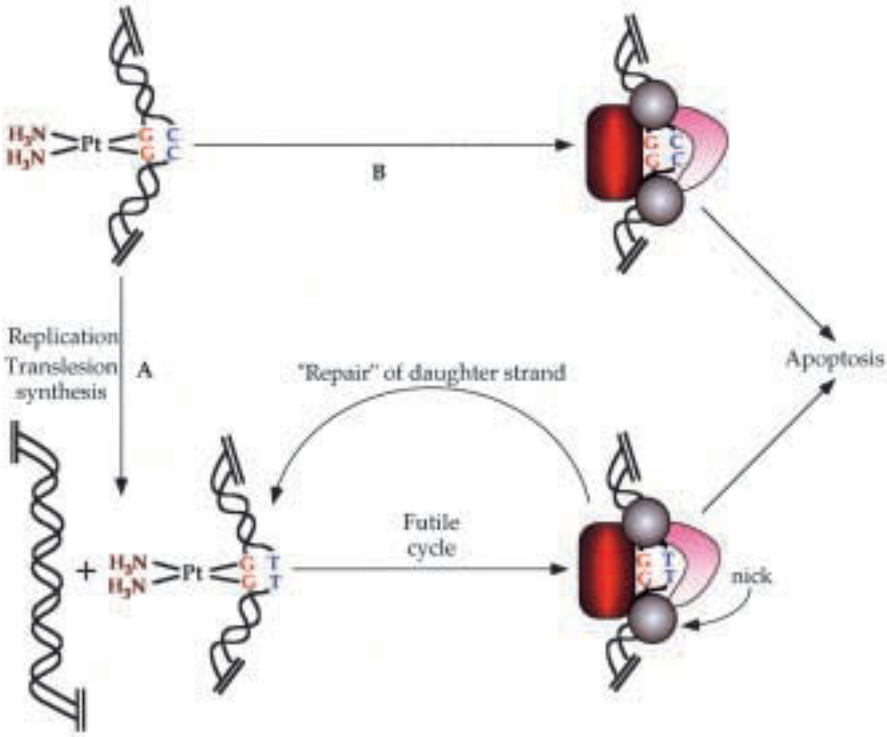

Fig. 4. *Possible role of mismatch repair in the cytotoxicity of cisplatin*. A) During replicative bypass, a mismatch is incorporated across from the cisplatin-DNA adduct. This compound lesion is bound by the mismatch repair proteins, which cut the DNA on the strand opposite the platinum. Repair synthesis would reproduce the same mismatch, resulting in a futile cycle and possibly the accumulation of DNA strand breaks which would activate apoptosis. B) Alternatively, the mismatch repair complex can recognize the cisplatin-DNA adduct alone and generate a signal that triggers apoptosis.

oligonucleotide containing one 1,2-d(GpG) cisplatin intrastrand cross-link but only poorly to 1,2-d(ApG) and 1,3-d(GpTpG) adducts and not at all to a *trans*-DDP 1,3-intrastrand cross-link [108]. Further work demonstrated that hMSH2, a component of MutSα, binds specifically to DNA globally modified with cisplatin but not with other, clinically ineffective platinum compounds [109]. This protein also displays a weak selectivity for a single 1,2-d(GpG) adduct in a 100 bp probe compared to the corresponding unmodified DNA.

The binding of mismatch repair proteins to cisplatin-modified DNA in the absence of mutations could activate cell death indirectly, by initiating the same futile cycle described above. Alternatively, recognition of DNA adducts by the repair apparatus could trigger cell cycle arrest and/or apoptosis by some mechanism (*Fig. 4, B*), such as activation of the JNK or c-Abl signaling pathways [110]. The absence of a functional complex to detect damage would increase cellular tolerance for cisplatin-DNA adducts and explain the drug resistance associated with diminished expression of these proteins. This hypothesis is interesting in light of the recent connection between mismatch repair and PCNA, a replication factor that has been implicated in a cell cycle checkpoint [111][112], and the observation that hMLH1-deficient cells do not exhibit the prominent G_2-arrest usually observed following cisplatin exposure [107].

Cellular sensitivity to different platinum compounds and the recognition of the platinum DNA adducts by mismatch repair protein complexes appear to be linked [103]. It may also be significant that hMSH2 is expressed to higher levels in testicular and ovarian tissue than in other organs such as heart, liver and colon [109]. Whether or not mismatch repair plays a general role in the anticancer activity of cisplatin still remains debatable, however. Mismatch repair proteins bind to cisplatin-DNA adducts *in vitro* with weak specificity [109][113]. Although specificity is enhanced when a platinum lesion is combined with a mutation [113], it is still less than the affinity of these proteins for the unplatinated mutation [63][108].

Mismatch repair mutants are more likely to afford a mechanism for acquired cisplatin resistance [105–107]. Exposure to cisplatin can select for mismatch repair deficiency both *in vitro* [114][115], and *in vivo* [107], and deleting MSH2 produces a small but significant level of cisplatin resistance in xenografts [115]. The fact that several ovarian carcinoma cultured cell lines develop a defect in this pathway during the acquisition of resistance to cisplatin is intriguing, because the growth of resistant tumors is a significant problem in the treatment of ovarian cancer [116]. The importance of overcoming resistance in cancer chemotherapy should further stimulate investigation of this area.

HMG-Domain Proteins and Cisplatin

As mentioned in the introduction, preliminary studies demonstrated the existence of cellular proteins which specifically recognize and bind to cisplatin-modified DNA. To determine what role these proteins might play in the cytotoxic mechanism of cisplatin, it was necessary to learn their identity and study them individually. To achieve this goal, a cDNA encoding one of these SSRPs (structure-specific recognition proteins) was isolated by expression library screening [117][118], and a data base search with the predicted amino acid sequence revealed homology with the non-histone chromosomal protein HMG1 [118]. The region of highest identity (47%) was an 80-amino acid DNA-binding motif known as the HMG domain, which occurs in a variety of proteins. In a complementary study, HMG1 and HMG2 were detected among a small group of nuclear proteins isolated from HeLa cell extracts by fractionation on cisplatin-modified DNA cellulose [119]. Gel mobility shift assays demonstrated that HMG1 binds specifically to DNA treated with cisplatin, but not to DNA modified with *trans*-DDP [120], and the use of site-specifically modified probes revealed that it is the 1,2-intrastrand adducts that induce binding. These results stimulated a much more extensive study of the family of HMG-domain DNA-binding proteins.

The canonical HMG domain is a positively charged, predominantly α-helical 80 amino acid polypeptide [121–123]. This L-shaped peptide occurs in a wide variety of minor groove DNA-binding proteins having little or no homology outside of the HMG domain. Members of this protein family lie on a continuum between two modes of DNA recognition. At one end are proteins which bind to a defined, AT-rich, consensus sequence. These proteins have only a single domain, usually function as transcription factors and regulators of cellular differentiation, and include the lymphoid enhancer binding factor LEF-1, the testis-determining factor SRY, and the Sox family of proteins. At the other extreme are proteins such as HMG1 and HMG2, which contain more than one HMG domain and display no DNA sequence specificity (for a review on HMG1/HMG2 see [122]). The exact cellular function of these proteins is unknown, but because they bend DNA and recognize pre-bent DNA structures like other HMG-domain proteins, it has been proposed that they act as architectural factors during nucleic acid processing. In this role, the HMG-domain protein would facilitate the assembly of multi-protein complexes responsible for activities which include the maintenance of chromatin structure, DNA recombination, replication, transcription and repair.

The sequence homology between HMG domains is quite low, identity typically being on the order of 25%, but recent modeling experiments indi-

cate that the tertiary structure is highly conserved [124]. This result may explain why HMG domain proteins are all able to bind specifically to distorted DNA structures such as four-way junctions. Recent solution structures of the SRY and LEF-1 HMG domains in complexes with their target sequences revealed a pronounced bend in the DNA accompanied by unwinding of the helix and widening of the minor groove [125][126]. Similar features have been observed in structures of DNA duplexes containing the cisplatin 1,2-d(GpG) intrastrand cross-link [8–11]. Accordingly, cisplatin-DNA adducts might provide an optimal framework for specific recognition by this DNA-binding motif.

Binding to Cisplatin-DNA Adducts

Most of the HMG-domain proteins examined have the capacity to bind specifically to DNA cross-linked with cisplatin; *Table 3* summarizes studies with site-specifically modified DNA (see also [119][127–131]). The binding affinity of these proteins to a site-specific 1,2-d(GpG) cisplatin intrastrand cross-link was determined by using gel mobility shift and other assays. The differences in experimental conditions employed, the method for calculating K_d, components of the reaction solutions, and the DNA probes themselves, prohibit any quantitative comparison of the results. Several points of interest have emerged from these studies, however. First, there is no doubt that these polypeptides bind to a single 1,2-intrastrand cross-link with higher affinity than to the corresponding unplatinated DNA, the specificity ratio (ρ) ranging from 3–235. The higher specificity occurs with smaller DNA substrates, which is not surprising given that longer stretches of undamaged DNA provides more opportunity to form non-specific complexes with any DNA-binding protein.

The second observation is that isolated HMG domains recognize the cisplatin lesion, establishing that it is this motif which confers such an unusual activity on the family of proteins (but see [132]). That the domains do not bind to the cisplatin-DNA adducts with the same affinity and specificity as the whole proteins supports the fact that regions outside the domain influence the DNA-binding activity ([133] and references within). This observation could explain why the separate HMG domains of HMG1 exhibit distinctive interactions with the same cisplatin-modified substrate [134], since the binding affinity might have been modulated by a short string of basic residues that was only included at the C-terminus of HMG1 domain A.

Finally, the sequence context of the Pt-DNA adduct can have a significant impact on the strength of the protein-DNA interaction [134]. In a study

of a series of 15 bp platinated probes, changing the identity of the two nucleotides flanking a 1,2-d(GpG)-cisplatin-DNA adduct modulated the binding affinity by more than 2 orders of magnitude. In particular, a striking effect was apparent for the base-pair 3′ to the lesion, a position which has unusual minor-groove accessibility (*Fig. 1*) [8][131][135]. It has not been determined whether this modulation of binding is due to differences in the structures of the cisplatin-modified duplexes, changes in the stability and flexibility of the duplex DNA, base-specific protein-DNA contacts, or some combination thereof.

A recent study demonstrated that HMG1 can also apparently bind to DNA containing cisplatin interstrand cross-links [129]. As described above, the duplex-DNA distortions induced by the interstrand cross-link are quite

Table 3. *HMG-Domain Protein-Binding Constants for Cisplatin-DNA Adducts*

Protein [a])	Probe [b])	K_d [M]	ρ [c])	Reference
HMG1	TGGT-100mer	$3.7 \pm 2.0 \times 10^{-7}$	100	[120]
HMG1 domain B	TGGT-100mer	3×10^{-7}	3–4	[239]
	AGGC-92mer	4×10^{-7}	2.5	[239]
	TGGT-20mer	5×10^{-7}	> 4	[239]
	AGGT-15mer	$4.8 \pm 0.9 \times 10^{-8}$	nd	[134]
	CGGC-15mer	$1.3 \pm 0.2 \times 10^{-6}$	nd	[134]
HMG domain A	AGGA-15mer	$6.8 \pm 0.8 \times 10^{-9}$ [d])	235	[134]
	CGGC-15mer	$5.2 \pm 0.6 \times 10^{-7}$ [d])	3	[134]
Ixr1	AGGC-92mer	$2.5 \pm 0.1 \times 10^{-7}$	8	[147]
tsHMG	TGGT-20mer	$3.0 \pm 0.5 \times 10^{-8}$ [d])	230	[240]
tsHMG domain A	TGGT-20mer	$5.9 \pm 3.4 \times 10^{-7}$ [d])	20	[240]
hSRY	TGGT-20mer	$1.2 \pm 0.2 \times 10^{-7}$ [d])	20	[146]
	SRY-20mer	$5.0 \pm 1.0 \times 10^{-8}$ [d])	40	[146]
hSRY domain	TGGT-100mer	$4 \pm 1 \times 10^{-9}$	5	[146]
	SRY-100mer	$3 \pm 0.7 \times 10^{-9}$	nd	[146]
	TGGT-20mer	$4 \pm 0.7 \times 10^{-9}$	5	[146]
	SRY-20mer	$3 \pm 0.4 \times 10^{-9}$	10	[146]
mSRY domain	AGGC-92mer	$\approx 10^{-6}$	nd	[137]
LEF-1 domain	AGGC-92mer	$\approx 10^{-7}$	nd	[137]
mtTFA	AGGC-92mer	$\approx 10^{-7}$	nd	[137]
hUBF	TGGT-100mer	6×10^{-11} [e])	nd	[145]
hUBF	rRNA promoter	1.8×10^{-11} [e])	nd	[145]

[a]) The HMG1 peptides were from rat, tsHMG and mSRY peptides were from mouse, hSRY, Lef-1, mtTFA, and hUBF peptides were from human.
[b]) The cisplatin-modified (underlined) and the flanking nucleotides are listed as well as the probe length.
[c]) Approximate specificity of binding ratio for cisplatin-modified DNA compared with unmodified probe. nd: not determined
[d]) Determined by competition assays.
[e]) Determined by quantitative DNaseI protection assays.

different from those resulting from a 1,2-intrastrand adduct, suggesting that there is no correlation between the bending or unwinding angles and HMG recognition. The authors propose that the proteins recognize fixed distortions in DNA, but that if the DNA is too denatured or flexible, as is the case following *trans*-DDP modifications, binding is inhibited. To date, this is the only reported example of such an interaction, and to appreciate its importance, a comparison of substrates containing the different types of cisplatin adducts will be required.

In addition to binding structural distortions in DNA, the ability to bend linear DNA is a common feature of this protein family [123]. Thus HMG-domain proteins could affect the mechanism of cisplatin by further distorting DNA at the site of the lesions [136]. Circular permutation assays demonstrated that HMG-domain proteins amplify the bend in a 1,2-d(GpG) site-specifically platinated probe [137]. In addition to determining the bend angles, which ranged from 50° to 90°, these experiments also revealed that the platinum was near the center of the bend locus. The enhanced bending of the cisplatin-modified DNA suggests one reason why the interaction with HMG-domain proteins is favored. As described above, the roll between guanine bases is such that the platinum is under considerable strain, which could be released if the bend angle were increased to ≈ 90° through the formation of a protein-DNA complex [137].

The Role of HMG-Domain Proteins in Modulating the Cisplatin Sensitivity of Cells

The binding of cisplatin-modified DNA by HMG-domain proteins, a consequence of features in common with the natural DNA targets, may fortuitously play a role in the drug cytotoxicity. It is clear that HMG-domain proteins do respond to cisplatin, since both HMG1/2 and hUBF relocalize in cells exposed to the drug [138][139]. Interruption of the gene for Ixr1, a yeast HMG-domain protein, resulted in a 2–6 fold desensitization of the cells to the drug [140][141], which correlated with a decrease in the number of platinum-DNA adducts [141]. In addition, a recent report suggests that overexpression of HMG2 can sensitize cells to cisplatin [142].

If HMG-domain proteins function as a determinant of cisplatin cytotoxicity, the levels of these proteins would be expected to reflect cellular drug sensitivity. This issue is difficult to address, however, because it is not known whether only one member of this family, and if so, which one, or all HMG-domain proteins should be examined. Nevertheless, several studies have searched for a connection between the quantity of expressed HMG-domain protein and cellular response to cisplatin. No correlation was de-

tected between hSSRP1 expression and the cisplatin sensitivity of several cell lines and tissue types [118]. In contrast, elevated levels of HMG1/2 bound to cisplatin-modified DNA cellulose were detected in nuclear extracts from HeLa cells with acquired cisplatin resistance [143]. HMG1 was also expressed in higher levels in human hepatocellular carcinomas than in the healthy tissue [144]. Further research is needed to evaluate the importance of HMG-domain proteins in determining the sensitivity of tumors to cisplatin.

Several models have been proposed to explain what specific role HMG-domain proteins could play in the cisplatin mechanism of action. When the ability of this family to recognize cisplatin-modified DNA was first detected, it was suggested that HMG-domain proteins might be factors that communicate the presence of the genetic damage to the repair pathways [117], but no evidence to date supports such a hypothesis.

In another potential scenario, the formation of tens to hundreds of thousands of genomic platinum lesions following a chemotherapeutic dose of cisplatin [86][90] could titrate HMG-domain proteins away from a much smaller number of natural binding sites (*Fig. 5, A*). Both hUBF and hSRY bind with similar affinity to the 1,2-d(GpG) intrastrand adduct and to their target sequences (*Table 3*) [145][146]. If gene regulation by an HMG-domain protein were essential for cell viability, then such a diversion could result in cell death. This hypothesis was tested in yeast by taking advantage of the function of Ixr1 [147], also known as Ord1 [148], which represses the transcription of Cox5b, an isoform of subunit V of cytochrome c oxidase. If cisplatin-DNA adducts can titrate Ixr1 away from its recognition sequence, the transcription of genes regulated by Ixr1 should increase specifically in the wild-type cells exposed to cisplatin when compared with *ixr1* mutants. No cisplatin-induced increase was observed either in the mRNA levels of the Cox5b gene or in the activity of a reporter gene placed downstream of the Ixr1 promoter. Thus, in this system at least, titration of Ixr1 cannot be invoked to explain the cisplatin resistance of cells with a mutant HMG-domain protein.

There is more experimental evidence to support an alternative, but not exclusive, model (*Fig. 5, B*), which proposes that HMG-domain proteins bind tightly to cisplatin-DNA adducts and block repair complexes from removing the damage. Such repair shielding would enhance the cytotoxicity of the drug by allowing the lesions to persist in the cell. Gel mobility shift assays demonstrated that the binding of HMG1 to cisplatin-modified DNA is slightly faster than binding by the repair factor RPA, and that a preformed HMG1-DNA complex is not disturbed by RPA [38]. Moreover, addition of HMG-domain proteins to the *in vitro* NER assay specifically inhibited excision of the 1,2-d(GpG) but not the 1,3-d(GpTpG) intrastrand cisplatin-

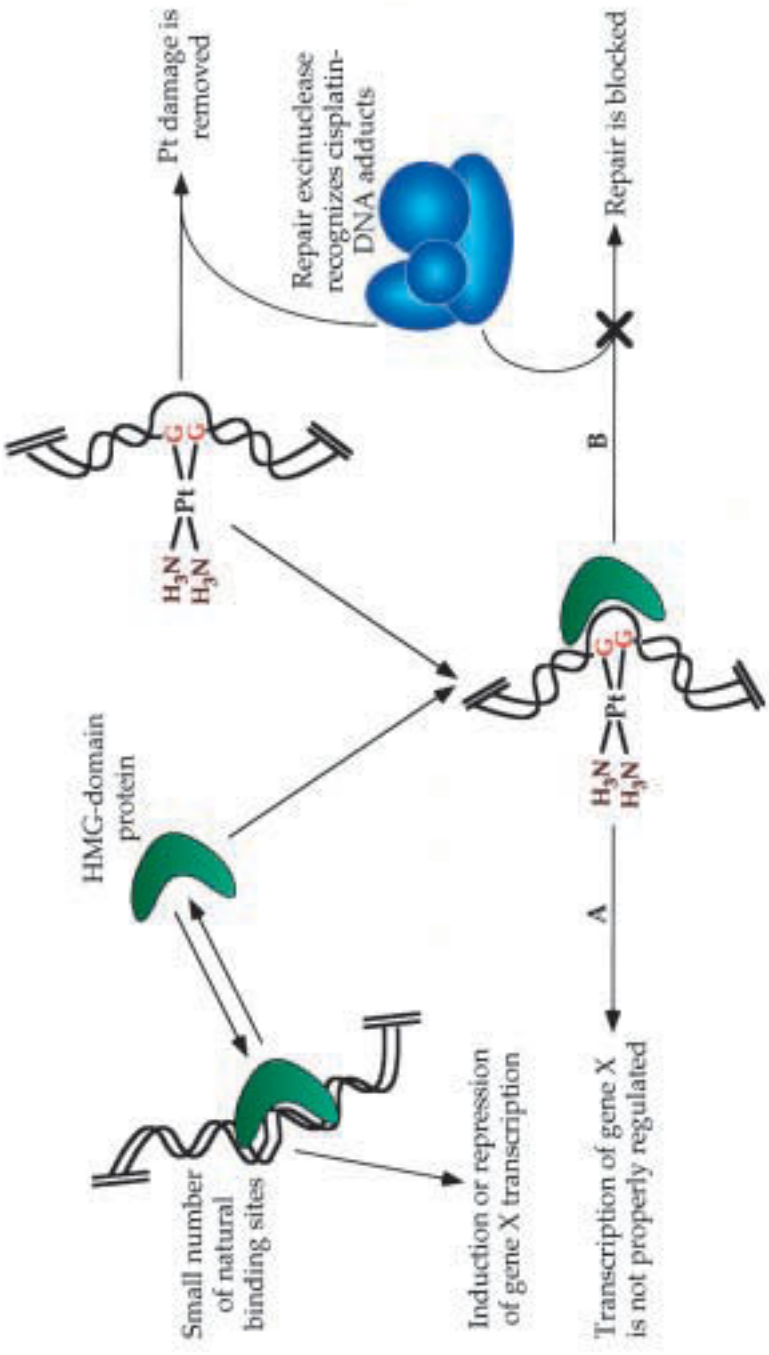

Fig. 5. *Models for HMG involvement in the cisplatin mechanism of action.* A) When a cell is exposed to a lethal dose of cisplatin, 10^4–10^5 DNA adducts are formed. If HMG-domain proteins bind with similar affinity to these lesions and to their natural binding sites, the proteins could be titrated away from their transcriptional regulatory function. B) The HMG-domain proteins could block access of the excision repair complex and shield the adducts from repair.

DNA cross-links [53][54][146]. *In vivo* evidence for this model was provided by yeast double mutants. In this system the differential cisplatin sensitivity caused by inactivation of Ixr1 was directly related to damage recognition and formation of the excision repair complex [140].

The endogenous HMG-domain proteins in HeLa cell free extracts do not seem to affect the relative rates of repair of cisplatin-DNA adducts [54][62]. Nevertheless, the hypothesis that HMG-domain proteins can enhance cellular sensitivity to cisplatin by blocking repair of the DNA adducts is still viable. Several HMG-domain proteins are specifically expressed in the testes ([146] and references cited therein), two of which, tsHMG and hSRY, inhibit the *in vitro* excision of cisplatin-DNA adducts at lower protein concentrations than any of the other HMG-domain proteins tested [54][146]. Selective expression of these or other such proteins in testicular tumors would provide an explanation for the unusual cisplatin sensitivity of this tumor type and the reduced repair of cisplatin-DNA adducts observed in testicular cell lines (discussed above).

Platinum Inhibition of Transcription

The formation of cross-links on DNA has the potential to affect directly two essential cellular processes, replication and transcription. Early work demonstrated that cisplatin could inhibit replication under conditions that did not block transcription or translation [2]. Adducts formed by *trans*-DDP can also inhibit DNA polymerases [4], however, and it has become evident that cisplatin lesions are not absolute blocks for replication (see the review by *Villiani et al.*, this book). Furthermore, cisplatin commonly causes an arrest in the G_2 phase of the cell cycle [149], suggesting that inhibition of gene expression, and not replication, determines whether the cell will live and divide, or undergo apoptosis.

Accordingly, some effort has been devoted to studying the effects of cisplatin on transcription. *In vitro* experiments with RNA polymerases demonstrated that productive elongation activity was prematurely terminated by the whole spectrum of cisplatin-DNA adducts, but not by the *trans*-DDP 1,3-intrastrand adducts [150–152]. Selective bypass of *trans*-DDP adducts was also demonstrated in XPA cells, suggesting that repair of the DNA lesions did not contribute to differential transcription inhibition by the platinum compounds [153]. *In vivo*, hormone-induced chromatin remodeling and subsequent transcription from the MMTV promoter was specifically inhibited by cisplatin [154]. In this case, platinum adducts seemed to cause a decrease in the DNA binding of one of the transcription factors, NF1. Several chromatin-associated proteins, such as the linker histone protein H1 or

HMG1/2, which modulate transcription [122], could produce these effects through their known binding affinity for cisplatin-DNA adducts [155]. This possibility was not thoroughly investigated, however, and it was suggested that NF1 is inhibited from binding by the cisplatin-induced distortions. This mechanism of cisplatin cytotoxicity could be countered by mutations which eliminate hot-spots for platinum binding, as observed in SV40 viruses selected for drug resistance [156].

It is possible that cisplatin cytotoxicity arises from more subtle effects than just general inhibition of transcription. The expression of some genes with a strong promoter is inhibited more in cultured cells by cisplatin than bulk RNA synthesis, and this differential sensitivity was not observed for *trans*-DDP [157][158]. On the other hand, the induction of several weak promoters by cisplatin is reminiscent of the bacterial SOS response [158][159]. Since the inhibition or activation of RNA polymerase II-mediated transcription by cisplatin is gene-specific and modulated by the promoter region, the end result would not be to shut down the cellular machinery so much as to create an imbalance. The selective inhibition, or induction, of gene expression by cisplatin is particularly relevant with respect to the expression of oncogenes, several of which have been implicated in cisplatin resistance [25].

As mentioned above, one consequence of stalled RNA polymerase II at a DNA adduct is activation of transcription-coupled repair [27]. This effect may depend on the type of polymerase, however, since the removal of some types of DNA damage is slower from RNA-polymerase I transcribed ribosomal DNA than from a nuclear gene [160]. The lower level of repair in the nucleolus could also reflect the influence of other transcription factors, such as the HMG-domain protein UBF, which bind to cisplatin-modified DNA [145]. When HeLa cells were exposed to cisplatin at concentrations which did not seem to affect nuclear transcription, inhibition of rDNA gene expression was associated with the redistribution of UBF, along with other factors responsible for rRNA transcription [138]. These observations indicate how cisplatin might exert a combination of effects. Transcription is stopped due to titration of essential factors by the platinum-DNA adducts, and the same proteins could shield the lesions from the repair activity.

p53 and the Cellular Response to Cisplatin

To avoid the propagation of mutations leading to malignant transformation, the cell has two options. It can arrest the cell cycle and attempt to repair the lesions or, if the damage is too extensive, it can initiate a suicide

program. One of the major factors required for maintenance of genomic stability is the product of the p53 tumor suppressor gene, a nuclear protein that exerts its effects through transcriptional regulation (for reviews see [161–163]). Upon exposure to genotoxic compounds, p53 protein levels increase due to several post-transcriptional mechanisms, resulting in the activation or repression of a variety of downstream genes. The loss of p53 activity removes a crucial barrier to unrestrained neoplastic growth. More than 50% of solid human tumors have mutations in this gene, usually in highly conserved regions such as exons encoding the DNA-binding domain. In addition, the status of the p53 gene in a tumor can be an important prognostic indicator [164][165]. A non-functional p53 could confer resistance to chemotherapy by protecting tumor cells from drug-induced apoptosis, or it could sensitize the cells owing to the absence of p53-dependent growth arrest and repair.

p53 and Cisplatin Sensitivity

Given the importance of p53 in cancer, it is not surprising that many researchers have tried to establish a connection between p53 and cisplatin sensitivity, but unfortunately a consensus has not been attainable. Introduction of a wild-type p53 gene by viral transfer into both a human non-small-cell lung cancer line and an ovarian cancer cell line selected for cisplatin resistance resulted in sensitization of the cells to cisplatin-induced apoptosis [166][167]. Similarly, ovarian carcinoma cell lines selected for cisplatin resistance had non-functional p53, associated with lower levels of apoptosis [168–170]. In contrast, a comparison of nine human ovarian cell lines did not demonstrate any correlation between p53 and cisplatin-mediated growth inhibition, suggesting that p53-independent pathways were responsible for cisplatin cytotoxicity [171]. In addition, in human breast cancer or foreskin fibroblast cells, which do not favor an apoptotic mechanism of cell death, inactivation of p53 sensitized the cells to cisplatin [172][173]. Finally, in a recent comparison of the drug sensitivity of 57 different human cell lines [174], on average p53-mutant cells were slightly less sensitive to cisplatin-induced growth inhibition, but there was a large range of responses, depending on the cell line. These results confirm the importance of cell type and cellular context in studying p53-mediated responses to cisplatin [164].

Testicular cancer is particularly interesting with respect to cisplatin because the addition of this drug to the chemotherapeutic regimen has had such a dramatic effect on patient survival [175][176]. In addition, testicular tumors are unusual because they rarely have mutations in the p53 gene

[177–182]. High expression levels of the p53 protein, usually a characteristic of a genetic mutation, are also observed in testicular tumors [178][181][183][184], although a recent study of mouse testicular teratocarcinoma cells suggested that the protein is not transcriptionally active until the cells are exposed to a DNA-damaging agent [185]. It is possible that the extreme sensitivity of testicular cancer cells to cisplatin is due to the induction of p53-regulated responses, such as apoptosis, but several *in vitro* studies failed to support this hypothesis. In an investigation of four testicular germ cell tumor cell lines, the p53 status was not a determinant of cisplatin sensitivity and p53-independent apoptosis was observed [186][187]. Similarly, although cisplatin-induced apoptosis observed in several murine testicular teratocarcinoma cell lines was dependent on a functional p53, the activation of a p53-independent cell cycle arrest resulted in comparably long-term survival for p53-normal and p53-mutant cells [188].

As discussed above, it is important to confirm *in vitro* results with *in vivo* studies. In an examination of several germ cell tumors from male patients who failed cisplatin-based therapy, either because of inherent or acquired resistance, mutations in the p53 gene were found in a subset of these tumors [189]. Defects in p53 could potentially cause some of the clinical resistance, due to the lack of an apoptotic response, but the majority of the resistance observed was not explained by p53 mutations. Together with the cell culture results, the clinical data suggest that p53 is not a key determinant of cisplatin cytotoxicity in testicular cancer. Ovarian cancer is another type of malignancy that is managed with cisplatin combination chemotherapy, but patient survival is significantly limited by the development of resistance [116]. For this type of tumor, p53 expression, either mutant or wild type, seems to be a marker for poor prognosis, which should be considered when planning the therapy regimen (for example, see [190–192] and references therein).

p53-Mediated Responses to Cisplatin

There are multiple pathways for p53 induction [161], but the specific mechanism for the activation of p53-mediated responses by cisplatin is still obscure. Details about the DNA-damage signal transduction pathway could be important for the mechanism of cisplatin resistance and must be provided by future research. In contrast, quite a lot is known about the downstream effects of p53. Several of these p53 activities have been implicated in the modulation of cellular sensitivity to cisplatin (*Fig. 6*).

Apoptosis. One of the consequences of p53 induction is apoptosis, a cellular response which is activated by many DNA-damaging agents including cisplatin [149]. Members of the Bcl-2 protein family, some of which are controlled by p53, can either accelerate or inhibit the programmed cell death pathway [193]. For example, the p53-regulated Bax protein usually promotes cell death, but its effects can be neutralized by Bcl-2. In several cases, cisplatin sensitivity was associated with the functional status of p53 and levels of the Bcl-2 proteins. In two different ovarian carcinoma cell lines, cisplatin resistance was acquired *in vitro* together with loss of p53-mediated transactivation of Bax and initiation of apoptosis [169][194]. The introduction of a wild-type p53 gene resulted in an increase in Bax expression and cisplatin-induced apoptosis [167], whereas exogenous Bcl-2 delayed the activation of programmed cell death [195]. A comparison of human testicular cell lines with more cisplatin-resistant bladder tumor cell lines revealed higher levels of apoptosis induced by the DNA-damaging agent etoposide in the testicular cancer cell lines [196]. Programmed cell death was

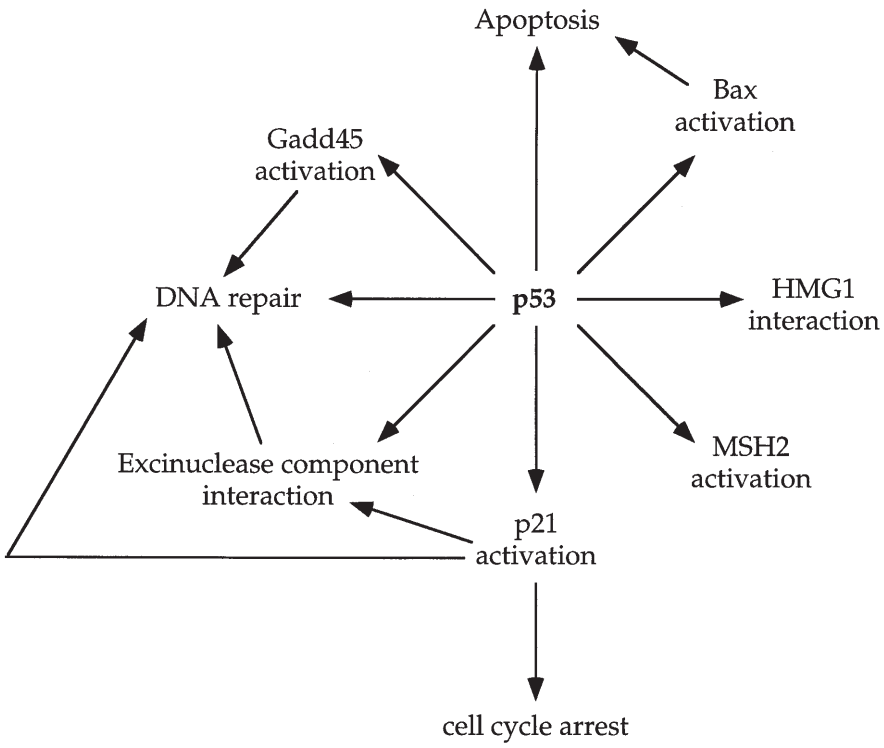

Fig. 6. *Downstream effects of cisplatin-induced p53*

associated with functional p53 and high levels of Bax, whereas in most of the bladder cancer cells p53 was non-functional and the Bax/Bcl-2 ratio was much smaller.

DNA Repair. A connection between p53 and DNA repair was observed in p53-deficient cells that exhibited less global DNA repair [197–199] (but see [200]), as well as a reduced capacity to reactivate cisplatin- and UV-damaged reporter plasmids [173][201][202]. Furthermore, pretreatment with low levels of UV activated a protective response in which the levels of repair activity were elevated, an effect not observed in p53-deficient cells [202][203]. It is possible that the p53 protein is directly involved in removing DNA damage since the protein recognizes both irradiated DNA and mismatches [162]. There is also evidence that p53 can interact with several components of the excinuclease, including RPA and the TFIIH-associated factors XPB and XPD [204][205]. So far, however, there is no evidence to demonstrate a direct role for p53 in the nucleotide excision repair pathway.

It is more likely that p53 influences repair in a regulatory capacity. One link between p53 and NER was made with the observation that the p53-regulated Gadd45 binds to PCNA, a component of both replication and repair [206]. Overexpression of Gadd45 provided a small level of protection from cisplatin [207], whereas Gadd45 antisense DNA sensitized human colon carcinoma cells to cisplatin, an effect which was associated with a decrease in repair [201]. It was hypothesized that the Gadd45 protein could interact directly with the repair proteins because it stimulated repair synthesis in nuclear extracts [206]; however, this result could not be reproduced under a variety of experimental conditions [208][209]. Thus, it seems likely that there are still some as yet unidentified factors which link p53 and the excision repair pathway.

The cyclin-dependent kinase inhibitor p21 is another downstream effector of p53 [161–163]. There is evidence for p53-independent induction of p21 [162], and under these conditions the protein may be responsible for cisplatin-induced apoptosis [210][211]. The p21 protein usually plays a protective role in response to cisplatin [212][213], however, an effect which correlated with enhanced repair of a damaged reporter plasmid [212–214]. These observations are consistent with the hypothesis that DNA-damage induced p53 activates a G_1 cell cycle arrest through p21, affording the cell time to repair the lesions and precluding the genetic instability produced by replication of damaged DNA. In accord with this model, the addition of p21 to cell free extracts blocked DNA replication but not excision repair [215], although since p21 does interact with PCNA, it has the potential to block the repair activity of PCNA in addition to its activity in replication [216].

In *E. coli*, DNA-damaging agents produce what is known as the SOS response, a coordinated up-regulation of genes which contribute to cell survival, at least in part, by repairing the DNA [27]. The p53 protein could provide a signal in a eukaryotic version of this type of general defense mechanism. The binding and activation of p53 by small oligonucleotides such as those generated by NER [162] suggests the existence of a feedback loop connecting DNA repair and p53-regulated gene expression. A p53-dependent increase in repair capacity has been reported for a variety of cell lines exposed to low levels of genotoxic agents [217]. Similarly, in a recent study a dinucleotide, mimicking a signal produced by UV-DNA damage, elicited a protective response to UV irradiation involving activation of the p53 pathway and enhanced repair [218].

Thus, there is substantial evidence to suggest that p53 plays a central role in the cellular response to DNA-damage. It is also clear that p53 can control the processing of Pt adducts. Additional experiments are needed to clarify exactly which function p53 fulfills in the management of the DNA repair pathway.

HMG1. Finally, a surprising finding that might connect cisplatin and p53 was made with the discovery that HMG1 enhances both p53 binding to DNA and its transactivation activity [219]. In particular, HMG1 was suggested to play an architectural role promoting the cooperative formation of protein complexes containing p53 at the site of transcriptional regulation. Although no direct interaction between p53 and cisplatin-modified DNA has yet been reported, the protein recognizes other types of DNA lesions and has been detected in association with some repair factors [162][204][205]. It is therefore feasible that HMG1 and p53 could encounter each other at the sites of cisplatin-DNA adducts in response to cellular exposure to the drug.

Another intriguing scenario is that cisplatin-DNA adducts could modulate p53 activity by some manifestation of the titration hypothesis (*Fig. 5, A*). For example, if p53 and HMG1 were to interact *in vivo* as they do *in vitro*, HMG1 could mediate the specific binding of this complex to cisplatin-modified DNA, keeping the p53 protein away from its natural targets. At least some of the p53-regulated activities are enhanced by treatment with cisplatin, however, providing evidence against this theory. In an inverse situation, cisplatin-induced p53 might attract HMG1 away from the platinum adducts, preventing it from shielding the lesions from repair. This hypothesis provides another possible link between p53 and repair. More detailed information about such an interaction *in vivo* is required before any conclusions can be drawn about how it contributes to the cisplatin mechanism of action.

Other Nuclear Proteins and Cisplatin-Modified DNA

DNA-PK

Several other proteins can bind cisplatin-modified DNA and hence might influence cellular processing of the damage. One example is the DNA-dependent protein kinase, DNA-PK. This protein plays a role in the repair of DNA double strand breaks, a product of ionizing radiation as well as V(D)J recombination [220][221]. There are two components of this complex. The first is the Ku heterodimer, a DNA-binding protein that specifically recognizes the ends of duplex DNA as well as single-strand-to-double-strand transitions, nicks, and hairpins. The second factor is the catalytic subunit, DNA-PK_{CS}, which functions as a kinase only when associated with DNA-bound Ku. Multiple phosphorylation substrates have been identified *in vitro*, including itself, p53, RPA, c-Jun, HMG1, and a variety of other transcription factors. DNA-PK might modulate cisplatin activity indirectly, since cells with a DNA-PK defect were sensitive not only to double-strand-break-inducing agents, but also to cisplatin and UV radiation [222]. These cells exhibited lower levels of nucleotide excision repair, which was restored by transfection with the wild-type DNA-PK gene. In an *in vitro* assay, however, the levels of repair synthesis were unaffected by addition of purified DNA-PK, or by clearing the extracts by immunoprecipitation. No complementation was observed when two extracts with deficiencies in different components of DNA-PK were mixed together. It was suggested that DNA-PK must play a regulatory role in the excision repair pathway, possibly activating the cell to the presence of DNA-damage by phosphorylating transcription factors.

DNA-PK can directly bind to cisplatin-modified DNA, but the interaction does not enhance its phosphorylation activity [223]. In fact the presence of the platinum adducts on the DNA substrates significantly decreased the phosphorylation activity when compared with undamaged DNA [223][224]. It is not immediately apparent how these results can be reconciled with the hypothesis discussed above, but only a few substrates were investigated, so it is possible that the full range of effects of cisplatin-DNA adducts on activity are still not known. In addition, since the affinity for damaged DNA was slightly less than for undamaged DNA, the significance of this interaction in the cisplatin mechanism of action may be minimal.

Histone H1

The histone H1 protein has higher affinity for cisplatin-modified DNA than for DNA damaged by *trans*-DDP or undamaged DNA [225]. This inter-

action is similar to that of other abundant chromosomal proteins, HMG1 and HMG2, and may reflect common functions involving the organization and maintenance of chromatin structure. Like HMG1/2, H1 preferentially binds to altered DNA structures, including supercoiled DNA and four-way junctions [226]. This lysine-rich protein is associated with the linker DNA of chromatin, and may bind to the DNA where it crosses itself as it enters and exits the core particles. H1 interacts with HMG1 *in vitro*, and it has been suggested that HMG1 functionally replaces H1 during the remodeling of chromatin that occurs during replication, transcription, or repair [226][227]. It is feasible that this protein could effect cisplatin cytotoxicity in the same manner as the HMG-domain proteins. This hypothesis is made more attractive by the existence of tissue-specific histone proteins, including testis-specific variants of H1 [226].

TBP

Another protein that binds to cisplatin-modified DNA is the TATA box-binding protein TBP [228][229]. The addition of either UV- or cisplatin-damaged DNA inhibited transcription in an *in vitro* assay, and activity was restored by the addition of the basal transcription factor TBP both in a reconstituted system and in whole cells. TBP Activity might be titrated away by the presence of cisplatin-DNA lesions, as postulated for HMG-domain protein (*Fig. 5, A*). Structural similarities were noted between the TBP-bound DNA target and the crystal structure of the 1,2-d(GpG) cisplatin intrastrand adduct, including the DNA bend as well as an opening and flattening of the minor groove. This correspondence may promote the formation of similar protein-DNA complexes, since TBP bound to the two types of DNA generated comparable DNase 1 footprints [229], and the transcription factor did not associate with DNA modified by other compounds, such as *trans*-DDP. These experiments suggest that TBP has the potential to play a major role in the mechanism of action of cisplatin.

Telomeres/Telomerase

The ends of chromosomes are protected from fusion, degradation or rearrangements by a repetitive DNA sequence known as the telomere [230]. In humans, telomeres comprise 500 to >2000 tandem repeats of the 6 bp sequence TTAGGG. Such a G-rich strand provides an attractive target for cisplatin. A recent study of cisplatin-treated HeLa cells suggested that a low dose of the drug causes telomere shortening due to incomplete replication

of the chromosome ends, followed by induction of the programmed cell-death pathway [231]. Whether such DNA adducts are important in the cytotoxic mechanism of cisplatin remains to be determined, but the unique telomere sequence does present a novel target for designing new platinum-based antitumor agents.

Telomeres also protect the cell from losing important genetic information due to the shortening of the chromosome ends at each replicative cycle. Since the telomeric sequence is finite, however, after a certain number of cellular divisions a critical length is reached and the cells enter senescence and die [232]. To avoid such a crisis, some cells maintain the telomere structure through the activity of the ribonucleoprotein telomerase, which synthesizes new repeats at the ends of the DNA. Telomerase activity has been detected in the majority of human cancers, but not in most normal tissue, implicating this enzyme in cellular immortality and suggesting novel approaches to cancer therapy [233][234]. Inhibition of telomerase activity by an antisense vector increased the sensitivity of a malignant glioblastoma cell lines to cisplatin-induced apoptosis [235]. A recent study investigated whether telomerase activity can be blocked by cisplatin. Exposure of testicular cancer cell lines to the drug inhibited telomerase activity, an effect not observed with bleomycin, doxorubicin or *trans*-DDP [236]. The authors suggest that cisplatin-DNA adducts can specifically inhibit the transcription of the G-rich gene for the RNA component of the enzyme.

Concluding Remarks

Cells exposed to cisplatin can respond either by attempting to fix the damage or, if the injury is too extensive, committing suicide. It is unlikely that any one factor in the cell controls this crucial decision and thereby the clinical efficacy of the drug. Much is known about proteins that interact with cisplatin-DNA adducts. We are beginning to understand how cisplatin-induced DNA distortions provide a structural basis for protein recognition, and the identity and function of many factors which mediate cisplatin cytotoxicity are now being defined. However, these proteins are involved in a complex, interwoven set of pathways. Many of the details of these systems, particularly the methods of communication between critical cellular mechanisms, are still obscure and must be delineated in order to understand how the processing of the DNA lesions differs in sensitive and resistant tissue. The information on these diverse cellular responses should provide the basis for new therapeutic protocols and rational drug design.

A recent experiment with radiolabeled cisplatin has afforded new insight into its pharmacology. When radioactive [^{191}Pt]cisplatin was admin-

istered to cancer patients, gamma camera imaging revealed selective accumulation of platinum in tumor tissue as well as in several organs such as the liver, kidney, bladder, gastrointestinal tract, uro-genital region and the neck (*Fig. 7*) [237]. What distinctive features of the tissue produce this platinum localization? It is likely that differential delivery to the tissue as well as efficient uptake by the cells could have a significant role. It is also possible,

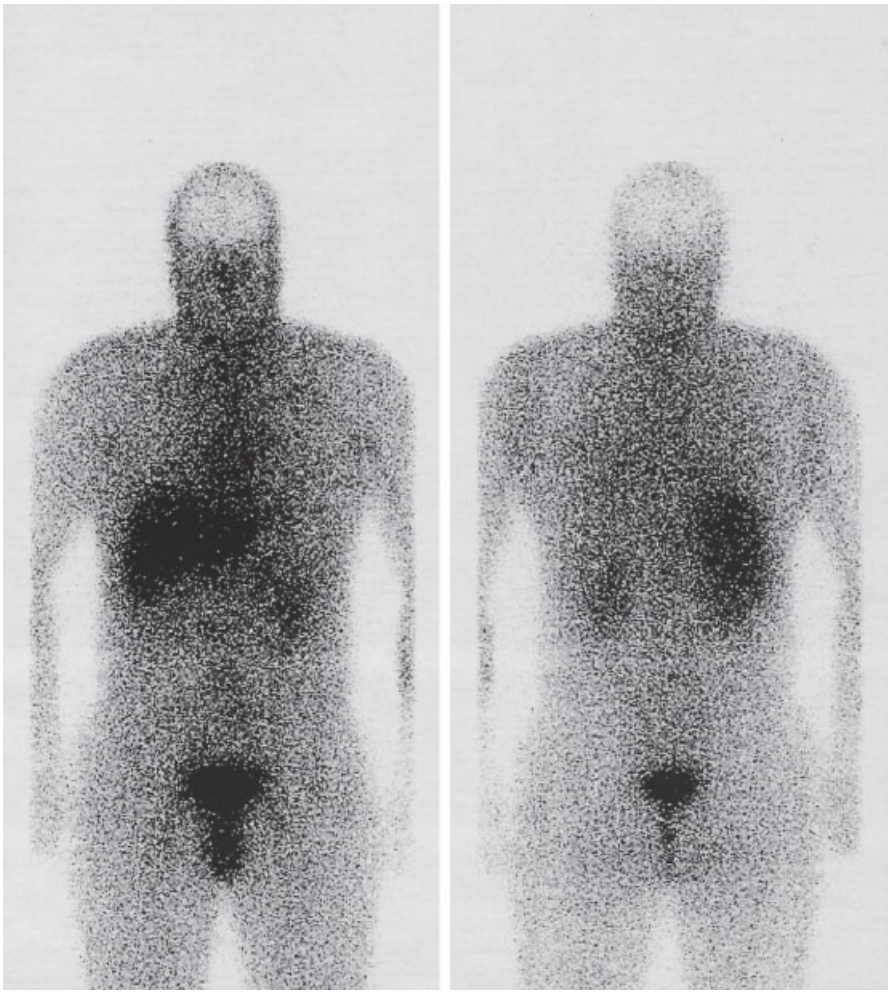

Fig. 7. *Whole-body image* (anterior to the left, posterior to the right) *of a testis cancer patient 1 h after the end of a [^{191}Pt]cisplatin infusion*. The platinum was still clearly detectable after 65 h but only weakly visible 7 days after the infusion. Chemotherapy was performed following surgery, so no macroscopic tumor tissue remained in the testis. In a different patient, however, platinum was detected in a metastatic tumor of the neck. Reprinted with permission from the Scandinavian University Press from [237].

however, that some of the proteins discussed above prevent rapid removal of the Pt-DNA adducts, allowing detectable levels of platinum to accumulate in the tissue. At this time, the relative importance of these two possibilities, uptake into the tissue *vs.* intracellular processing, is unclear, but these preliminary studies suggest that this issue is worthy of further pursuit.

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The Mechanism of Action of Cisplatin: From Adducts to Apoptosis

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Cisplatin is well known as a DNA-damaging agent, and the specific adducts produced in DNA have been well characterized. However, the mechanism by which these adducts kill cells is less well understood. Cisplatin causes cells to arrest at either the G1-, S- or G2-phase of the cell cycle in an attempt to repair the damage. Failing adequate repair, the cells eventually undergo an aberrant mitosis followed by apoptosis. Apoptosis can be described as multiple pathways converging from numerous different initiating events and insults such as cisplatin; these pathways converge on a common irreversible execution phase in which proteases and nucleases digest the doomed cell. Studies of apoptosis have identified many cellular factors that play a role in the decision as to whether a cell lives or dies. These factors include the p53 tumor suppressor, the Bcl-2 family of proteins, and intracellular signal-transduction pathways mediated by mitogen-activated protein kinases and phosphatidylinositol 3-kinase. Understanding cell-cycle regulation and apoptosis provides new targets that can be exploited to enhance the therapeutic activity of cisplatin. It has already been shown that the activity of cisplatin can be enhanced by preventing cell-cycle arrest, or by inhibiting protein kinase cascades, and these effects may be selective for the tumor. If these approaches are as effective in patients, cisplatin should continue to have a significant impact on the treatment of cancer.

DNA as the Critical Target for Cisplatin

The discovery in 1965 by *Barnett Rosenberg* that cisplatin caused filamentous growth of *E. coli* was the prelude to testing cisplatin as an anticancer agent [1][2]. *Rosenberg*'s results in Sarcoma 180 and Leukemia L1210 aroused interest at the National Cancer Institute, and soon cisplatin was tested and established as a drug with curative ability in testicular cancer and high potency in numerous other cancers. In 1979, *Roberts* and *Thompson* wrote a comprehensive review on the 'Mechanism of Action of Antitumor Platinum Compounds' [3]. For many people, including myself,

Cisplatin. Edited by Bernhard Lippert

this review was well-used and frequently cited. Possibly one of its major contributions was the discussion of the possible targets in a cell, and the conviction that damage to DNA was critical for the cytotoxic activity of cisplatin. This conclusion was consistent with the original observation in *E. coli*, the filamentous growth being explained as continued growth of the bacteria in the absence of DNA synthesis. The damaged bacteria still transcribed genes and synthesized proteins suggesting that DNA synthesis was the process critically affected. Other evidence for DNA as the critical target for cisplatin is the hypersensitivity of cells with defective DNA repair. For example, patients with Xeroderma pigmentosum are hypersensitive to sunlight due to defective repair of UV-induced DNA damage. Cells from these patients are also hypersensitive to cisplatin.

In early studies, it was evident that cisplatin caused DNA interstrand cross-links and DNA-protein cross-links. Both of these lesions were easy to study, not because they were the most predominant, but because they caused dramatic changes in biophysical properties: the apparent molecular weight of denatured, single-stranded DNA is increased because an interstrand cross-link prevents separation of the two complementary strands. Analysis of both DNA-interstrand cross-links and DNA-protein cross-links was further facilitated by the development of the alkaline elution technique which permitted rapid analysis of numerous samples. Accordingly, in many experiments, the production of DNA interstrand cross-links was correlated with cytotoxicity [4–6]. Our initial foray into this subject was an attempt to correlate DNA interstrand cross-links with the resistance observed in various sublines of murine L1210 cells [7]. It became evident that interstrand cross-links could not explain the response of these cells. Evidence for other lesions in DNA came from studies on the inactivation of bacteriophage. For example, interstrand cross-links in bacteriophage T7 were reported to be too rare to account for the observed inactivation of phage [8]. Similar studies on lambda phage reported that there were approximately 5 inactivating lesions for each DNA interstrand cross-link [9]. In their review, *Thompson* and *Roberts* provided evidence that cisplatin produced approximately 1 interstrand cross-link in 400 platinations [3]. They went on to discuss the potential sites in DNA that might be damaged by cisplatin, but it was evident that a definitive analysis of the chemical structure and frequency of all the lesions in DNA was required.

Characterization of DNA Adducts Produced by Cisplatin

In 1979, we began the characterization of the chemical structures of all of the adducts produced in DNA with the goal of establishing their contri-

bution to cytotoxicity [10–13]. Concurrently, *Fichtinger-Schepman et al.* performed similar work in the Netherlands [14][15]. The basic strategy employed by both groups involved enzyme digestion of *in vitro* platinated DNA with separation of the products by HPLC. The difference between this laboratory and *Fichtinger-Schepman et al.* was in the end-point of the digestion. Deoxyribonuclease I and P_1 nuclease were used to degrade DNA to deoxyribonucleotides, the end-point for *Fichtinger-Schepman et al.*, whereas further digestion with alkaline phosphatase yielded deoxyribonucleosides, our endpoint. These two complementary approaches eventually led to the definitive identification of the structures of the cisplatin-derived adducts in DNA. This work was reviewed in 1987 [16], and will only be briefly summarized here.

We initially characterized the reaction of cisplatin with deoxyribonucleosides which gave various mono- and diadducts (*e.g.*, dG-Pt and dG-Pt-dG) that were used as chromatography standards. To determine the structures of adducts in DNA, we decided to use a radioactive analog to facilitate discrimination of a low level of adducts against a high background of unmodified deoxyribonucleosides. This turned out to be an important decision when it was subsequently found that some sites in DNA are preferentially modified and therefore become saturated at high levels of platination. The radiolabeled analog used was *cis*-dichloro([^{3}H]ethylenediamine)platinum(II). This analog is also an effective antitumor agent in experimental models and produces adducts at identical sites in DNA as cisplatin. This analog became invaluable later for confirmation that the same adducts also occurred in cells.

The major site of platination in double-stranded DNA (65%) derives from intrastrand cross-links between two neighboring deoxyguanosines (GG) (*Fig. 1*). About 20% of the DNA platination derives from intrastrand cross-links at an AG sequence, but no adducts were detected when these two nucleosides were in the opposite order (*i.e.*, GA). Another 9% of the platination derives from a cross-link between two deoxyguanosines separated by a third nucleoside (*i.e.*, in a GNG sequence where N is any nucleoside). All of these modifications are through the N(7) position on the purine ring. Following incubations of more than a few hours, there was no evidence for monofunctional platination of DNA. However, following an incubation of 15 min, over 40% of the platination of DNA was in the form of monofunctional modification of deoxyguanosine; these adducts rearranged rapidly to the various bifunctional adducts. Note that no monofunctional modifications were found at deoxyadenosine suggesting that the initial reaction at an AG sequence is with the deoxyguanosine. The N(7) position of the deoxyadenosine in the orientation AG is very close to the second leaving group of cisplatin, thereby favoring reaction with this site; this may explain the lack of cross-linking in the sequence GA [12].

The high frequency of cross-links at GG sequences deserves further explanation. If cisplatin randomly reacted with every deoxyguanosine, and subsequently cross-linked to a neighboring base, there would be no more than 36.8% of the adducts at GG sequences, that being the frequency at which GG occurs in the human genome. To obtain 65% of the platination at GG requires that cisplatin preferentially targets this sequence for reaction. This has been described as the most electronegative region in double-stranded DNA [17] and therefore the most reactive to the positively charged, aquated cisplatin intermediate. Interestingly, the electonegativity of single-stranded DNA is much more dissipated throughout the molecule, and the frequency of adducts approaches much more closely that expected for an initial reaction randomly with any deoxyguanosine [16].

DNA interstrand cross-links were also purified and found to be formed between two deoxyguanosines, but this requires a major contortion of the DNA structure and may only occur when an alternate purine is not in close proximity on the same strand [12]. This presumably explains why interstrand cross-links occur at less than 1% of the total platination of DNA. One other adduct that was shown to form *in vitro* was a cross-link between deoxyguanosine and glutathione [18]. This adduct could be produced when

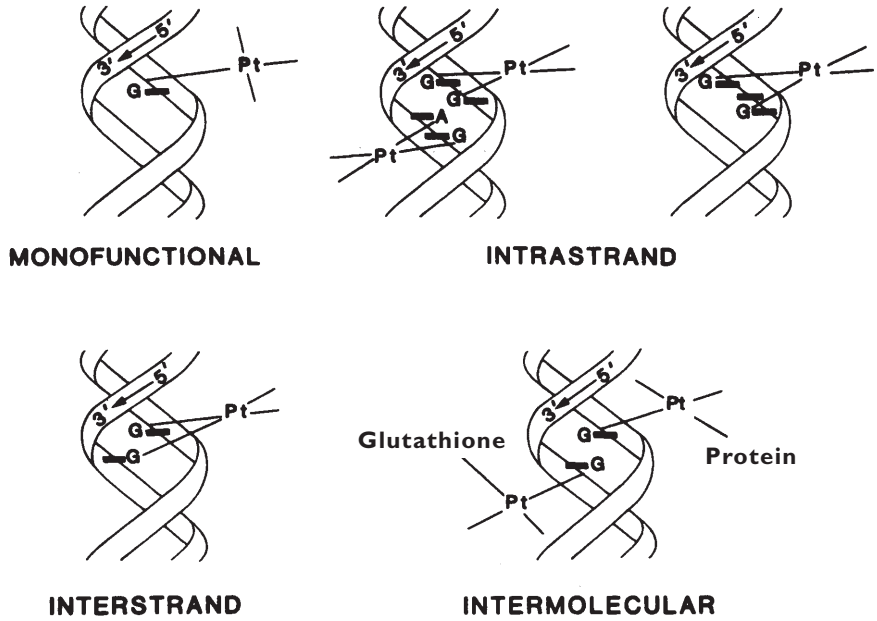

Fig. 1. *Structures of the various adducts produced in DNA by cisplatin.* Reproduced with permission from [16].

the DNA was first platinated for a short time to give monofunctional adducts, and then glutathione was added. Glutathione at concentrations found in cells (1–5 mM) can also reduce the overall level of DNA platination, but our results showed that glutathione can prevent the formation of cross-links even after the DNA has been initially platinated. This observation also explains the relative inactivity of *trans*-diamminedichloroplatinum(II) which rapidly produces monofunctional adducts, but which rearrange very slowly to bifunctional adducts. These long-lived monofunctional adducts are readily cross-linked to glutathione which prevents them from producing cross-links within DNA [19].

The same techniques used for characterization of DNA adducts *in vitro* were also applied to the analysis of cells treated with the radioactive cisplatin analog [20][21]. Again, > 60% of the DNA platination occurred as a cross-link at GG sequences, while the frequency of cross-links at AG and GNG sequences represented about 10% each. No other major adducts were detected as might result from DNA-protein or DNA-glutathione cross-links, but such lesions could not be ruled out as a low level of radioactivity was always recovered on the HPLC in the areas where such adducts would be expected. Similar results were obtained by *Fichtinger-Schepman* in cell culture and in human blood of patients receiving cisplatin, but in their case, they used immunochemical detection with antibodies developed to the defined adducts [22][23]. Hence, these results confirmed that the same adducts occurred in cells as in pure DNA, although the frequency of the lesions may be slightly different.

How Much Cisplatin Does It Take to Kill a Cell?

Despite knowing the structures of all of the DNA lesions, it remains to be determined if any one lesion is more or less toxic to the cells. As discussed above, it was originally thought that DNA interstrand cross-links were the critical lesion. Once it was realized that these lesions are very rare, opinions shifted to suggest that DNA intrastrand cross-links are more cytotoxic. Unfortunately, there is no specific data that implicates either type of lesion in cytotoxicity. For some drugs like nitrosoureas, DNA repair pathways that remove only selected lesions (*i.e.*, *O*(6)-methylguanine DNA methyltransferase) have helped to define the role of a particular lesion [24]. No separate pathway has been found for repair of a specific cisplatin adduct, so this approach has not been informative. A number of experiments have been performed in which specific adducts on defined DNA sequence, have been transfected into cells. This approach has shown that an adduct inhibits replication or transcription, but this does not directly address the question of mechanism of cytotoxicity.

One way to gain a perspective on the relative importance of specific cisplatin adducts is to determine how many lesions it takes to kill a cell. *Roberts* measured the amount of DNA platination in many cell lines and compared this to the degree of cytotoxicity. He concluded that cytotoxicity occurs when there are around 2–10 nmoles of Pt/g DNA, which reflects about 1 Pt/100,000–500,000 nucleotides [25][26]. Cytotoxicity in these assays was defined as 63% loss of colony-forming ability, that is, an average of one lethal hit per cell based on a *Poisson* distribution. As discussed below, these cytotoxicity assays may not truly reflect cell killing as cells frequently arrest for several days before recovering, and they may not have grown to countable colonies within the time frame of the experiment. We performed similar experiments in human HL-60 cells using 50% inhibition of growth over 5 days as our measure of cytotoxicity [27], which is open to the same criticism that we have not defined a concentration that kills cells. However, our values for the level of DNA damage were very similar to those of *Roberts*. Specifically, we reported that 1Pt/250,000 nucleotides inhibited growth of HL-60 cells, a number that reflects 48,000 DNA adducts per cell. When one considers the number of interstrand cross-links at a predicted frequency of 1% of total platination, it is realized there are 480 interstrand cross-links/cell. Hence a cell clearly needs to contend with a large number of these lesions. In contrast, ultraviolet radiation produced 1,080,000 lesions/cell to attain the same degree of toxicity [27]. Perhaps the lack of potency of ultraviolet damage is due to the lack of interstrand cross-links, the relative lack of potency of intrastrand cross-links, or it could simply be due to far more effective repair of the damage. These numbers do not answer the question as to which lesion is critical, but they do show that there are large numbers of every type of cross-link in a cell treated with cisplatin, and any or all of the lesions could be important for the cytotoxicity of the drug.

Cell-Cycle Perturbations Following Cisplatin

The binding of cisplatin to DNA is not in itself sufficient to cause cell death. Cells usually take several days to die after incubation with cisplatin, and it is during this time that decisions are made that impact on the survival or death of the cell. Cisplatin is generally considered a cell-cycle-phase non-specific drug, but it is still more toxic to dividing cells than to resting cells. Furthermore, cisplatin can be up to 10 times more toxic to cells that are about to enter S-phase than cells that have just exited S-phase. In one series of experiments, normal human fibroblasts were incubated with cisplatin while arrested at confluence; at various times thereafter, they were analyzed for the amount of DNA-bound platinum, and plated to score cell

survival [25]. The cloning efficiency of cisplatin-treated cells increased from 1% to 30% over 6 days consistent with the observed rate of repair of DNA damage during this period. These results suggested that cytotoxicity is a function of the amount of unrepaired damage remaining in DNA at the time cells enter S-phase. Accordingly, it appeared that disruption of DNA synthesis is critical for cytotoxicity.

In 1983, *Salles at al* showed that inhibition of DNA synthesis did not correlate with cytotoxicity [28]. This paper showed that much higher levels of cisplatin were required to inhibit DNA synthesis than were needed to inhibit cell growth. Unfortunately, DNA synthesis was measured for only 90 min after drug treatment. We attempted to explain this discrepancy in studies of leukemia L1210 cells [29]. Following a 2 h incubation with cisplatin, cell cycle perturbation was measured by flow cytometry for up to 10 days. At low concentrations of cisplatin, cells accumulated in the G_2-phase for 1–3 days and then recovered. Such cells clearly had the capacity to replicate on damaged DNA, but arrested in G_2 to repair critical damage before continuing to proliferate. At higher drug concentrations, the cells still progressed to G_2, although this was often preceded by a slow passage through the S-phase. Cell death as assessed by loss of membrane integrity occurred between 4 to 6 days. These results show two reasons for the observed inhibition of DNA synthesis, first cisplatin causes a direct block to DNA synthesis at high concentrations, and second, that cisplatin arrests cells out of the S-phase of the cell cycle. The arrest of cells at G_1, S or G_2 is now known to be regulated by complex pathways described as cell-cycle checkpoints which are discussed in more detail below.

Analysis of cell-cycle perturbation following incubation with cisplatin was studied further in Chinese hamster ovary (CHO) cells either proficient or deficient for DNA repair [30]. Repair-proficient CHO/AA8 cells responded in a manner similar to L1210 cells. Cisplatin caused S, then G_2-phase accumulation of the cells followed by recovery at low concentrations. At higher concentrations of cisplatin, the cells died after a prolonged G_2 arrest. The repair-deficient CHO/UV41 cells died at much lower concentrations of cisplatin; these concentrations caused no apparent perturbation of S-phase, but still caused G_2 arrest prior to death. In one series of experiments, cells synchronized in G_2 were incubated with cisplatin and found to cycle to the next G_2 before arresting. These results show that inhibition of DNA synthesis may not always be apparent, although passage through S-phase still appears to be a prerequisite for G_2 arrest and the subsequent cell death. This suggests that cells may eventually die because of secondary damage inflicted while trying to replicate on a damaged DNA template.

Continuing these studies still further, we established that CHO cells undergo mitosis prior to dying, and that inhibition of protein synthesis with

cycloheximide prevented both mitosis and cell death [31]. Following a prolonged G_2 arrest, the cells eventually condensed their chromosomes as if entering mitosis, but the chromosomes were scattered rather than neatly organized as a metaphase plate (*Fig. 2*). In some cases, it appeared that the chromosomes were segregating to more than the usual two poles of the cell. Staining for β-tubulin showed that more than 80% of the cells had three or four mitotic spindle poles. Hence, it appeared that the spindle pole continued to divide even while the DNA replication cycle was arrested. A G_1 pop-

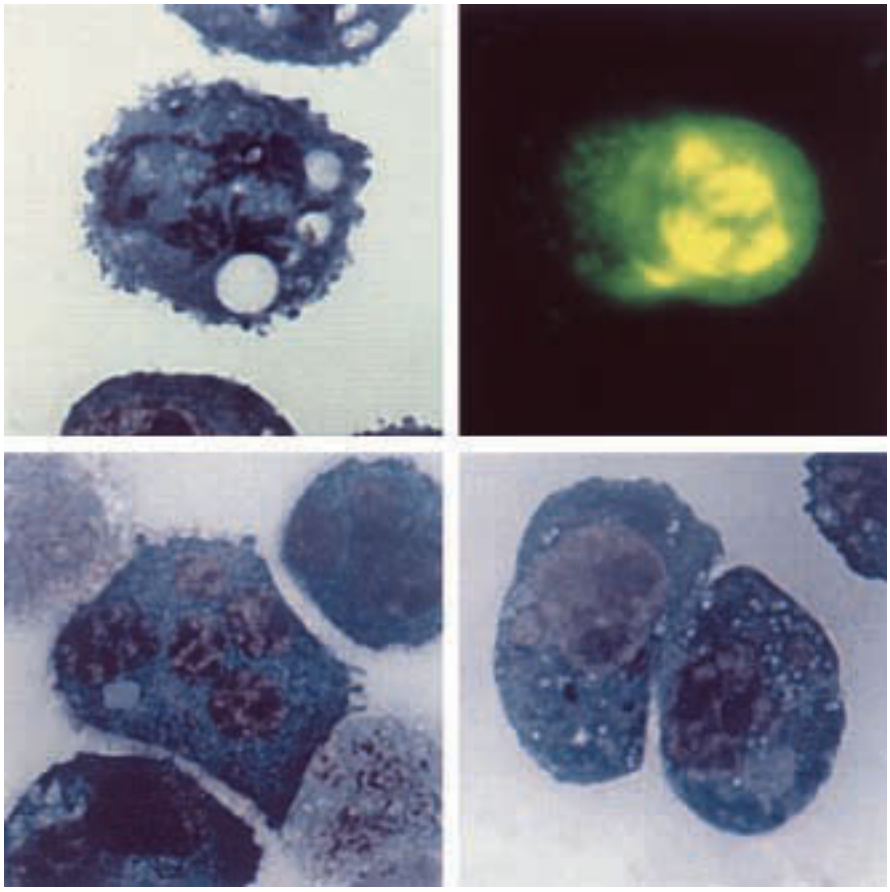

Fig. 2. *Morphology of aberrant mitosis induced by cisplatin*. Following incubation with cisplatin, cells progress to G_2 and eventually enter mitosis. The top left panel shows a cell undergoing mitosis, but the chromosomes appear to be pulled to three different loci in the cell. The top right is a mitotic cell stained with an anti-β-tubulin antibody and shows the presence of multipolar mitotic spindles. The bottom panels show the consequence of aberrant mitosis; cells form nuclear membranes around scattered chromosomes giving either many large nuclear particles (*right*), or a few micronuclei (*left*). The cells were stained with Giemsa so that the nuclei stain purple and the cytoplasm blue.

ulation of cells still resulted but the cells had a heterogeneous DNA content; many of these cells showed micronuclei where individual chromosomes appeared to have been surrounded by a new nuclear membrane. Some cells appeared to die immediately as judged by loss of membrane integrity and detachment from the culture dish, while others reattached to the dish after mitosis, and died over the following 24 h. This line of investigation has not yet been performed in as much detail in other cell lines, so the generality of the appearance of lethal mitosis remains to be determined. However, results to date in numerous cell lines suggest that cisplatin-damaged DNA causes cell-cycle perturbation, an arrest in the G_2-phase to repair damage, and in the absence of adequate repair, the cells eventually undergo an abortive attempt at mitosis that results in cell death.

Overcoming Cell Cycle Arrest as a Therapeutic Strategy

In 1989 *Hartwell* and *Weinert* coined the term 'cell-cycle checkpoint' to describe how a cell arrested in the face of environmental signals [32]. Perhaps the best known cell-cycle checkpoint is that mediated by the tumor-suppressor protein p53 [33][34]. When cellular DNA is damaged, the p53 protein is stabilized causing transcriptional activation of $p21^{waf1}$, an inhibitor of cyclin-dependent kinase, and arrest of the cell at the G_1-phase of the cell cycle. The fact that more than 50% of human tumors are defective in this p53 response sets them apart from normal tissue, and these tumors exhibit a marked arrest in the S- and G_2-phase as discussed above (the CHO cells discussed above are defective for p53). Many yeast mutants have been identified with defective 'cell-division cycles' and a number of these are defective in response to DNA damage [32]. Of particular interest is the RAD9 mutant which fails to arrest in G_2 following DNA damage; such cells have no time to repair DNA damage but rather pass directly into a lethal mitosis [35].

The above discussion suggests a potential therapeutic strategy in which drugs might overcome cell-cycle arrest in tumors and enhance cytotoxicity. Such a strategy has been known for almost 30 years, although the mechanism was not initially realized. The prototype drug is caffeine which is capable of enhancing the cytotoxicity of DNA-damaging anticancer agents [36]. In 1982, *Lau* and *Pardee* showed that caffeine abrogated the G_2 arrest elicited by DNA damage, thereby limiting the time available for DNA repair [37]. Many subsequent papers, including our own [31], have confirmed this observation. This strategy has the potential to selectively target cells that are defective in the p53 tumor-suppressor gene. Tumors with mutant p53 fail to arrest in G_1, but instead arrest in S and G_2 where the addition of

caffeine causes a rapid abrogation of the cell-cycle arrest, and enhances cytotoxicity (*Fig. 3*). Further impetus for this therapeutic strategy came when it was realized that p53 could also modify G_2 arrest. Although p53 is not required for G_2 arrest, it does appear to prevent caffeine from abrogating the arrest [38–40]. Caffeine seemed like a promising drug with the ability to enhance cisplatin therapy preferentially in the absence of p53. Unfortunately, the concentration of caffeine required to achieve these effects in cell culture (5 mM) exceeds by more than 50-fold that tolerated by a patient.

Recently, we established that 7-hydroxystaurosporine (UCN-01) is 100,000 fold more potent than caffeine at overcoming the G_2 arrest, and dramatically enhances the cytotoxicity of cisplatin in Chinese hamster ovary cells at exactly the same concentrations that bypass the G_2 checkpoint [41][42]. UCN-01 also enhanced the activity of cisplatin in human cell lines, and furthermore, this occurred preferentially in cells with disrupted p53 function [43]. Toxicology experiments have shown that the required doses of UCN-01 are well tolerated in both mice and dogs [44]. Accordingly, UCN-01 would appear to have great potential to be used effectively in combination with cisplatin to enhance cell killing specifically in the tumor. The importance of this strategy for the current discussion is that it emphasizes the

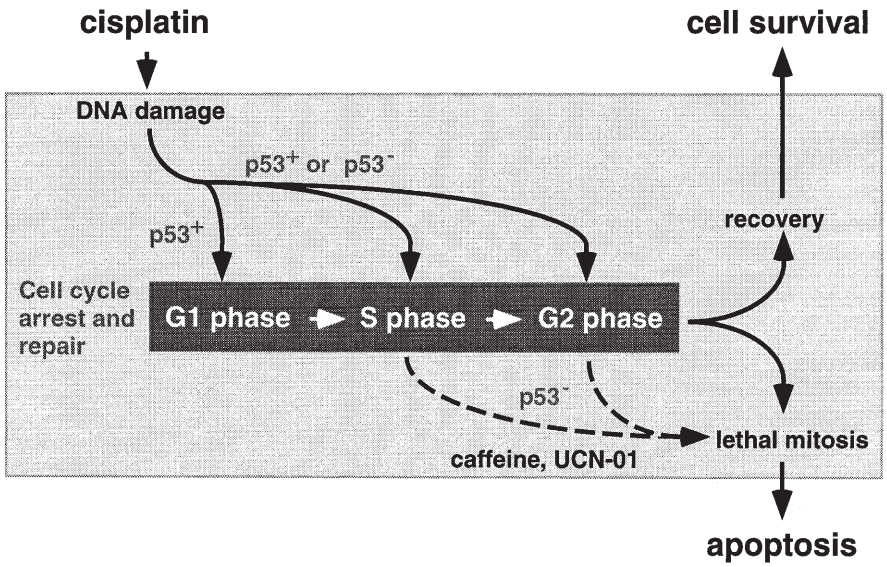

Fig. 3. *The cell-cycle perturbations that occur as a consequence of DNA damage induced by cisplatin*. The dark box represents the time period during which cells arrest at various phases of the cell cycle with the intent to repair the damage. Once the DNA is repaired, cells may recover and continue to grow. The dotted arrows imply that caffeine and UCN-01 can overcome S- and G_2-phase arrest and drive the cells into a lethal mitosis.

importance of G_2 arrest for cell survival, and that cisplatin-induced cytotoxicity is usually the consequence of undergoing mitosis before DNA is adequately repaired. In ongoing experiments, we have provided yet further evidence for the importance of mitosis for cytotoxicity. Cisplatin-arrested cells were incubated concurrently with UCN-01 and the mitotic inhibitor nocodazole; these cells remained arrested in mitosis rather than dying. Accordingly, it appears that cells need to complete mitosis and probably enter G_1 before they die.

A word of caution is required. Very high concentrations of cisplatin will rapidly arrest cells at all phases of the cell cycle, and they die without ever progressing to G_2/M-phase. However, this is unlikely to be relevant to the *in vivo* situation where significant G_2 arrest has been observed. Nude mice carrying a human ovarian carcinoma showed a marked accumulation of cells in the G_2-phase for up to 114 h following treatment with 10 mg/kg cisplatin [45]. In a transplantable murine mammary tumor, we have observed a dramatic accumulation of cells in G_2 between 24 and 96 h following 5 mg/kg cisplatin (unpublished observations). This cell-cycle arrest is consistent with the observed 5 day tumor growth delay in this model. Accordingly, it appears that cell-cycle arrest at G_2 is very relevant to the *in vivo* action of this drug, and that a subsequent lethal mitosis may be the most relevant mechanism of cell death induced by cisplatin.

Apoptosis

Apoptosis is the term coined by *Kerr*, *Wyllie*, and *Currie* in 1972 to describe a distinct morphology of dying cells [46][47]. Apoptotic cells appear in histological sections as isolated shrunken cells that have lost contact with their neighbors. Within these dying cells, chromatin can be seen condensed at the nuclear membrane, while other organelles appear normal. Apoptotic cells often produce membrane protuberances known as blebs, and are rapidly engulfed by neighboring cells thereby avoiding an inflammatory response. It was hypothesized that this death represented tissue homeostasis in which there is a balance between cell replication (mitosis) and cell death (apoptosis). In 1980, *Wyllie* showed that apoptosis was characterized by digestion of chromosomal DNA in the inter-nucleosome spacer region giving a 'ladder' of fragments of 180 base-pair multimers [47][48]. The subject of apoptosis received very little interest until around 1990 when oncogenes and tumor-suppressor genes were discovered to regulate the process. The first report that apoptosis also occurred in response to anticancer drugs occurred in 1975 [49], but this observation was also overlooked until 1990, the same year that cisplatin was established as a drug that induces apopto-

sis [50–52]. Since then, there has been an explosion of new information and understanding of the events that regulate cell survival and apoptosis, and many of these events will be highlighted below.

Apoptosis is induced during numerous physiological processes including development, tissue remodeling, and regulation of the immune system, while defects in the process can lead to many human disorders such as cancer, autoimmune diseases, neurodegeneration and AIDS [53]. At the cellular level, the stimuli for apoptosis include engagement of certain receptors, such as the Fas/CD95 or glucocorticoid receptors; disengagement of a receptor, such as occurs upon removal of serum growth factors; or exposure to numerous stressful environmental conditions. Considering that numerous cytotoxic agents also induce apoptosis, one can see the potential complexity in the regulatory networks required to integrate this information and to decide the fate of a cell. To help understand apoptosis, it is necessary to discriminate three different stages (*Fig. 4, A*): 1) an initiation phase, in which

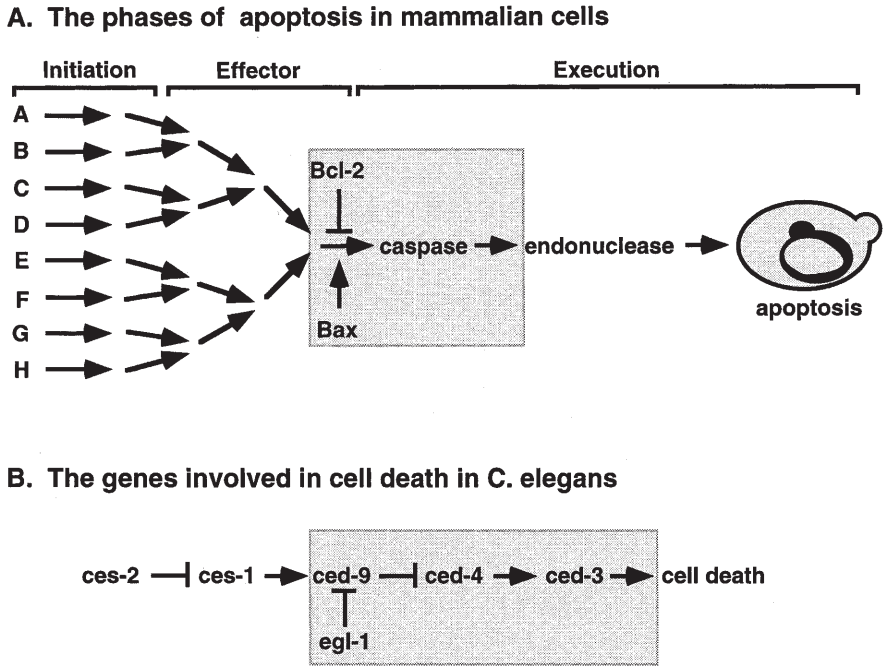

Fig. 4, A. *A diagrammatic representation of the converging pathways leading to apoptosis in mammalian cells*. Multiple different insults can initiate the pathway through numerous different means. The effector phase integrates these signals leading to the decision of life or death. The execution phase of apoptosis is irreversible and common to all insults. B. *The genetic pathway of programmed cell death defined in* C. elegans. The shaded boxes reflect the area of the pathways that have been shown to be highly conserved between species. The endonuclease has not yet been defined in *C. elegans*.

a signal is received and one of numerous possible pathways specific to that particular signal are engaged; 2) an effector phase, in which the many initiating signals are integrated and a decision to live or die is made; and 3) a common irreversible execution phase, in which the cell undergoes autodigestion of proteins and DNA. Following the execution phase, the cell corpse is engulfed by neighboring cells and eventually destroyed so that no evidence remains.

The Execution Phase of Apoptosis

The simplest model organism that has provided a wealth of information on apoptosis is the nematode *Caenorhabditis elegans*. During normal development of the hermaphrodite form of the nematode, a process that involves exactly 1090 somatic cells, it is known that 131 cells die at precise times. Many mutants have been obtained that have defects in this process and that exhibit a number of phenotypes such as survival of all of these 131 cells, survival of 130 cells, survival of only a few of these cells, or death of the entire nematode. This has led to a model whereby cell and tissue-specific genes such as *ces-1* and *ces-2* regulate transcription of *ced-9* whose expression causes cell survival (*Fig. 4, B*). Downstream of *ced-9* are *ced-4* and *ced-3*, both of which are required for cell death. The most intriguing part of this model is that homologs are found in human cells, and some of these homologs can even function when expressed in the nematode.

The CED9 protein that protects the cells of *C. elegans* from death is homologous to the human oncogene Bcl-2. Bcl-2 was originally identified at the t(14;18) breakpoint in follicular B cell lymphoma [54]. Unlike other oncogenes, Bcl-2 does not stimulate cell proliferation, but rather protects cells from apoptosis induced by many stimuli. Hence, Bcl-2 appears to be at the convergence of many pathways of apoptosis, and may be the final determinant as to whether a cell enters the execution phase. Subsequent to the discovery of Bcl-2, a family of homologs have been discovered that can also protect cells (*e.g.*, Bcl-X_L, Mcl-1, Bcl-w and A1). There are also members of this family that have the opposite function in that they can either induce apoptosis or antagonize the protective activity of other members (*e.g.*, Bax, Bak, Bad, BclX_S). Recently, the *C. elegans* protein EGL-1 was identified as a member of this pro-apoptotic family [55]. These negative regulators of survival differ from the protective forms in lacking one or more essential domains present in Bcl-2 [56]. Bcl-2 appears to function by forming ion channels in membranes, particularly the outer mitochondrial membrane, and by interacting with other cell signaling proteins, but exactly how these functions suppress cell death remains to be determined.

The cloning of the *ced-3* gene led to clues as to how Bcl-2 could regulate cell survival. The CED3 protein was found to be homologous to the human interleukin 1β-converting enzyme (ICE) [57]. ICE is a cysteine protease with an unusual cleavage specificity in that it cuts its substrate interleukin 1β on the carboxy side of an aspartic acid. More than 10 mammalian homologs of ICE have been identified, and this family of proteases is now known as *c*ysteine-*asp*artate prote*ases*, or caspases [58]. Interestingly, each caspase is itself activated by proteolysis at aspartic acid either in an autocatalytic mode or more often as part of a caspase cascade (*Fig. 5*). This leads to two important questions: what initiates activation of the first caspase, and what are the critical substrates for caspase cleavage?

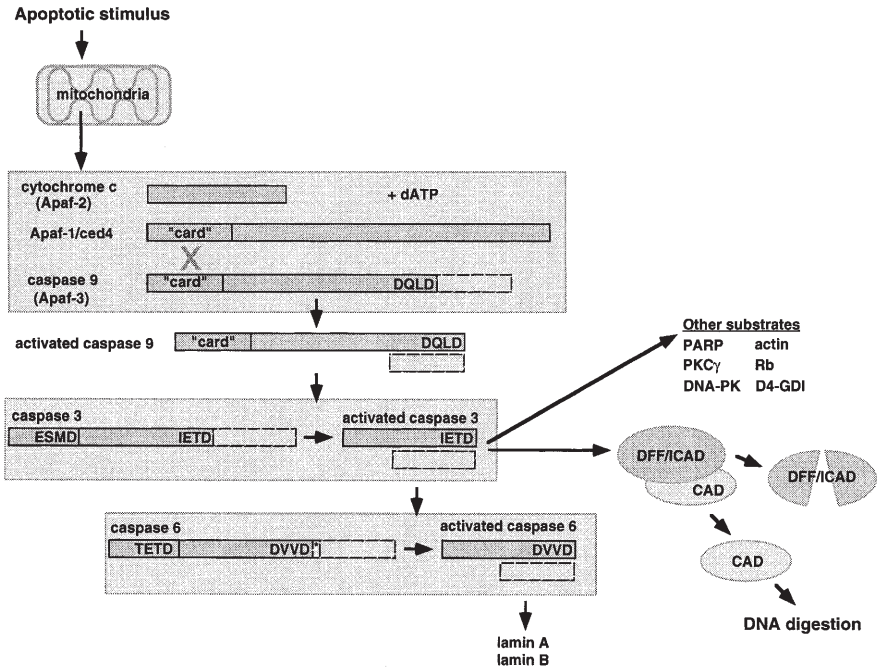

Fig. 5. *The execution phase of apoptosis.* An apoptotic stimulus causes the release of cytochrome *c* from mitochondria. The first box contains the components required to activate caspase 9; 'card' refers to the caspase-recruitment domain. Caspase 9 then activates caspase 3 (second box), which in turn activates caspase 6 (last box). The amino-acid sequences at the cleavage sites are shown. Caspases 3 and 6 also have a prodomain that is not present in the active protease; in caspase 6 an additional cleavage removes a small portion of the middle of the protein. Various substrates of the caspases are shown, including the pathway by which caspase-activated deoxyribonuclease (CAD) is activated which in turn leads to DNA digestion.

The answer to the question of how caspases are activated came from studies in cell extracts. It was found that cytosol isolated from undamaged cells can spontaneously activate caspases when dATP was added [59]. The cytosol was separated into three fractions defined as Apaf-1, Apaf-2 and Apaf-3 for *a*poptotic *p*rotease *a*ctivating *f*actors. Apaf-2 was identified as cytochrome *c*. Cytochrome *c* is normally located in mitochondria and should not have been present in the cytosolic fractions. Subsequently, cytosol was prepared using a method that retained mitochondria intact; these fractions now required the addition of both cytochrome *c* and dATP to activate caspases. It is now recognized that cytochrome *c* is released from mitochondria during apoptosis, and this is required for activation of the caspase cascade. Subsequently, Apaf-1 was identified as a homolog of CED4 [60], and Apaf-3 was identified as caspase 9 [61]. It is currently thought that Apaf-1 binds to caspase 9 which is then activated by cytochrome *c* plus dATP (*Fig. 5*), although the exact mechanism of this activation remains to be resolved. The trigger appears to be the release of cytochrome *c* from the inner mitochondrial space, a process blocked by Bcl-2, but the stimulus for this release also remains to be identified.

Once caspase 9 has been activated by cleavage, it in turn activates caspase 3, a central player in apoptosis in as much as it is responsible for cleaving numerous intracellular proteins such as poly(ADP-ribose) polymerase, DNA protein kinase, protein kinase C, actin, the Rho dissociation inhibitor D4-GDI, and the retinoblastoma susceptibility protein Rb (*Fig. 5*). Only one substrate has been clearly associated with the DNA fragmentation that eventually results. The cleavage of a 45 kDa protein designated DNA fragmentation factor, or DFF, appears essential for DNA digestion [62]. Subsequently, it was shown that the uncleaved murine homolog of DFF (called ICAD) is a suppressor of DNA digestion [63][64]. Cleavage of DFF/ICAD releases a novel endonuclease called caspase-activated DNase (CAD) that translocates to the nucleus and digests DNA. Cells expressing a cleavage-resistant form of ICAD no longer digest their DNA despite demonstrating other morphological characteristics of apoptosis [64].

The involvement of CAD as the endonuclease in apoptosis contradicts previous suggestions of other endonucleases involved in apoptosis. CAD is a Ca^{2+}-independent endonuclease whereas many groups have implicated a Ca^{2+}-dependent endonuclease in apoptosis [65–69]. However, we have presented considerable evidence that Ca^{2+} is not required for DNA digestion, and furthermore, that depleting Ca^{2+} is a stimulus for DNA digestion [70][71]. We have implicated deoxyribonuclease II (DNase II) as an alternate endonuclease involved in apoptosis [72]. DNase II requires low pH for activity, and we have established that intracellular acidification is a common occurrence in apoptosis [73–76]. Intracellular acidification is a conse-

quence of caspase activity by as yet unknown mechanisms. We have recently cloned the human DNase II cDNA and shown that it induces DNA fragmentation upon reintroduction into cells [77]. This was unexpected as the intracellular pH should be too high for DNase II activity. This suggests that cells may contain an endogenous inhibitor of DNase II whose inhibitory activity has been exceeded by expression of the transfected gene; perhaps such an inhibitor is also cleaved by caspases. The current hypothesis is that DNase II may be one of several endonucleases that can mediate DNA fragmentation during apoptosis.

The Effector Phase of Apoptosis

The previous section discussed the common events that occur in a cell dying in response to numerous stimuli or insults. Upstream of mitochondria, there are numerous converging pathways that transmit the initiating signals from the various targets to the mitochondria, causing activation of the caspase cascade. This part of the apoptotic signaling pathway is much less well understood, although a number of players have been identified, most notably components of normal intracellular signal transduction cascades.

Ever since the first successful attempts to maintain cells in culture, it has been known that serum is required for cell growth. Many growth factors in serum may be more appropriately defined as survival factors, and upon withdrawal, the cells undergo apoptosis. Survival is also promoted by contact with extracellular matrix components such as fibronectin that signal through integrin-type receptors. Cells also receive survival signals from their neighbors through cadherin-type receptors. Cells are exposed to many of these signals simultaneously, and rarely has any one signal been studied in the absence of all others. Realizing that all of these survival stimuli converge on the Raf/MEK/MAP kinase signaling pathway, I proposed in 1995 that activation of this pathway protected cells [78]. A major complexity to the role of MAP kinase in cell survival was realized with the discovery of multiple parallel MAP kinase pathways (*Fig. 6*). The MAP kinase homologs are termed Jun N-terminal kinase (JNK) and $p38^{Mpk2}$. The original MAP kinase pathway (now described as ERK1 and ERK2) is still found to protect cells, while a sustained activation of JNK has the opposite effect and is pro-apoptotic [79][80].

As an example of the integration of signals by this pathway, I will refer to some of our own ongoing research. We have found that many cytotoxic agents activate JNK, but the decision to undergo apoptosis can be influenced by the activity of ERK. For example, incubation of human ML-1 cells with anisomycin caused potent activation of JNK and onset of apo-

ptosis within 2 h. In contrast, incubation with vincristine caused milder activation of JNK, and little apoptosis resulted over 16 h. Selective inhibition of ERK activation with the MEK inhibitor PD98059 dramatically enhanced the rate of apoptosis induced by vincristine. However, cells grown in culture without other insults are very tolerant of PD98059, suggesting that suppression of ERK alone is insufficient to induce apoptosis. Confirmation that activation of JNK is important for apoptosis has been shown by a number of investigators using genetic approaches to inhibit the JNK-signaling pathway [81-83]. These results suggest that ERK can enhance cell survival in the face of a moderate level of JNK activation. It has been suggested that the critical function for JNK is activation of the transcription factor c-Jun; expression of a dominant-negative form of c-Jun can protect cells [84][85]. However, because transcription and new protein synthesis are not required for apoptosis in most cases, the importance of c-Jun seems difficult to reconcile. Hence, the critical substrates for ERK and JNK activity remain to be determined.

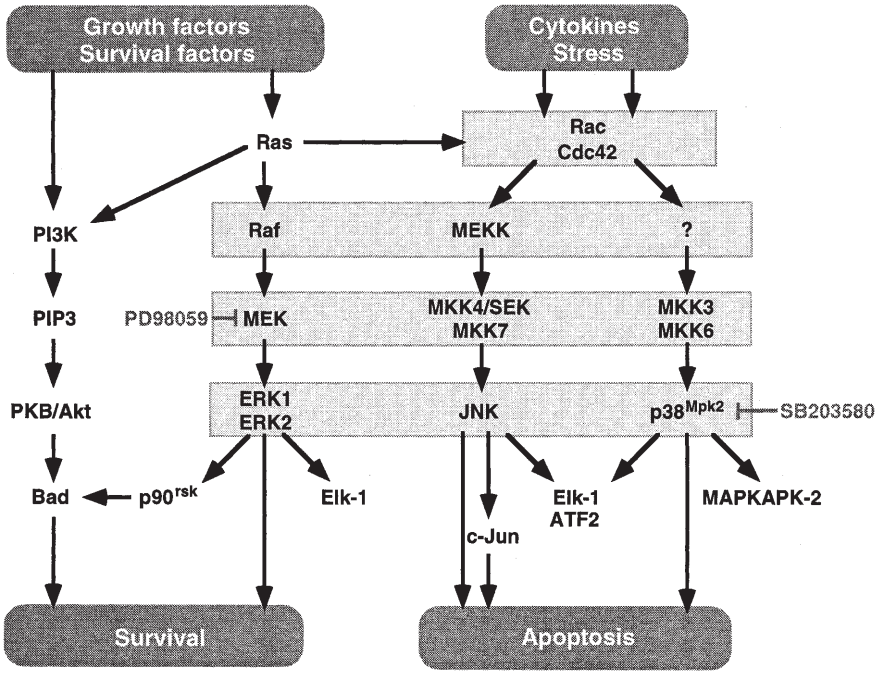

Fig. 6. *The signal-transduction pathways involved in regulating the effector phase of apoptosis*. Survival is enhanced by activation of the phosphatidylinositol 3-kinase (PI3K) pathway and the ERK1/2 pathway, while apoptosis is enhanced by activation of the JNK and/or $p38^{Mpk2}$ pathways. The proteins named in each box are homologs, although they are activated by distinct pathways as shown. The various components are discussed in the text.

The third MAP kinase pathway involving $p38^{Mpk2}$ has a more ambiguous role in apoptosis. In some models it appears that inhibition of $p38^{Mpk2}$ with SB203580 can suppress apoptosis [79][86], but more often it has been found to have no role in apoptosis or cell survival. Furthermore, in some models, suppression of neither JNK nor $p38^{Mpk2}$ appears to protect cells from apoptosis [86][87]. In most of these papers, attempts were made to inhibit only one of these pathways rather than inhibiting both pathways simultaneously, hence it is possible that both pathways may contribute in a redundant manner to the induction of apoptosis.

Another pathway stimulated by survival factors and that appears to protect cells from apoptosis is mediated by phosphatidylinositol 3-kinase (PI3K) [88–90]. The protective role of this pathway has been confirmed using Ras dominant-negative constructs that selectively inhibit signaling through PI3K [90]. The immediate downstream signaling event is the activation of protein kinase B (PKB), the human homolog of the transforming v-Akt, which is also essential for cell survival [90][91]. Intriguingly, one substrate for PKB/Akt is the Bcl-2 family member Bad; phosphorylation of Bad prevents its pro-apoptotic ability [92]. The same site in Bad can also be phosphorylated by $p90^{rsk}$, a downstream effector of the ERK1/2 pathway. Hence phosphorylation of Bad represents a convergence of two recognized survival pathways. However, these pathways also appear to protect cells in the absence of Bad suggesting that other important targets must also exist.

What Has Apoptosis Got To Do With Cisplatin?

Experiments in this laboratory identified apoptosis as a consequence of the action of cisplatin and many other anticancer agents [50–52]. Many authors have subsequently misquoted these results when suggesting that cisplatin kills by apoptosis, and that suppression of apoptosis is a mechanism of resistance. It should be evident by now that apoptosis is better defined as a consequence of the mechanism of action of cisplatin and a failure of the mechanisms of resistance. Apoptosis is certainly not an alternative to the formation of DNA cross-links, nor to the cell-cycle perturbations that result; these are still essential events in the initiation phase of apoptosis. The mechanisms of resistance to cisplatin still include reduced drug accumulation, reduced DNA platination, and altered DNA repair. However, apoptosis provides a framework for understanding the complete pathway from initial insult to eventual death of a cell. It provides the realization that there are additional factors that influence cell survival and death. Expression of Bcl-2 family members or changes in signal transduction pathways impact

on the response of cells to cisplatin as well as numerous other insults. This knowledge will provide new areas that can be exploited to enhance the therapeutic index of cisplatin.

The execution phase of apoptosis seems an unlikely area in which cisplatin cytotoxicity could be modified. By the time this part of the pathway is engaged, the cells have already committed to death. Furthermore, the mechanisms for the execution phase of apoptosis are present in all cells, so targeting these steps is unlikely to provide any selective action against the tumor. In contrast, modulation of the effector phase of apoptosis represents an intriguing area for exploitation. Tumor cells have modified their signal-transduction pathways in numerous ways, most notably by constitutively activating receptors such as $p185^{erbB2}$ (Her2/neu), or intracellular components such as Ras. Several attempts to interfere with these signaling pathways have been found to sensitize cells to cisplatin. For example, antibodies to the epidermal growth factor (EGF) receptor lead only to quiescence, but the combination with cisplatin causes a markedly synergistic cell kill [93]. The same has been seen when antibodies that antagonize $p185^{erbB2}$ were combined with cisplatin [94]. Finally, synergy has been seen by the combination of protein-kinase-C inhibitors and cisplatin [95][96]. These observations are consistent with the role of ERK1/2 in enhancing cell survival, and that inhibition of the ERK1/2 pathway may result in cell death if it occurs at the same time as an insult activates JNK. However, this hypothesis remains to be fully proven.

Cisplatin has been shown to activate JNK in a number of systems [83][97][98]. Furthermore, the suppression of JNK signaling with a dominant-inhibiting SEK suppresses cisplatin-induced apoptosis [83]. However, in all these papers, JNK activation was only measured up to 3 hours after addition of cisplatin. Considering the discussion above on cell-cycle progression following cisplatin, the importance of JNK activation so soon after treatment is difficult to rationalize. This activation of JNK occurs before the cells have undergone cell-cycle arrest, and several days before the cells pass through a lethal mitosis and die. This is far too early to have a direct effect on release of cytochrome *c* from mitochondria and activation of the caspase cascade. It has also been suggested that JNK regulates DNA repair following cisplatin [99], and ERK may contribute to cell-cycle arrest [100]. Hence, it remains to be established whether JNK has any role in lethal mitosis and the subsequent apoptosis induced by cisplatin. It also remains to be established whether suppression of ERK pathways with antibodies to the EGF receptor or $p185^{erbB2}$ still depends on passage through the cell cycle or whether the cells die rapidly without undergoing a lethal mitosis.

One other area of apoptosis that has received considerable attention is the role of the p53 tumor-suppressor protein in response to DNA-damaging

agents. As discussed above, DNA damage induces p53 leading to expression of $p21^{waf1}$ and G_1 arrest. This G_1 arrest is intended to protect cells, so it would be expected that cells with defective p53 would no longer arrest and therefore be sensitized to DNA-damaging agents. Cells derived from $p21^{waf1}$ knock-out mice are indeed more sensitive to cisplatin and other DNA-damaging agents [101], but this is frequently not the case for cells with defective p53.

Another function for p53 appears to be the induction of apoptosis as demonstrated when the wild-type p53 gene is reintroduced into cells with mutant p53 [102][103]. It should be noted that the unregulated overexpression of p53 that occurs in these experiments is very different from the highly regulated endogenous p53 that rarely induces apoptosis in normal cells. Subsequently, it was shown that tumors lacking p53 were resistant to radiation and adriamycin [104][105]. However, it has frequently been overlooked that these experiments were performed in cells that had been transformed with Ras and the adenovirus gene E1A; the latter was already known to sensitize cells to apoptosis [106]. A later paper showed that tumors derived spontaneously in p53 knock-out mice did not exhibit resistance to radiation [107]. The explanation for these conflicting observations is that the p53-induced apoptosis depends on the phenotype of the cell in which it is expressed; for example, it is equally appropriate to consider apoptosis as E1A-dependent in these model systems as it is to consider it p53-dependent. There are many genes regulated by p53, one of which is Bax, a pro-apoptotic member of the Bcl-2 family. In many cases, p53-mediated induction of Bax enhances sensitivity to DNA-damaging agents, while in other cases p53 fails to induce Bax. It is possible that the ability to induce Bax is dependent on expression of a second gene such as E1A. Further work is required to define the regulation of Bax expression and the endogenous genes that produce this E1A-like phenotype.

Many publications consider apoptosis induced by DNA-damaging agents as p53-dependent, but this is clearly an exaggeration. Some agents may initiate apoptosis through a pathway modulated by p53, but p53 is rarely required for apoptosis. Cisplatin induces apoptosis in cells expressing either wild-type or mutant p53, and there is conflicting data on the significance of p53 function for response to cisplatin [101]. The largest comparison of p53 expression and cisplatin response has been performed in the National Cancer Institute's panel of 60 human tumor cell lines [108]. This panel has been used to screen more than 60,000 potential anticancer drugs, and the cell lines have also been screened for many molecular determinants such as expression of wild-type or mutant p53. Analysis of this database shows that cells with mutant p53 are, on average, more resistant to the effect of cisplatin [109]. However, there is considerable variation in the response with

some mutant cell lines being more sensitive than some p53 wild-type cell lines. This demonstrates that there are numerous other parameters that determine the sensitivity of cells to cisplatin.

The mechanism by which cisplatin kills a cell represents a complex story. This review has identified numerous determinants of cellular response to cisplatin. DNA platination is an essential first step in the eventual demise of a cell, but the final outcome is also dependent upon the capacity for DNA repair, the ability to arrest cell cycle progression, the p53 status, the activity of intracellular protein kinase cascades, and the expression levels of pro- and anti-apoptotic members of the Bcl-2 family. It is likely that many other determinants remain to be identified. Many investigators are trying to predict the response of a tumor based on one or several of these parameters, but the above discussion would suggest it may be difficult if not impossible to predict sensitivity of a tumor to cisplatin without directly measuring it.

Dedication: For many years, I admired the work of *John J. Roberts*, a pioneer in understanding the mechanism of action of cisplatin. His 1979 review in 'Progress in Nucleic Acid Research and Molecular Biology' was my entry into this exciting field of investigation. I not only highly regarded *John* as a scientist, but he also became a good friend. I fondly recall the final time I saw *John*; his wife *Gaynor* was dragging him across the runway in Venice to catch their plane, yet *John* continued to talk back across the runway to me about cisplatin, his words slowly fading as the distance between us increased. Unfortunately, *John* died of mesothelioma in 1990, a reminder of how slow our progress is in conquering this disease. I considered it a privilege to be invited to write this review and hope it is a worthy sequel to *John*'s. *John Robert*'s inspiration lives on and I am proud to dedicate this review to his memory.

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Replication of Platinated DNA and Its Mutagenic Consequences

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The platinum compound *cis*-diamminedichloroplatinum(II) or cisplatin is one of the most effective and broadly used anticancer drugs, and it is particularly useful for treatment of testicular cancer. Cisplatin is believed to exert its cytotoxic effects by interacting with DNA, where it inhibits both replication and transcription and induces programmed cell-death. However, much data has been accumulated in recent years indicating that the replication machinery can elongate past cisplatin-DNA lesions in a mutagenic way. Intervention of specific DNA polymerases and protein-protein interactions between replicative enzymes and DNA-damage-recognition proteins may lead to occasional mutagenic translesion synthesis. The consequences of cisplatin-induced mutations may severely alter the fate of the cell. When occurring in proto-oncogenes, they can result in their activation leading to a key step in the process of tumorigenicity, or in the acquisition of a cisplatin-resistant phenotype. In this work we review recent research dealing with both the effect of cisplatin on DNA replication and the mutagenic consequences of translesion synthesis of cisplatin-DNA adducts. The identification of the mechanisms of cisplatin translesion synthesis should allow the refinement of strategies aimed at minimizing the adverse effects of this cellular process.

Introduction

Cisplatin is one of the most effective and broadly used anticancer drugs and it is particularly useful for the treatment of testicular cancer [1]. Cisplatin interacts with cellular DNA, RNA and proteins [2–4]. Interaction of cisplatin with DNA forms several classes of DNA adducts [3]. DNA adducts are generally considered to be responsible for the toxicity and mutagenicity of cisplatin, although its biological activity cannot be solely explained by its ability to damage DNA [4].

Cisplatin-DNA lesions have been shown to interfere with DNA replication and transcription [2–4]. Inhibition of DNA replication can produce

Cisplatin. Edited by Bernhard Lippert

major effects on rapidly dividing cells, among which are cancer cells. Cisplatin has been shown to generate local distortions in the DNA [5][6]. For instance, binding of cisplatin to the N(7) atoms of two adjacent guanosine residues, the most abundant adduct produced by the drug *in vivo* and *in vitro*, results in a 35–40° bending [7] and 21° unwinding [8] of DNA. These conformational changes may represent a severe impediment to the action of most DNA polymerases, RNA polymerases and other proteins involved in DNA replication and transcription.

The molecular basis of cisplatin cytotoxicity is not well understood. In the past, cytotoxicity was believed to be the result of inhibition of DNA synthesis, but, as cell death does not correlate directly with the extent of inhibition of DNA replication [9], the process appears to be more complex than initially thought. This complexity is illustrated by the results of a recent publication showing that cisplatin concentrations which severely prevent DNA synthesis result in an early S-phase arrest in Chinese hamster ovary-cells, while drug concentrations that are unable to completely inhibit DNA synthesis lead to cell death through apoptosis after G2/M arrest [10]. The transduction pathway responsible for this apoptotic induction remains unknown, but one may postulate that a pause of the replication fork at cisplatin lesions, although not sufficient *per se* to completely abolish DNA synthesis, may provide the cell with a signal to induce programmed cell death. Similarly, another apoptosis-inducing signal could be provided by the alteration of DNA transcription caused by cisplatin. It has been shown that cisplatin affected the level of DNA transcription *in vivo* [9], and that cisplatin adducts, particularly the intrastrand lesion implicating two adjacent guanines (Pt-d(GpG)), blocked RNA elongation *in vitro* [11]. Moreover, recent findings indicate that cisplatin-DNA lesions could act as a decoy for a transcription factor involved in rRNA synthesis [12] and for TATA-box-binding protein (TBP), a crucial component of the transcription machinery [13]. The almost perfect match between the structures of an oligonucleotide bearing the Pt-d(GpG) lesion and the DNA in the TBP-TATA-box complex could explain the hijacking of TBP, leading to the proposal that recognition of cisplatin-damaged DNA by TBP may divert it from its normal functions [14]. Thus, direct inhibition of RNA polymerases by cisplatin lesions and/or competition between cisplatin-damaged DNA and TATA-box-containing promoters could result in a significant decrease of transcription and contribute to the drug's toxicity.

However, much data has been accumulated in recent years indicating that the replication machinery can elongate past cisplatin-DNA lesions in a mutagenic way [15]. Intervention of specific DNA polymerases and protein-protein interactions between replicative enzymes and DNA damage-recognition proteins may lead to occasional translesion DNA synthesis. This translesion synthesis can occur in an error-prone fashion, leading to induc-

tion of mutations which can be fixed by subsequent rounds of replication. The consequences of cisplatin-induced mutations may severely alter the fate of the cell. When occurring in proto-oncogenes, they can result in their activation leading to a key step in the process of tumorigenicity, or in the acquisition of a cisplatin-resistant phenotype.

A number of mechanisms allow a cell to become resistant to cisplatin. The most commonly acknowledged ones include decreased drug uptake [16], increased levels of sulfur-containing macromolecules reacting with cisplatin [17], and increased DNA repair [18]. Increased tolerance to cisplatin adducts may also play a role in the appearance of a resistant phenotype. This is as suggested by data obtained in some ovarian carcinoma cells in which resistance is accompanied by a reduced rate of adduct removal when compared to the sensitive parental cell-line [19]. The molecular mechanism of this phenomenon is unknown but has been correlated with an increased replicative bypass of platinum-DNA adducts.

The purpose of this work is to review recent research dealing with both the effect of cisplatin on DNA replication and the mutagenic consequences of translesion synthesis of cisplatin-DNA adducts. Our review will cover both studies performed in prokaryotes (or with prokaryotic proteins) and with eukaryotes (or eukaryotic proteins).

Effects of Cisplatin on DNA Replication: *In Vitro* Studies

Effect on DNA Elongation by Purified DNA Polymerases and Mutagenic Consequences of Translesion Synthesis

One early study examined the capacity of *E. coli* DNA polymerase I *Klenow* fragment to replicate primed single-stranded bacteriophage M13 mp8 DNA modified with cisplatin [20]. Sites of replication blockage were precisely identified on sequencing gel allowing a 'replication mapping' of the arrest sites. Inhibition of DNA synthesis occurred principally at $(dG)_n$, $n = 2$ sequences, now known to be the major cisplatin-binding site in DNA. Blockage, at runs of two or more guanines, was also observed by attempting to replicate platinated SV40-virus DNA by *E. coli* DNA Polymerase I *Klenow* fragment [21]. In a subsequent study, replication-mapping experiments on primed platinated M13 mp10 DNA by the eukaryotic replicative DNA polymerase α showed arrest sites which included those seen with the prokaryotic enzyme DNA Polymerase I, namely at potential d(GpG), d(ApG), and d(GpNpG) crosslinks, plus other minor sites [22].

Cisplatin initially reacts with the N(7) of guanine to create a monofunctional adduct. In a second step, these monoadducts chelate to another pu-

rine base to form bifunctional lesions [23]. Replication of platinated single-stranded DNA by both *E. coli* DNA polymerase I and *Drosophila* or calf thymus DNA polymerase α was less affected by monofunctional than by bifunctional cisplatin adducts [24], indicating that the bifunctional lesions play the predominant role in inhibiting *in vitro* DNA elongation by these DNA polymerases. Interestingly, DNA adducts formed by a new class of monofunctional platinum antitumor drugs have been subsequently reported to inhibit the progression of both T7 bacteriophage DNA polymerase and *E. coli* DNA polymerase I *Klenow* fragment [25].

It should be pointed out that the gel methodology employed to perform the 'replication mapping' experiments described above was aimed almost exclusively at the detection of arrest sites of DNA replication and that a quite high ratio of platinum adducts per total nucleotides in template DNA was necessary to characterize the stop sites. In addition, since a number of different platinum-DNA adducts are formed both *in vivo* and *in vitro*, the use of randomly platinated templates made it impossible to assess the relative contribution of each individual adducts on the inhibition of DNA replication. In order to address this problem, the construction of DNA substrates containing a single platinum adduct at specific, chemically defined sites has been achieved by several groups, and the capacity of a number of prokaryotic and eukaryotic DNA polymerases to replicate such substrates was investigated. One study monitored the capacity of T7 bacteriophage DNA polymerase, T4 DNA polymerase, *E. coli* DNA polymerase I *Klenow* fragment and *E. coli* DNA polymerase III holoenzyme to replicate site-specifically platinated, linearized single-stranded M13 DNA. These polymerases were all stopped by cisplatin-d(GpG), -d(ApG), and -d(GpCpG) intrastrand adducts; however the inhibition of DNA elongation was not complete for any of the lesions and varying degrees of bypass replication, which resulted when the polymerase was able to translocate past the adduct, were observed [26]. The authors found that the extent of bypass varied from 2 to 6% for the d(GpG) adduct, from 2 to 19% for the d(ApG) adduct and from 2 to 25% for the d(GpCpG) adduct, the *Klenow* fragment of DNA polymerase I being, on average, the most efficient in performing translesion synthesis. In a subsequent study, replication of site-specifically platinated single-stranded oligonucleotides was investigated using both *E. coli* DNA polymerase III holoenzyme and *E. coli* DNA polymerase I *Klenow* fragments [27]. The *Klenow* fragment was found to be quite efficient in bypassing a d(GpG) adduct (25% translesion synthesis) and to poorly bypass a d(ApG) adduct, while DNA polymerase III holoenzyme was severely inhibited by both lesions. Interestingly, data from both articles [26][27] suggest that *E. coli* DNA polymerase I can perform some polymerization past the d(GpG) cisplatin intrastrand adduct; the discrepancy in the efficiency of the observed

translesion synthesis may be due to the difference in sequence context of the adduct and/or to the different length of the templates used.

The replication of oligonucleotides containing single cisplatin-lesions by the eukaryotic DNA polymerase ε, purified from calf thymus, has been examined. This polymerase, like polymerases α and δ, is required for chromosomal DNA replication and is also involved in DNA repair. The action of both ε activity and its associated $3' \rightarrow 5'$ exonuclease was blocked by the d(GpG)-cisplatin intrastrand adduct and by its monofunctional form [28]. Furthermore, with some of the substrates used it was noticed that the polymerase was sequestered on the platinated DNA and therefore prevented from beginning replication on other templates. These results indicate that, in addition to the block in DNA elongation, the d(GpG)-cisplatin adduct may exert its toxic action by sequestering a DNA polymerase. On the other hand, in the same study it is pointed out that, with other substrates, this hijacking of the enzyme by the d(GpG) lesion was not observed, leading to the proposal that in such cases the adduct affected the initial binding of DNA polymerase ε.

The direct comparison of the capacity of calf thymus DNA polymerases α, ε, δ, and β to replicate oligonucleotides containing a single d(GpG)-cisplatin adduct has been done [29]. The templates contained the same part of H-*ras* gene sequence bearing the lesion on codon 13 that was used for a previous study [30]. It was found that DNA synthesis catalyzed by replicative DNA polymerases was blocked at the base preceding the lesion. Addition of accessory proteins such as Proliferating Cell Nuclear Antigen (PCNA) to DNA polymerase δ or Replication Protein A (RPA) to DNA polymerase α did not restore their capacity to elongate past the adduct. On the other hand, DNA polymerase β, which appears to be mainly implicated in cellular base-excision repair [31], efficiently bypassed the cisplatin adduct. It should be noted that given the highly distributive mode of replication of long stretches of DNA by DNA polymerase β *in vitro*, high amounts of enzyme were necessary to achieve translesion synthesis; however, this situation may not be unphysiological since a substantial increase in cellular DNA polymerase β level is provoked by treatment with alkylating or oxidative-stress-inducing agents [32][33]. In addition, a ninefold increase of *in vitro* DNA synthesis activity attributed to DNA polymerase β has been reported in extracts from Human Malignant Glioma cells following *in vivo* cisplatin therapy [34]. Some of the experiments in the article by *Hoffmann et al.* [35] provided an initial glimpse into the possible molecular mechanism underlying the capacity of DNA polymerase β to bypass the d(GpG) adduct. In fact, it was found that DNA polymerase β was the only polymerase among those examined capable of primer extension from a 3′-OH located opposite to the base preceding the lesion, indicating its unique capacity to reinitiate

DNA replication at the site opposite to the adduct. In addition, DNA polymerase β was able to elongate the arrested replication products of the other three DNA polymerases in their presence, thus showing its capacity to successfully compete with them at a stalled replication complex. These results suggest that only DNA polymerase β, possibly because of its distributive mode of action and simple subunit composition, can productively associate with the primer/template junction formed at the base preceding the d(GpG) adduct and continue DNA elongation in a reaction which includes the replicative DNA polymerases.

The molecular mechanisms of cisplatin-induced mutagenesis are not yet understood. The established capacity of DNA polymerase β to efficiently bypass the defined cisplatin-d(GpG) lesion *in vitro* [29] offered a unique possibility to investigate the mutagenic consequences of such translesion synthesis. This study [35] was conducted by identifying, on denaturing gel, the products of bypass, which were subsequently excised, purified and PCR-amplified. As a control, products generated on an undamaged template were also purified and amplified in the same way. It was found that 42% of the replication products by calf thymus DNA polymerase β contained mutations; 26% were single-base deletions in the cytosine 5′ of the two guanines implicated in the cisplatin adduct, while 60% were multiple mutations located four to seven bases downstream from the adduct. It is of interest to compare the mutation spectrum of this *in vitro* study with results obtained *in vivo*, where a single-stranded vector bearing the same cisplatin-modified H-*ras* sequence was replicated in Simian COS-7 cells. In the *in vivo* study [30], the observed mutation frequency was 21%, but in variance with the results with purified DNA polymerase β, the most frequent modifications were base substitutions, and 92% of the mutagenic events occurred at one or at both of the platinated guanines involved in the intrastrand crosslink. However, a direct comparison of *in vivo* and *in vitro* data is difficult since DNA repair after translesion replication may substantially alter *in vivo* results. In addition, the discrepancy between the *in vivo* and *in vitro* mutagenesis spectra might be explained by the different stability and topology of the DNA templates used (60-mer oligonucleotides *vs.* single-stranded DNA). Finally, it is possible that the bypass replication of the platinated H-*ras* sequence *in vivo* requires the action of DNA polymerases other than β, or the involvement of yet unidentified accessory proteins. A further point of interest of the *in vitro* study by *Hoffmann et al*. [35] was the capacity of DNA polymerase β to produce an unusual mutagenic event in replicating the platinated substrate: the tandem replication of a twelve-base-pair sequence. If reproduced with other DNA sequences and lesions, these results suggest that tandem replication by DNA polymerase β could contribute to damage-induced genetic instability.

Effect on DNA Unwinding by Purified DNA Helicases

DNA helicases are a class of enzymes necessary for fundamental DNA transactions such as DNA replication, transcription, repair, and recombination. Moreover, among the components of the DNA replication, repair, recombination or transcription apparatus, the first that may encounter a site of DNA damage are the DNA helicases. Thus, a complete understanding of the effect of cisplatin lesions on DNA metabolism requires a biochemical analysis of their interaction with this class of proteins. At least three reports have investigated the effects of cisplatin intrastrand lesions on the activity of DNA helicases implicated either in repair or in recombination.

As far as DNA-repair enzymes are concerned, one study dealt with the capacity of the yeast Rad3 DNA helicase to unwind cisplatinated DNA [36]. In *Saccharomyces cerevisiae* the product of the Rad3 gene is a $5' \rightarrow 3'$ helicase required for damage-specific incision of DNA [37]. M13 mp18 single-stranded DNA (template strand, the strand on which the enzyme translocates) was randomly platinated to obtain an increasing number of cisplatin adducts bound to DNA, varying from one to 520, to one to 20 nucleotides; the template strand was then annealed to a 206-nucleotide long complementary sequence (primer strand) to construct a suitable substrate to monitor DNA-helicase action. The results showed that Rad3-helicase unwinding was inhibited when cisplatin adducts were located on the template strand, while the cisplatin-modified primer strand was displaced with the same efficiency as the unmodified one. Competition experiments suggested that Rad3 helicase could have been sequestered at platinated sites on the template strand. In a second study, the activity of a $3' \rightarrow 5'$ DNA helicase purified from calf thymus was examined on a series of oligonucleotide substrates containing a unique, specific d(GpG)-cisplatin adduct [38]. This DNA helicase, termed Helicase E, is also thought to be involved in DNA repair [39]. The results obtained with Helicase E on site-specifically modified substrates resembled, in part, those obtained with Rad3, in the sense that Helicase E did not appear to be able to traverse the cisplatin-d(GpG) adduct placed on the template strand to which it bound, but could efficiently displace a cisplatin-modified primer strand. However, complete inhibition of the helicase activity by the adduct occurred only when the modified site was placed just upstream of the primer, meanwhile some unwinding took place if the adduct was on the template strand within the annealed region, suggesting that, in the latter case, the lesion did not represent an absolute stop to the helicase progression. The mechanism by which cisplatin adducts inhibit the unwinding by the two helicases may also differ, since Helicase E did not appear to be sequestered at the d(GpG)-cisplatin adduct. The inhibitory action of the major cisplatin-d(GpG) lesion was also tested

on the unwinding of RecB, the helicase subunit of the RecBCD complex. RecBCD plays a key role in homologous recombination in *E. coli* [40]. It was found that both the DNA helicase and DNA-dependent ATPase activities of the RecB protein were inhibited by the presence of the lesion on the template strand [41].

At present, among the numerous eukaryotic DNA helicases discovered, only very few have been proved to be directly implicated in DNA replication [42][43]. In the case of *Herpes Simplex* Virus type 1 DNA replication, two of the seven proteins required for viral origin-specific DNA replication are DNA helicases [44]. Their direction of unwinding is either $3' \rightarrow 5'$ or $5' \rightarrow 3'$, and their activities, together with those of other replicative proteins, ensure initial strand separation at the replication origin and the opening of the replication fork necessary for the synthesis of leading and lagging DNA strands. Therefore, the study of the effect of the major d(GpG)-cisplatin intrastrand adduct on the strand-displacement capacity of these two proteins should be informative on the effect of the drug on the action of replicative DNA helicases. The first of the two helicases examined was the product of the UL9 gene, a protein that recognizes elements within the Herpes origin-of-replication and functions in a $3' \rightarrow 5'$ direction [44]. The presence of the cisplatin adduct on the template strand within the annealed region of the substrate significantly reduced, but did not abolish unwinding nor DNA-dependent ATPase activities of UL9 [45]. This effect was reminiscent of the one produced by the same adduct on the calf thymus DNA Helicase E [38]; however, in the case of UL9, addition of the *Herpes* single-stranded-DNA-binding protein, ICP8, the product of the UL29 gene, greatly stimulated the capacity of the helicase to unwind platinated DNA. This stimulatory effect was species-specific, since other single-stranded-DNA-binding proteins could not substitute for ICP8. Furthermore, it appeared to be the result of the functional and physical interaction that is known to exist between UL9 and ICP8, and not due to the preferential interaction of ICP8 with the cisplatin-d(GpG) adduct. Interestingly, results from a recent article show that ICP8 stimulates the DNA-helicase activity of the UL9 protein by increasing its processivity, thus facilitating its translocation along DNA and through regions of secondary structure [46]. Based on the findings of this study it is tempting to speculate that ICP8 enables the UL9 protein to bypass the cisplatin-d(GpG) lesion by tethering it to the DNA substrate, thereby preventing its dissociation.

The second *Herpes*-virus replicative DNA helicase is the product of the UL5, UL8 and UL52 genes. This heterotrimeric $5' \rightarrow 3'$ helicase is also endowed with a DNA-primase activity and it is responsible for concomitant DNA unwinding and primer synthesis at the viral replication fork [44]; therefore, its role in viral replication is distinct from the one of the UL9 helicase.

To study the effect of the d(GpG)-cisplatin lesion on the progression of the helicase-primase holoenzyme, its activity was examined on substrates in which one of the two strands was partially unwound, thus resembling a replication fork [47]. As for all the helicases examined so far, it was found that the lesion affected the helicase-primase only when located on the DNA strand along which it translocated, although the extent of inhibition observed was greater here than for calf thymus Helicase E and *Herpes* UL9 helicase. As with the UL9 helicase, addition of ICP8 also specifically stimulated unwinding of platinated DNA by the helicase-primase, but, in variance with what was found for UL9, DNA-coating concentrations of ICP8 were necessary for optimal unwinding of damaged substrate. Addition of competitor DNA to helicase reactions led to a substantial reduction of DNA unwinding by the helicase-primase, suggesting that the enzyme is distributive. Contrary to what was observed for UL9, addition of ICP8 did not affect competition, indicating that it did not stimulate the helicase-primase by increasing its processivity. Rather, ICP8 may stimulate DNA unwinding and enable bypass of the cisplatin intrastand-crosslink by recruiting the helicase primase to the DNA.

These studies [45][47] suggest that specific protein-protein interactions between a single-stranded-DNA-binding protein and two replicative DNA-helicases allows substantial unwinding of substrates containing the major cisplatin lesion, but the mechanisms of stimulation of the helicases' activities by the ICP8 protein appears to be different for the two enzymes.

Effect on DNA Synthesis by Cellular Crude Extracts

In vitro replication of DNA templates by cellular crude extracts is expected to reproduce events taking place in the cell more faithfully than replication accomplished by purified DNA polymerases. An SV40-based replication system has been used to examine the effects of cisplatin lesions on DNA replication by cytosolic extracts prepared from human cell lines [48]. Double-stranded plasmid-DNA templates containing the SV40 origin-of-replication were randomly modified with cisplatin to an extent ranging roughly from one to six lesions per template molecule. Under these conditions, inhibition of DNA synthesis by cytosolic extracts was observed, although residual replication (estimated at 20% of the control) occurred in the presence of 2.6 adducts per plasmid molecule. Interestingly, the platinum lesions appeared to be particularly inhibitory when placed within the SV40 replication-origin. This is demonstrated by the fact that replication of recombinant plasmid templates also contains 2.6 adducts per molecule, yet an unmodified origin is only inhibited to 52%. Recently, oligonucleotides con-

taining a unique d(GpG)-cisplatin intrastrand lesion were used as templates for DNA synthesis by CHO and HeLa-cell extracts [49]. Two types of substrates were compared: the first was a 90-mer single-stranded oligonucleotide primed with a 17-mer, while the second was modeled by annealing an additional oligonucleotide to the single-stranded primed template. To construct the second substrate, an oligonucleotide partially complementary to the 5′-end of the single-stranded DNA was hybridized to form a fork-like structure containing both a double-stranded region, within which the cisplatin adduct was located, and a 5′ single-strand tail. Appearance of full-length products in a reaction catalyzed by cell extracts was observed only with altered fork-like substrate, whereas complete inhibition of DNA synthesis occurred on damaged single-stranded template. This result suggested a role for additional accessory factors that could permit DNA polymerases to bypass lesions when present in fork-like oligonucleotides. Interestingly, in a subsequent study it was found that High-Mobility-Group protein 1 (HMG1 protein) [50][51] bound preferentially *in vitro* to the forked DNA containing the cisplatin adduct and not to the undamaged substrate. Binding of HMG1 protein to the platinated substrate reduced the appearance of full-length product catalyzed by cellular crude extracts [52], suggesting that the protein affected the capacity to replicate across the lesion and implicating HMG-binding as a potential mechanism of cisplatin toxicity. However, caution should be taken in interpreting the results of theses studies since recent fractionation experiments aimed at identifying the proteins implicated in the *in vitro* bypass replication have shown that ligation events are implicated in the appearance of at least part of the full-length products catalyzed by cellular crude extracts on damaged fork-like substrates (*N. Tanguy Le Gac*, *J. S. Hoffmann,* and *G. Villani*, unpublished results). Experiments are underway to evaluate the part of full-length products due to ligation and to identify the proteins capable of transforming the fork-like template into a substrate that can be ligated.

Effects of Cisplatin on DNA Replication: *In Vivo* Studies

Effect on DNA Initiation and Elongation

As stated in a recent review [53], the development of efficient cisplatin chemotherapy has brought an unexpected challenge: as many patients survive longer, they find themselves at risk of late complication in their antineoplasic therapy. Indeed, although unambiguous data on the capacity of cisplatin to induce secondary cancers in humans is still lacking, its carcinogenic properties in rats and mice has been reported [53][54]. Treatment-

induced secondary cancer may depend on the ability of the replication machinery to synthesize DNA through cisplatin-DNA adducts in a mutagenic manner. The identification of the mechanisms of bypass may allow the refinement of strategies aimed at minimizing the capacity of the replication machinery to perform translesion synthesis.

Progress in molecular biology has led to the development of strategies for elucidating the processing of damages introduced in DNA by cisplatin. Since the traditional procedure, which consists of determining the toxic effect or the mutation spectrum induced by randomly treating a target DNA sequence, was not sensitive enough to measure the relative contribution of the different adducts formed, the use of site-specifically modified substrates has been introduced to precisely monitor their toxicity and mutagenicity. In recent years, the mechanistic links between cytotoxicity, resistance, and mutagenicity induced by cisplatin have been examined in a number of studies.

One early study used bacteriophage M13 DNA containing a single Pt-d(GpG) intrastrand crosslink to study survival of single- and double-stranded modified genomes in *E. coli* [55]. This work revealed that there were no differences in survival between the platinated and unplatinated double-stranded vectors. This lack of difference was thought to result from adduct repair occurring before the replication of cisplatin-modified DNA, or by a bias existing towards the replication of the undamaged strand. Therefore, in the same work the genotoxic potential of Pt-d(GpG) adduct was examined on single-stranded vectors where either excision repair or switch mechanisms to replicate undamaged strand should not occur. Survival of the single-stranded modified vector appeared to be 10–12% of that of the corresponding unmodified genome. This result indicated that a single Pt-d(GpG) adduct can be almost lethal for the replication of a single-stranded bacteriophage. A related study was performed in eukaryotic cells using a SV40 single-stranded shuttle vector bearing a unique Pt-d(GpG) lesion. The data showed that survival of single-stranded vectors was about 26% [30]. Thus, COS-7 cells seem to be able to perform translesion synthesis of DNA through the major cisplatin-DNA somewhat more efficiently than *E. coli* cells. In another set of studies, the effect of induction of the SOS-response system [56] on cisplatin toxicity was examined. Induction of SOS response in *E. coli* by UV irradiation before cisplatin treatment enhanced the survival of Pt-d(GpG) modified duplex genomes to 38% relative to that of unplatinated control genome [57]. In contrast, this value was 22% when the SOS response was not induced. Therefore, the reduced toxicity observed following induction of the SOS response could be related to an acquired ability of the replication machinery to perform translesion synthesis. Another interesting finding is that different adducts formed by cisplatin did not seem to have the same toxic effect. In *E. coli*, it was shown that the major adduct

formed by cisplatin on DNA, Pt-d(GpG) had a greater lethal potential than Pt-d(ApG) [58], suggesting that perhaps the latter lesion is more easily bypassed. It should be noticed that in *E. coli* mutagenesis, provoked by Pt-d(ApG) and Pt-d(GpG) adducts, it has also been found to be dependent on induction of the SOS response [57][59].

The studies cited above did not precisely indicate which step of DNA replication was affected by the lesions. It would be of interest to know if the initiation or elongation steps of DNA synthesis are differently affected in order to evaluate their respective contribution to the overall toxicity of cisplatin. However, only a few reports exist in which the respective contribution of the initiation and elongation components of DNA replication have been studied since there are no simple and reliable methods to study the inhibitory effect of the drug on these processes separately. One study [60] presented results which showed that cisplatin only had a slight effect on initiation of DNA synthesis; the authors concluded that, under their experimental conditions, inhibition of initiation of DNA replication was essentially dependent on the ability of a genotoxic agent to induce double-strand breaks. Another study [61] examined the effect of ethylenediamine- or diaminocyclohexane-platinum compounds on the inhibition of DNA initiation. Based on data obtained with velocity-sedimentation analysis of DNA synthesized in treated cells, it was suggested that initiation of DNA replication was inhibited by the platinum compounds. However, since the authors did not use cisplatin, direct comparison with the formerly described study cannot easily be made. Data suggesting inhibition of DNA-replication initiation by cisplatin lesions placed at the origin of an SV40-replication system has also been obtained *in vitro* [48].

Clearly, further studies are needed to identify precisely the effects of cisplatin on the different phases of DNA replication *in vivo*.

Effect on Toxicity

The difference in cell sensitivity to cisplatin as a function of the cell cycle can be a useful tool to examine the role played by the inhibition of DNA replication in the drug-induced toxicity. This point was addressed by a study [62] which showed that cells treated with 6 μg/ml in the G1 phase, before the start of DNA replication, exhibited a ten-hour cell-division delay during the first cell cycle after treatment, whereas the delay was only of three hours if the treatment occurred in the late S-phase when most of the DNA replication was terminated. In addition, more chromosomal aberrations were detected following treatment in the G1 phase. The different responses of the cells treated with cisplatin in G1 *vs.* cells treated in the late

S-phase, underlined the primary role of inhibition of DNA replication in influencing the drug toxicity. Understanding the mechanisms which link cisplatin-DNA damages to cell death is an important challenge. Initially, it was believed that cisplatin cytotoxicity was solely the result of DNA-replication inhibition [63]. Subsequent studies did not directly correlate cisplatin-induced cell death with inhibition of DNA replication [9] but led to the conclusion that induction of apoptosis was the major route in the toxicity induced by the drug [64][65]. Nevertheless, an indirect role of DNA replication in cisplatin-induced cytotoxicity could be invoked in the initiation of an apoptotic response to DNA damage. It has been shown that cisplatin-damage tolerance was the fundamental mechanism that caused increased cisplatin resistance in some ovarian carcinoma-cell lines [66]. A likely explanation for this resistance is that these cell lines require higher levels of DNA damage to activate programmed cell-death pathways. Consistent with this hypothesis are the results of a study suggesting that the mechanism of cisplatin resistance in A2780 ovarian-carcinoma-resistant derivative cell lines, is an upstream event that signals the initiation of apoptosis but not the apoptotic process itself [67]. After cisplatin treatment, resistant cell lines exhibited a tenfold to 40-fold increase in survival when compared to the wild-type cell line which showed only a twofold to threefold increased repair of cisplatin-DNA adducts [68]. Therefore, the resistance towards cisplatin displayed by these cells could not solely be accounted for by enhanced repair of the lesions, but was more likely related to some mechanisms of damage tolerance. One can then hypothesize that inhibition of the replication machinery following DNA damage is alleviated in these resistant cell lines and renders them more tolerant to cisplatin-DNA lesions than the sensitive parental cell-line, supporting the hypothesis that a modulation in the inhibition of DNA replication plays a role in the induction of apoptotic events. This theory is in agreement with previous data in which damage tolerance was associated with enhanced replicative bypass in a cisplatin-resistant derivative of the A2780 ovarian carcinoma-cell line [19]. However, generalization of this hypothesis may be inappropriate since it was observed that, although unrepaired cisplatin-induced DNA damage in the S phase could be important for the induction of apoptosis [65], apoptotic cells were detected in response to cisplatin treatment at any point in the cell cycle [69]. Moreover, replicative bypass is unlikely to be involved in the observed cross-resistance of several cancer-cell lines to drugs that do not exert their cytotoxic effects through binding to DNA [66]. Therefore, induction signals of apoptotic pathways are probably the result of several molecular events including inhibition of DNA replication.

Consequences for Resistance

Drug resistance is a major obstacle for the successful treatment of cancer with cisplatin. Although high response rates are often initially observed in patients, resistance frequently occurs, rendering subsequent therapy largely ineffective. Cell lines exhibit different mechanisms that account for their acquired cisplatin resistance: these mechanisms include a) decreased platinum accumulation, b) elevated levels of proteins such as glutathione or metallothionein which can sequester cisplatin before it reaches its pharmacological targets, c) enhanced repair capacity to remove Pt-DNA lesions, d) alteration in the types of Pt-DNA lesions formed, and perhaps, e) DNA sequence modifications in regulatory regions rich in guanine residues [70]. An additional mechanism of resistance may be the consequence of an increased capacity of the cell to tolerate platinum-DNA lesions. This has been shown to be the primary mechanism that caused decreased cisplatin sensitivity in a series of cell lines [66]. This damage tolerance could be associated with either enhanced replicative bypass or defective apoptotic processes. Enhanced replicative bypass, ranging from twofold to fivefold compared to sensitive cell lines, has been found to play a clear role in the resistance of human A2780 cell lines [19], which have a functional apoptotic pathway in response to cisplatin-DNA damage [67]. The mechanisms of replicative bypass that take place *in vivo* are largely unknown, although it appears that most cells are capable of some degree of replicative bypass of cisplatin-damaged DNA [19]. Among the eukaryotic DNA polymerases, DNA polymerase β could be a candidate for participating in the *in vivo* translesion synthesis of cisplatin lesions. This DNA polymerase has been found to be overexpressed in resistant cell lines [34][71] and to be induced by genotoxic treatment [32][33]. Its main role in cisplatin resistance has been related to enhanced repair of the cisplatin-DNA damages [34] but, given its ability to replicate DNA containing the Pt-d(GpG) lesion *in vitro* [29][35], one can postulate that overexpression of polymerase β could contribute to replicative bypass.

Survival through S phase as a result of replicative bypass should give the cells an additional period of time to repair DNA adducts in an arrested G2 phase [72], suggesting a link between translesion synthesis and resistance as a consequence of the augmented capacity of the cell to repair DNA lesions. An additional link between replicative bypass and cisplatin resistance may be inforced by the finding that overexpression of several proto-oncogenes are correlated with cisplatin resistance following drug exposure [73]. Although the molecular mechanisms that could relate proto-oncogene induction to cisplatin resistance have not yet been explored, *in vivo* mutagenic replication of a single-stranded vector bearing the major cisplatin lesion Pt-(GpG) placed on codon 13 within the human H-*ras* has been report-

ed [30]. Interestingly, the mutation observed with the highest frequency in this system results in the amino-acid substitution that is known to be a key step in the activation of the H-*ras* proto-oncogene (see below).

Recent observations lend support to the hypothesis of a direct role for mismatch repair in coupling cisplatin damage to apoptotic response, and the lack of mismatch repair to cisplatin resistance. First, cisplatin-resistant ovarian cell lines have been shown to acquire a microsatellite instability (RER+) phenotype [74] and to be defective in strand-specific mismatch repair [75]. Second, it has been shown that the mismatch-repair protein complex hMutSα can recognize and bind to cisplatin-d(GpG) and -d(ApG) crosslinks [76][77]. Third, resistant cell lines which acquire an RER+ phenotype and lose mismatch-repair activity also lose the ability to undergo cisplatin-induced apoptosis [74][75]. Finally, human colon and endometrial cancer-cell lines that are deficient in hMLH1 or hMSH2 protein function are more resistant to cisplatin than sublines in which the mismatch-repair deficiency is complemented by chromosome transfer [78]. The development of drug resistance through loss of mismatch-repair ability has a precedent in the case of DNA-methylation damage [79]. In the case of *O*(6)-methylation, it has been proposed that inappropriate attempts of mismatch correction could lead to cell death [79]. Thus, the cell would acquire resistance in absence of futile repair. However, in the case of cisplatin, an additional model has been proposed whereby cisplatin adducts cause DNA replication to stall in hMLH1 proficient cells leading to cell death. Conversely, the absence of hMLH1 allows replication bypass of the lesion and cell survival [80]. Cellular proliferation and, presumably, DNA replication, has been shown to be required for induction of apoptosis [81]. hMutSα protein recognizes cisplatin crosslinks in a duplex DNA in which the complementary DNA strand contains two C residues opposite the d(GpG) crosslink [77]. However, this is a relatively poor substrate for hMutSα and a duplex molecule in which non-complementary bases are situated opposite the platinated guanines are bound with much greater affinity [82]. Such structures can arise in the cell if platinum-damaged DNA can undergo mutagenic bypass replication. Inability to replicate through damages could contribute to the drug toxicity in sensitive cells by generating an intrinsic lethal event or a signal which activates pathways leading to cell death. Thus, cisplatin resistance may be acquired by reducing the probability of occurrence of either lethal events or preapoptotic signals. The capacity of mismatch-repair proteins to bind cisplatin-DNA lesions and to potentially inhibit translesion synthesis may implicate these proteins directly in the antitumor activity of the drug. In accordance with this hypothesis it has been reported that hMutSα is overexpressed in testicular and ovarian tissues which are the most successfully treated by cisplatin [81].

Mutagenesis Induced by Cisplatin: *In Vivo* Studies

Pattern of Mutations Produced

The mutagenic properties of cisplatin have been demonstrated in a variety of prokaryotic and eukaryotic systems. In this paragraph we will focus on data concerning the molecular bases of this mutagenicity, the pattern of mutations induced with regard to the lesions produced, and the biological consequences for the cell.

Cisplatin mutagenicity in *E. coli* has been well documented in several forward-mutation assays. An early study [83] examined mutations induced within the *E. coli* LacI gene and concluded that GpApG and GpCpG adducts were the major hotspots for cisplatin-induced base-substitution mutations. However, their system could only identify base-substitution mutations resulting in nonsense codons and was therefore limited in its detection capacity. In another study [84], after *in vitro* treatment of a DNA sequence containing the Tet^R gene followed by transfection of *E. coli* cells, mutations were primarily located at ApG and GpG sequences with only a minority appearing at GpCpG or GpApG sequences. In the same study it was reported that, when the relative proportions of ApG and GpG adducts were taken into account, the ApG-intrastrand crosslink was at least five times more mutagenic than the GpG adduct. GpG, ApG and GpXpG sequences are the targets of roughly 90% of the DNA adducts reported to form when cisplatin damages DNA, and mutagenesis *in vivo* revealed excellent correlation between the location of mutations and the sites of platination [85]. Further studies investigated the spectrum of mutations displayed by unique adducts placed on these particular sequences. In general, all these different studies reported that ApG and GpG lesions induced A $\rightarrow$ T or G $\rightarrow$ T transversions, nearly all located at the 5′-modified base [57–59]. This asymmetry between the capacity of the two nucleotides bound by cisplatin in generating mutagenic events could be due to the structure of the lesions involving adjacent purines. In fact, NMR data [8] as well as crystallographic studies [7], indicated that the distortion induced in the helix by the Pt-d(GpG) adduct is more pronounced on the 5′-side of the lesion than on the 3′-side. The Pt-d(ApG) lesion is believed to share the same structural characteristics, in agreement with the asymmetric pattern of mutagenesis induced by both adducts [86][87]. These data predicted that DNA polymerases would have more difficulties to synthesize DNA in front of the 5′-nucleotide than in front of the 3′-nucleotide. Therefore, the 5′-platinated nucleotide could act as the primary misinformational site. In all the prokaryotic studies examined, cisplatin-induced mutagenesis was clearly dependent on the induction of the SOS response. Surprisingly, the use of site-specifically modified ol-

igonucleotides containing the Pt-(GpTpG) adduct revealed that no mutations were induced by this lesion even in SOS-induced cells [58]. Similarly, no significant mutagenesis was detected in another study when the Pt-d(GpCpG) adduct was present [88]. Apparently, the mutagenic potential of Pt-d(GpNpG) adducts was severely influenced by the sequence context surrounding the lesions, which is clearly different for these studies and the previous ones [83][84] where such adducts were found to be mutagenic.

The pattern of mutations induced by cisplatin in eukaryotic cells has been studied in different systems, including yeast, CHO, monkey and human cells. Similar to what has been found in *E. coli*, most mutations were base substitutions located at the ApG or GpG sites and in ApGpG and GpApG sequences [89–91]. The locations of these mutations highly correlated with the theoretical binding sites of cisplatin and with *in vitro* DNA-polymerase-inhibition assays. However, some differences exist between the pattern of cisplatin mutagenicity in eukaryotic and prokaryotic cells. Cisplatin-associated mutational hotspots were studied in the supF gene propagated in XP or normal human cells [91]. Quantification of the cisplatin-DNA adducts by replication-mapping analysis revealed that, although mutations occurred at target sites for *cis*-DDP-adduct formation, there was no correlation between sites of mutation and the most frequent sites of adduct formation. The lack of correlation between mutational hotspots and sites of adduct formation found by *Bubley et al.* may be due to the DNA sequence or chromatin-structure context surrounding the lesions, since such a mutational pattern was not detected in the CHO *aprt* gene [89]. A significant proportion of the mutations induced by cisplatin in eukaryotic cells involved deletions of DNA fragments [91][92]. These deletions could result from the sequence context encompassing the cisplatin-DNA adduct. The presence of sequence repeats on both sides of the deleted fragment suggested that deletion of large DNA fragments could be derived from heterologous recombination between these sequences after slippage and mispairing of the DNA. Induction of recombination events may be triggered by single-strand or double-strand breaks introduced during processing of cisplatin lesions. Such mechanisms were suggested to be responsible for the major mutagenic events observed in the *white* and *vermilion* genes of *Drosophila melanogaster* [92]. In the yeast *SUP4-o* gene, tracts of three to five consecutive GC base pairs were the preferred sites for cisplatin-induced single base-pair insertions or deletions [90]. These events were presumably associated with slippage and mispairing of the template-DNA strand within the run. The stability of such a misalignment could be increased by the formation of a cisplatin crosslink between the residues flanking the looped-out nucleotide [90]. Moreover, in contrast to prokaryotic cells, in which most mutations occurred at the 5′-side of the cisplatin adduct, it has also been found that cisplatin-induced mutation at codon 13 of the H-*ras* gene

was located at the 3′-position of the adduct in COS7 or mouse cells [30][93]. The discrepancy between the observed mutation sites at the Pt-d(GpG) adduct in prokaryotes or eukaryotes could result from the influence of the sequence context and/or from a different processing of the lesion by prokaryotic or eukaryotic replication machinery.

Thus, it appears that mutagenesis induced by cisplatin *in vivo* shows a correlation with the theoretical DNA-binding sites of the drug but can also be influenced by the sequence context of the adducts as well as the chromatin structure of the DNA region.

Biological Consequences

The possibility of inducing secondary cancers should be listed among the most important undesired effects of cisplatin treatment. The observation that cisplatin causes a variety of mutagenic effects in mammalian cells has raised concerns about its potential as a human carcinogen. Cisplatin tumorigenicity has been studied in several rodent systems and revealed that cisplatin could initiate or induce preneoplasic and neoplasic lesions in multiple tissues [54][94]. Cisplatin given to pregnant rats is a transplacental carcinogen for fetal liver, kidney, nervous system and lung [95]. It was suggested that genotoxic mechanisms may play an important part in the drug-induced tumor incidence as the highest DNA-adduct levels were observed in the most tissues susceptible after transplacental administration of cisplatin [96]. Transplacental mutagenicity of cisplatin in mice was monitored through appearance of H-*ras* mutations initiated in the skin of fetal mice [93]. Most mutations observed in this study were G → T transversions in codons 61, 12 and 13. Interestingly, mutations on codon 13 occurred at the 3′-nucleotide of the adduct which was consistent with the previously described mutagenicity of a single cisplatin-DNA adduct placed in the same location [30]. This particular mutation has been reported to be a key factor in the activation of the proto-oncogene [97] and could therefore be part of the process of tumorigenicity induced by cisplatin. The results of *Pillaire et al.* and *Munoz et al.* provide the first evidence that cisplatin can initiate a unique and specific spectrum of *in vivo* mutations in two different experimental systems.

Conclusions and Perspectives

The development of new drugs capable of extending the clinical effectiveness of cisplatin necessitates a further increase in our knowledge of the molecular basis of cisplatin activity.

The data accumulated over the last two decades point to cellular DNA as the likely target for cisplatin action and the chemistry of the interactions between the drug and the DNA has been the subject of a great deal of studies. Among the important outcomes of such investigations is the fact that the crystal structure of the major intrastrand cisplatin-DNA adduct and the solution structure of an interstrand crosslink formed on DNA by the drug are now known [6][7]. Another aspect of cisplatin-DNA binding for which essential information has been collected at the molecular level, is the interaction between cisplatin adducts and proteins which recognize these adducts on DNA. A comprehensive knowledge on both the types of adducts formed and the proteins which can modulate their effect through specific binding should improve our understanding of how cisplatin cytotoxicity is mediated.

One essential function of the cellular metabolism affected by cisplatin-DNA lesions is DNA synthesis. Intuitively, it is clear that absolute inhibition of DNA replication by unrepaired cisplatin lesions should be a major constituent of the drug toxicity, as postulated by a number of early studies. However, mechanisms of cisplatin toxicity appear now to be more complex, since apoptotic cell-death has been induced at doses of cisplatin which did not permanently inhibit DNA replication [9]; consequently, induction of apoptosis by cisplatin cannot be related exclusively to direct inhibition of DNA synthesis. Nevertheless, inhibition of DNA replication, even if transient, may contribute one of the starting signals for programmed cell-death. We have reviewed here some data indicating that an increased capacity of the cell to tolerate cisplatin-DNA lesions can contribute to cisplatin toxicity and resistance, perhaps by modulating the apoptotic signals induced by the drug. Understanding the transduction mechanisms which link cisplatin-DNA damages and cell death is an important challenge for future research.

One of the mechanisms by which a cell can tolerate cisplatin-DNA damages is its capacity to replicate through such lesions (DNA translesion-synthesis). A number of studies have shown that cisplatin adducts can be replicated in a mutagenic way *in vivo* and *in vitro* [15]. In addition to influencing cytotoxicity and resistance, mutagenic translesion synthesis could contribute to one of the most undesired side effect of cisplatin treatment, the induction of secondary cancers. Recent *in vivo* data [30][93] have provided evidence that the major intrastrand cisplatin-lesion, the Pt-d(GpG) adduct, produces the same proto-oncogene-activating mutations in different experimental systems. *In vitro*, the same lesion can be bypassed in a mutagenic way by the eukaryotic DNA-polymerase β [29][35]. High amounts of DNA polymerase β were necessary to accomplish mutagenic cisplatin translesion-synthesis; nevertheless, the physiological significance of the observation may still hold since this particular polymerase has been shown to be

induced by a number of genotoxic treatments. The question of whether the *in vitro* capacity of DNA polymerase β to replicate past the cisplatin-d(GpG) lesion is relevant to cisplatin mutagenesis *in vivo* and has been addressed very recently [98]. In this study, it is shown that the frequency of mutations induced by cisplatin increases significantly in CHO cells overexpressing rat DNA-polymerase β compared to control cells displaying a normal level of enzyme, thus indicating that overexpression of DNA polymerase β affects cisplatin mutagenesis *in vivo*.

In addition to DNA polymerase β, other DNA polymerases could participate, together with proteins directly implicated in DNA replication, in translesion-synthesis processes leading to cisplatin mutagenesis. DNA helicases are a class of enzymes necessary for cellular DNA replication and are among the first proteins of a DNA-replication complex to encounter a DNA damage. Thus, a comprehensive study of the effect of cisplatin lesions on DNA synthesis should include a biochemical analysis of the interaction of cisplatin-DNA damage with DNA helicases. Two recent studies [45][47] suggest that specific protein-protein interaction between the *Herpes* single-stranded DNA-binding protein and the two replicative *Herpes* DNA-helicases allow substantial *in vitro* unwinding of substrates containing the Pt-d(GpG) adduct. As in the case of Herpes virus, protein-protein interactions have been demonstrated between eukaryotic DNA-polymerases, helicases and single-stranded-DNA-binding proteins, leaving open the possibility that complexes including DNA polymerases, DNA helicases and single-stranded-DNA-binding proteins may function at the replication fork and may eventually lead to occasional replicative bypass. The construction of appropriate cisplatin-damaged substrates and the availability of purified proteins should enable this hypothesis to be tested experimentally.

The identification of the mechanisms of cisplatin translesion synthesis should allow the refinement of strategies aimed at minimizing the adverse effects of this cellular process.

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Interstrand Cross-Links in Cisplatin- or Transplatin-Modified DNA

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This chapter deals with the interstrand cross-links in cisplatin- or transplatin-modified DNA and focuses on three main aspects: the formation of the interstrand cross-links, the distortions induced in the DNA double helix and the potential use of the interstrand cross-linking reaction in the context of antisense and antigene strategies. In the reaction between cisplatin and DNA the interstrand cross-links are preferentially formed between two guanine residues at the d(GpC) · d(GpC) sites. The X-ray structure at 1.6-Å resolution of a double-stranded decamer containing a single interstrand cross-link locates the water molecules surrounding the platinum residue, which offers an explanation about the chemical instability of the interstrand cross-links. In the reaction between transplatin and DNA, interstrand cross-links are formed between complementary guanine and cytosine residues. The solution structure of a double-stranded DNA dodecamer containing a single interstrand cross-link by nuclear magnetic resonance shows that the base pairs on each side of the adduct are pushed away by the ammine ligands of the platinum residue. Another pathway to form interstrand cross-links is to take advantage of the rearrangement of two kinds of adducts in which the platinum residues have four am(m)ine ligands. These adducts are either the monofunctional *cis*-$[Pt(NH_3)_2(dG)(Am)]^{(n+1)+}$ adducts (where Am is an heterocyclic amine) or the transplatin 1,3-intrastrand cross-links at the d(GpNpG) sites (where N is a nucleotide residue). They are stable within single-stranded oligonucleotides. The pairing of the platinated single-stranded oligonucleotides with their complementary strands triggers the rearrangement of the adducts into interstrand cross-links. Parameters interfering with the linkage-isomerization reaction of the transplatin 1,3-intrastrand cross-links are analysed. Conditions have been determined for a fast, specific and irreversible cross-linking reaction in cell-free medium and in cells. The cross-linking of platinated oligonucleotides to RNA *via* the rearrangement of the transplatin 1,3-intrastrand cross-links into interstrand cross-links offers a new possibility to modulate gene expression by steric blocking of the cellular machinery.

Introduction

The pioneering work of *Rosenberg* [1][2] on the effect of *cis*-diamminedichloroplatinum(II) (cisplatin) on bacteria and mice has led to the discov-

Cisplatin. Edited by Bernhard Lippert

ery of one of the most powerful antitumor drugs used in human chemotherapy. These early results led many laboratories to devote their work to the aim of elucidating the mechanism of action of cisplatin. There is strong evidence showing that cellular DNA is the target of the drug [3–7]. The reaction between DNA and cisplatin results in the formation of different kinds of adducts. Although the adducts block DNA replication and transcription, there is not yet a clear understanding of the antitumor activity of cisplatin. Subsequent to the formation of the damaging lesions, which is the first necessary step, it is likely that cell death results from multi-step reactions involving several pathways [8][9].

Cisplatin reacts preferentially with purine residues in DNA and forms mainly bifunctional lesions [3][10–12]. *In vivo* and *in vitro*, the major adducts are 1,2-intrastrand cross-links at the d(GpG) and d(ApG) sites (*cis*-{$Pt(NH_3)_2$[d(GpG)-*N7*(1),*N7*(2)]} and *cis*-{$Pt(NH_3)_2$[d(ApG)-*N7*(1), *N7*(2)]}) and they represent about 65 and 25% of the bound platinum respectively. Among the minor adducts are the interstrand cross-links between two guanine residues on opposite strands at the d(GpC)·d(GpC) sites. The 1,3-intrastrand cross-links at the d(GpNpG) sites (N being a nucleotide residue) have been found, but their rate of formation is very slow [13][14]. The 1,2-d(GpG)-intrastrand cross-links are the most abundant and are often assumed to play a key role. Indeed, this hypothesis is strongly supported by several recent results showing that cisplatin-modified DNA containing 1,2-d(GpG)-intrastrand cross-links is recognized by several proteins such as HMG box proteins [15–17], TATA box-binding protein [18], histone H1 [19], the DNA mismatch-repair protein hMutS-α [20][21], the protein Ku [22], recognition which could interfere with the cellular machinery at different levels [23–30]. However, whether one or several kinds of adducts are involved in the cytotoxicity of cisplatin is still under debate. Resistance of cells to cisplatin has been reported to be associated with increased gene-specific DNA repair efficiency of interstrand cross-links [31–33].

Another fascinating aspect of the Pt^{II} complexes is that *trans*-diammine-dichloroplatinum(II) (transplatin), the geometrical isomer of cisplatin, has much lower cytotoxicity potency than cisplatin although it also binds to DNA [3][10][34]. The nature of the adducts formed in the *in vivo* reaction between DNA and transplatin is not yet completely elucidated, and *in vitro* there were some controversies. Sterical constraints preclude formation of 1,2-intrastrand cross-links in DNA which might explain the clinical inefficiency of transplatin. However, recent findings show that some Pt^{II} complexes having the *trans* geometry are cytotoxic [35–37]. Interestingly, platinum iminoether complexes *trans*-[$PtCl_2${(*E*)-HN=C(OMe)Me}$_2$] and derivatives have *in vivo* antitumor activity towards both lympho-proliferative and solid metastasizing murine tumors [38][39]. Moreover, Pt^{IV} complex-

es with *trans* geometry for the leaving groups have an antitumor activity and are able to overcome cisplatin resistance of ovarian cancer cells [40][41].

Numerous studies support the view that the formation of the adducts in the reaction between DNA and cisplatin or transplatin proceeds in a two-step solvent-assisted reaction [10][12]. It is generally accepted that, once formed, the intrastrand and interstrand cross-links are stable. However, there are several reports dealing with the rearrangement of the adducts [42][43]. Some of these rearrangements offer the possibility to form specific interstrand cross-links. In this chapter we intend to focus on cis- and transplatin interstrand cross-links. We describe the conformational changes induced in DNA by the interstrand cross-links. We also present two reactions leading to the formation of interstrand cross-links. In both reactions the starting products are single-stranded oligonucleotides containing a single adduct in which the Pt^{II} residue has four am(m)ine ligands. The pairing of the platinated oligonucleotides with their complementary strands triggers the rearrangement of the adducts into interstrand cross-links. The potential use of these reactions to modulate gene expression is discussed.

Cisplatin

Distortions Induced in DNA by the Interstrand Cross-Links

Interstrand cross-links are preferentially formed between the two N(7) of guanine residues [13][44] on the opposite strands in the sequences d(GpC)·d(GpC) [45][46]. The reactivity of the d(GpC)·d(GpC) sites with cisplatin is, in first approximation, independent of the nature of the flanking base pairs, although these base pairs modulate the rate of closure of the monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$adduct into interstrand cross-link [14]. It has been reported that, at very low level of platination of circular DNA, the amount and nature of interstrand cross-links depends upon the DNA topology [47].

The distortions induced in the DNA double helix by the interstrand cross-links have been characterized by several techniques. As judged by chemical probes (diethyl pyrocarbonate, hydroxylamine, osmium tetroxide), antibodies to cisplatin-modified poly(dG-dC)·poly(dG-dC), natural (DNase I) and artificial (1,10-phenanthroline-copper complex) nucleases, the cytosine residues are accessible to the solvent, and the distortions are located at the level of the adduct [48–50]. From the electrophoretic mobility of the multimers of double-stranded oligonucleotides containing a single interstrand cross-link [50] it is deduced that the DNA double helix is unwound (79°) and its axis is bent (45°).

2D Nuclear magnetic resonance (NMR) studies of two double-stranded DNA decamers containing a single interstrand cross-link have confirmed that the cytosine residues no longer paired with the cross-linked guanine residues are extruded from the double helix [51][52]. This extrusion allows a rotation of 180° of the platinated guanine residues which brings them into the minor groove. The phosphodiester backbone forms a kind of chicane such that the local geometry is left-handed with a large unwinding, and the axis of the double helix is bent towards the minor groove. In this model, electrostatic interactions between the oxygens of the phosphate groups of the platinated guanine residues and the square planar Pt^{II} atom lead to a pseudo-octahedral geometry around the metal atom. This interaction, and the stacking of both platinated guanines with the adjacent base pairs, contributes to the stabilization of the structure.

One of the two platinated duplexes used in the NMR experiments has been crystallized. The good quality of the crystals allows diffraction beyond 1.6 Å resolution [53]. The crystals belong to space group C_2 with one molecule per asymmetric unit. The structure has been solved by multi-wavelength anomalous dispersion at 100 K by using the anomalous contribution to the scattered amplitudes of the constitutive platinum residue as an unique source of phase information. The electron-density map at 1.7-Å resolution computed from these MAD phases is directly interpretable at the atomic level. Two ribbon representations are shown in *Fig. 1*. Several features are in good agreement with the data in solution such as the extrusion of the two cytosines, the position of the platinum residue in the minor groove, the direction of the bending (47°) towards the minor groove, and the large unwinding of the double helix (110°). In addition, the hydration of the platinum residue is determined. Two water molecules are located at 3.6 Å from the platinum residue with their oxygens completing a nearly regular tetragonal bipyramid with the four nitrogens bound to platinum. They contribute to the widening of the minor groove. A cage is formed by these two water molecules, seven other water molecules, the two NH_3 ligands bound to platinum and the two 0(6) from the cross-linked guanines. This cage, which is bridged to two phosphate groups on the opposite strands by other water molecules, contributes to the stabilization of the structure. One of the extrahelical cytosine residues makes a *Hoogsteen* base pair with a terminal G · C pair from another duplex. The second extruded cytosine residue makes an intermolecular contact with a phosphate group.

The sequence of the events (platination, conformational changes) leading to the migration of the N(7) of guanine residues from the major groove of the double helix to the minor groove is not yet known. It seems unlikely that the two guanine residues, separated by 7.1 Å in B-DNA, react simultaneously with cisplatin. The subsequent formation of a monofunctional *cis*-

$[Pt(NH_3)_2(dG)Cl]^+$, which is assumed to be the first step of the reaction, leads to the DNA conformation modification. It resembles, to some extent, the conformation of DNA containing a monofunctional $[Pt(dien)(dG)]^{2+}$ adduct (where Pt(dien) stands for diethylenetriamineplatinum(II)). The distortions induced in DNA by $[Pt(dien)(dG)]^{2+}$ exhibit a sequence-dependent variability as judged by chemical probes and artificial nucleases, but do not drastically alter the overall shape of the double helices as judged by gel mobility [54][55]. It should be noted that the $[Pt(dien)(dG)]^{2+}$ adducts facilitate the B-Z transition of poly(dG-dC).poly(dG-dC) [10][11]. On the other hand, the rate of closure of *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ into an interstrand cross-link depends upon the nature of the base pairs flanking the site of platination. The values of the monofunctional adduct half-life ($t_{1/2}$) in duplexes whose central sequences are d(TG*CT)·d(AGCA), d(CG*CT)·d(AGCG), and d(AG*CT)·d(AGCT) (where G* stands for *cis*-$[Pt(NH_3)_2(dG)Cl]^+$) are 12, 6 and 2.8 h, respectively [14]. The differences in the rates originate in either the rate of formation of the aquated species or/and the local deformability of the double helix. Surprisingly, at the

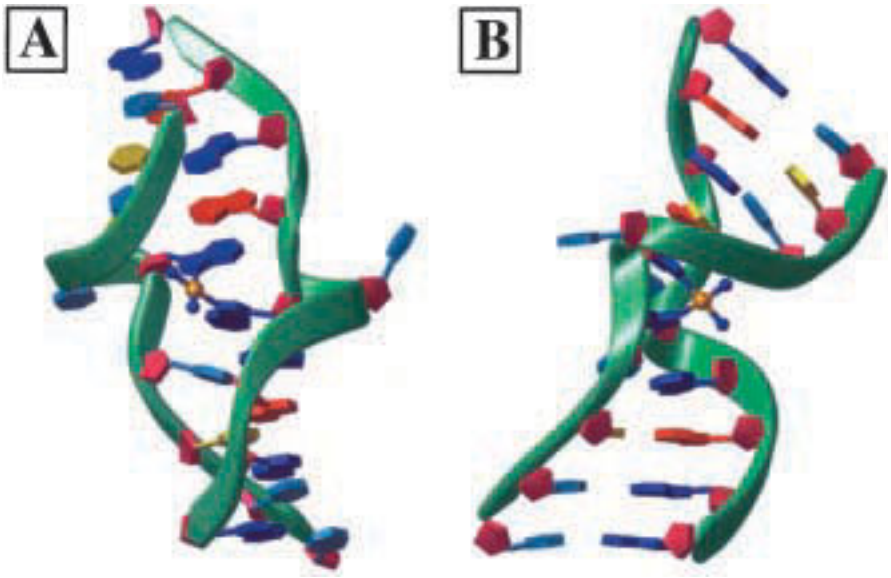

Fig. 1. *Two ribbon representations of the crystal structure of the DNA decamer d(CCTCG*-CTCTC/GAGAG*CGAGG) containing a unique cisplatin interstrand cross-link at d(GpC)·d(GpC) site* (asterisks indicate the chelated bases in the adduct). A front view (*A*) allows to see the structure with the lesion in the minor groove. A side view (*B*) shows the chicane of the backbone with the helix-sense reversal. Pt^{II} atom, yellow; ammine groups, navy blue; sugars, pink; guanines, navy blue; adenines, red; thymines, yellow; cytosines, light blue; phosphodiester backbone, green.

d(AGCT)·d(AGCT) site, the *interstrand* cross-linking reaction is as fast as the *intrastrand* cross-linking reaction at the d(GpG)·d(CpC) site ($t_{1/2}$ is equal to 2.1 ± 0.3 h [56]), and faster than that at the d(GTG)·d(CAC) site ($t_{1/2}$ larger than 20 h [14]).

Instability of the Interstrand Cross-Links

Interstrand cross-links are unstable in conditions close to physiological conditions [57]. The bonds between platinum and the N(7) of guanine residues are cleaved spontaneously, with essentially one cleavage reaction per cross-linked duplex in either of both DNA strands ($t_{1/2}$ for the cleavage reaction is about 29 h). As shown in the reaction scheme (*Fig. 2*), the cleavage generates monofunctional adducts which can react further to yield interstrand and intrastrand cross-links. The distorted local conformation could allow the formation of adducts which are not usually formed in double-stranded DNA containing a monofunctional adduct. An attractive hypothesis to explain the instability of the interstrand cross-links is that one of the two water molecules, in apical position with respect to the square of the platinum atom, labilises the G-Pt bond in solvolysis reaction. When the local

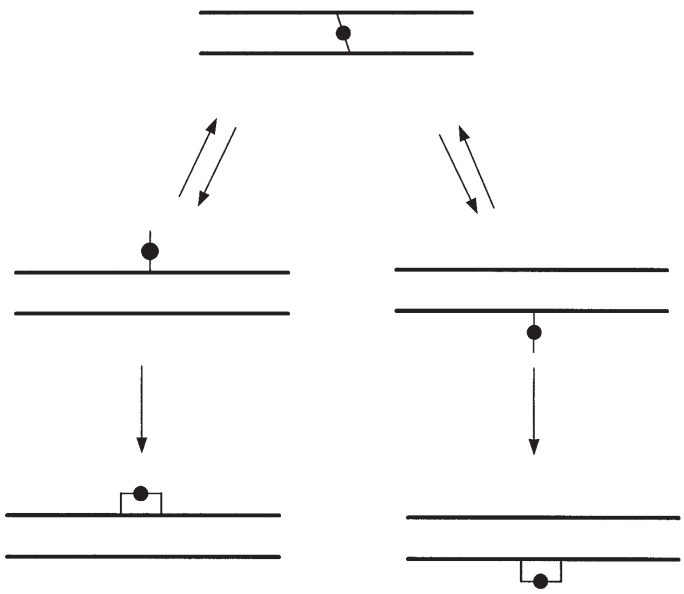

Fig. 2. *Rearrangement of cisplatin* (●) *interstrand cross-link into intrastrand cross-links* via *the formation of monofunctional* cis-$[Pt(NH_3)_2(dG)(H_2O)]^{2+}$ *adducts*

structure is modified as in ternary complexes, in which one of the two cross-linked strands is displaced by another non-cross-linked strand, the interstrand cross-link becomes stable (unpublished results).

Instability of the Monofunctional cis-*[Pt(NH$_3$)$_2$(dG)(Am)]$^{(n+1)+}$ Adducts*

Trisubstituted Pt^{II} complexes offer another way to form interstrand cross-links. These complexes of general formula *cis*-[Pt(NH$_3$)$_2$(Am)Cl]$^{n+}$, where Am is an heterocyclic amine such as pyridine, pyrimidine, ellipticine, etc., react with single- and double-stranded DNAs and form monofunctional *cis*-[Pt(NH$_3$)$_2$(dG)(Am)]$^{(n+1)+}$ adducts [58][59]. These adducts are kinetically inert as long as the platinated DNA is single-stranded. Hybridization of the single-stranded DNAs with the complementary strands triggers two concomitant reactions [59][60]. One is the cleavage of the bond between the platinum and the guanine residues with the release of *cis*-[Pt(NH$_3$)$_2$(H$_2$O)(Am)]$^{(n+1)+}$. This complex can react further with the same or another guanine residue and yields the monofunctional *cis*-[Pt(NH$_3$)$_2$(dG)(Am)]$^{(n+1)+}$ adduct. The consequence is a migration of the *cis*-[Pt(NH$_3$)$_2$(dG)(Am)]$^{(n+1)+}$ adduct along the same or another double helix. In fact, this migration ends finally because of the other reaction. In this reaction the bond between the platinum and Am residues is cleaved, which generates a monofunctional *cis*-[Pt(NH$_3$)$_2$(dG)(H$_2$O)]$^{2+}$ adduct. This adduct can react further and form intrastrand or interstrand cross-links. The relative yields of the two concomitant reactions (cleavage of the Pt-G and Pt-Am bonds) depend upon the conformation of the hybrids and the nature of the phosphodiester backbone (ribo, deoxyribo, *etc*.) and of Am [61]. It should be possible, by the right choice of Am, to favor the formation of intrastrand or interstrand cross-links in the reaction of DNA and the trisubstituted platinum(II) complexes. Some of these trisubstituted Pt^{II} complexes are active against a number of murine and human cancer cell lines [58][62]. Whether this activity is related essentially to the monofunctional adducts or to the rearrangement of the monofunctional adducts into bifunctional cross-links is not known.

Transplatin

Reaction between Transplatin and Double-Stranded DNA

The first step of the reaction between DNA and cisplatin or transplatin yields monofunctional adducts *cis*- or *trans*-[Pt(NH$_3$)$_2$(dG)Cl]$^+$ and the $t_{1/2}$

values for both reactions are of the same order of magnitude (2–3 h) [4][10]. Recently, it has been proposed that the transplatin monofunctional adducts evolve slowly ($t_{1/2} \approx 40$ h), and that the bifunctional lesions are mainly interstrand cross-links between the complementary guanine and cytosine residues [63][64]. Different conclusions were drawn from other studies. In one study [65][66], the closure of the monofunctional adducts is slow, being 80% complete in 48 h, but several kinds of intra- and interstrand cross-links are formed. In another study [56], the monofunctional adduct closure is fast ($t_{1/2} = 3.1$ h) with formation of 1,3- and longer range intrastrand cross-links. Discrepancies between these studies are only apparent in the sense that they originate not from the techniques used to analyse the samples, but from the nature of the platinated DNAs (molar ratio of bound platinum per nucleotide, length of the DNA fragments) and the experimental conditions (salt concentration, temperature). This has been proved by looking at the interstrand cross-linking reaction in duplexes containing a single monofunctional *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct in the upper strand, and zero to two monofunctional $[Pt(dien)(dG)]^{2+}$ adducts in the lower strand at defined positions [67]. A schematic representation of the platinated duplexes is given in *Fig. 3*. In these duplexes, the inert $[Pt(dien)(dG)]^{2+}$ adducts mimic the presence of transplatin monofunctional adducts [54].

After [^{32}P] labeling at 5′-end the duplexes were incubated at 37 °C and at various times analysed by gel electrophoresis under denaturing conditions.

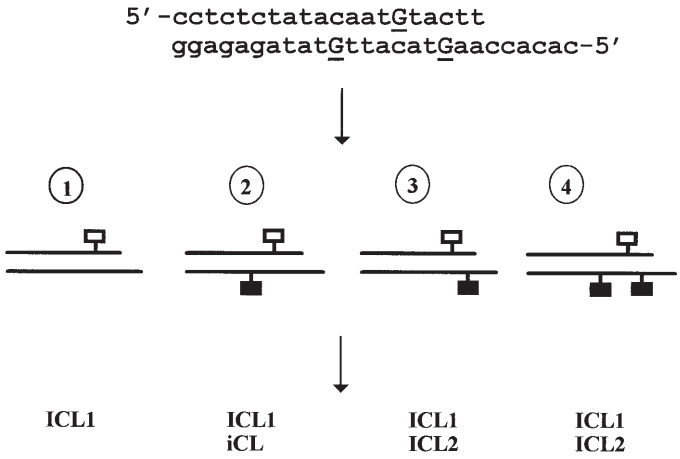

Fig. 3. Top: *sequence of the duplex with the underlined characters indicating the location of the monofunctional* trans-*$[Pt(NH_3)_2(dG)Cl]^+$ adducts* (□) *in the upper strand and that of the monofunctional $[Pt(dien)(dG)]^{2+}$ adducts* (■) *in the lower strand.* Four duplexes (1–4) have been prepared, and they contain 1, 2, or 3 adducts, respectively. ICL stands for interstrand cross-link and iCL for intrastrand cross-link.

Oligonucleotides containing intrastrand or interstrand cross-links are easily separated by this technique. Over a period of 50 h, in the platinated duplex *1*, only interstrand cross-links between complementary guanine and cytosine residues in agreement with previous results [64], are detected. In the platinated duplex *2*, which contains a $[Pt(dien)(dG)]^{2+}$ adduct in the lower strand three base pairs away from the *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct and on its 5′-side, both intrastrand and interstrand cross-links are detected. In the platinated duplex *3*, which contains a $[Pt(dien)(dG)]^{2+}$ adduct in the lower strand two base pairs away from the *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct and on its 3′-side, mainly two kinds of interstrand cross-links are detected. In the platinated duplex *4*, which contains two $[Pt(dien)(dG)]^{2+}$ adducts, two kinds of interstrand cross-links are formed, but in a different ratio to that in duplex *3*. In duplexes *3* and *4*, there is one interstrand cross-link between the complementary guanine and cytosine residues, and the other is between the guanine and an adenine residue located four or five base pairs away on the 3′-side of the guanine residue.

From these experiments it can be concluded that the closure of the monofunctional adducts depends upon the presence of other adducts in its vicinity. Although a systematic study has not yet been done, the interference between two or more adducts is expected to be a function of several parameters, such as the nature and the number of base pairs between the adducts, the DNA supercoiling, the local environment of the DNA, in addition to the distortions of the DNA double helix induced by the adducts, which are also function of these parameters. DNA has to be platinated at a low drug-to-nucleotide residue ratio when *in vitro* and *in vivo* experiments are compared. This holds also for cisplatin-modified DNA, but is masked by the preferential binding of cisplatin to runs of guanine residues and the ability of the monofunctional adducts to react with the adjacent residues.

Taking into account these conclusions and the high reactivity of the transplatin monofunctional adducts with glutathione [56][66], it is likely that in cells transplatin forms a low level of interstrand cross-links because of the slow closure of the monofunctional adducts coupled to their trapping by intracellular sulfur nucleophiles.

Distortions Induced in DNA by the Interstrand Cross-Link

As in the case of the cisplatin interstrand cross-links, several techniques have been used to characterize the distortions induced in the DNA double helix by the transplatin interstrand cross-links. From gel electrophoresis [68] it has been deduced that the DNA double helix is unwound (12°) and its axis is bent (26°) toward the major groove. Chemical probes and DNase I footprinting indicate that the distortion of the double helix spreads over four-

five base pairs, but there is no evidence for a local denaturation of the DNA [49][68]. A preliminary 2D-NMR study confirms that the guanine and cytosine residues are cross-linked with the guanine residue in the *syn*-conformation and without unpairing of the flanking base pairs [69]. The two NH_3 groups of the platinum residue are not in the plane of the cross-linked bases but are respectively above and below this plane. They interact with the adjacent base pairs and push them away from the cross-linked residues along the axis of the double helix (*Fig. 4*). The axis of the double helix is almost straight. This model could explain the slow closure of the monofunctional adduct into interstrand cross-link. In order to locate the platinum residue near the N(3) of the cytosine residue complementary to the monofunctional adduct, two events have to occur concurrently which are the rotation of the platinated guanine residue from the *anti*- to the *syn*-conformation and the displacement of the adjacent base pairs along the axis of the double helix.

Rearrangement of the (G1,G3)-Intrastrand Cross-Links in Single-Stranded DNA

In the reaction between transplatin and single-stranded DNA at pH 5.5 several kinds of bifunctional adducts are formed, 60% between two guanine

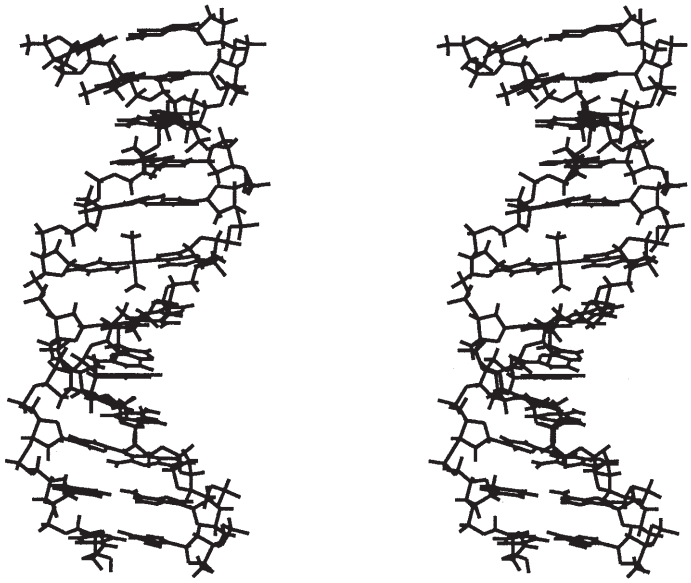

Fig. 4. *Stereoscopic view derived from 2D-NMR data of the duplex d(CTCTCG*-AGTCTC)·d(GAGACTC*GAGAG) containing a transplatin interstrand cross-link*

residues, 35% between guanine and adenine residues and 5% between guanine and cytosine residues respectively [65]. The flexibility of the single-stranded DNA and the accessibility of several sites allow the formation of adducts not formed in the reaction between transplatin and double-stranded DNA. It is generally considered that the bifunctional adducts are stable. Although a systematic study has not been done, this has been verified in the case of the (G1,G3)-intrastrand cross-links at the GNG sites (N being a nucleotide). There is one exception concerning the intrastrand cross-links having a cytosine residue adjacent to the 5′-side of guanine (sequence CGNG) [70][71]. In this case the metal migrates from the 5′-side of guanine to the 5′-side of cytosine and an equilibrium between the two isomers ((G2,G4)- and (C1,G4)-intrastrand cross-links) is attained (*Fig. 5*). The reaction is rather slow ($t_{1/2} \approx 120$ h at 30 °C). The rate of the reaction depends upon temperature ($t_{1/2} \approx 1.2$ h at 80 °C), but is independent of pH in the range 5–9 and on the nature and concentration of the salt (NaCl or $NaClO_4$) in the range 10–400 mM. The fact that the rate is the same in NaCl or $NaClO_4$ is not in favor of an intermediate step involving an aquated species. The rearrangement is sequence-specific since it does not occur when the cytosine residue is replaced by a guanine residue, or when the cytosine residue is on the 3′-side of the adduct.

Fig. 5. *Rearrangement of the transplatin (–□–) (G2,G4)-intrastrand cross-link into (C1,G4)-intrastrand cross-link within a single-stranded oligonucleotide.* N stands for a nucleotide residue.

Rearrangement of the (G1,G3)-Intrastrand Cross-Links in Duplexes

The pairing of the platinated oligodeoxyribonucleotides containing a single (G1,G3)-intrastrand cross-link with their complementary strands not only prevents the intrastrand rearrangement but also triggers another linkage isomerization reaction which consists in the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links. This rearrangement occurs whatever the nature of the base pairs on each side of the intrastrand cross-link [64][72–74]. A schematic representation of the rearrangement is given in *Fig. 6*.

The interstrand cross-link is between the 5′ guanine residue (and not the 3′ guanine residue) and the complementary cytosine residue. The formation of an intermediate monoaqua species during the rearrangement can

be excluded for two main reasons. The rate of the rearrangement is independent of the nature (NaCl or $NaClO_4$) and concentration of the salt up to 500 mM. Moreover, the rate is faster than that found in the case of the same duplex containing a monofunctional adduct. An explanation of this reaction is a direct nucleophilic attack of the Pt-G(3′) bond by the cytosine complementary to the 5′ guanine residue. This implies that the attacking residue is located near the platinum residue and along its *z*-axis.

The local conformation of the duplex containing a single 1,3-intrastrand cross-link is not fully characterized. NMR data is not available. From gel electrophoresis experiments [70] it has been deduced that the double helix is unwound (26°) and its axis is bent (45°). Chemical probes suggest a local distortion of the double helix over four to five base pairs, including the three base pairs at the level of the adduct and the 5′ base pair adjacent to the adduct [75]. By means of molecular mechanics modeling techniques, three families of stable and distorted three-dimensional structures were generated [76]. The three families have a different backbone geometry but, in the three cases, the location of the cytosine residue is compatible with the requirement for an attack of the platinum residue (an example is given in *Fig. 7*). Molecular modeling does not indicate which family induces the fastest rearrangement and one cannot yet predict the most favorable structure for a fast rate. Although a systematic study is still in progress, experiments done on various samples have led to the determination of some of the factors governing the rearrangement [64][72–74].

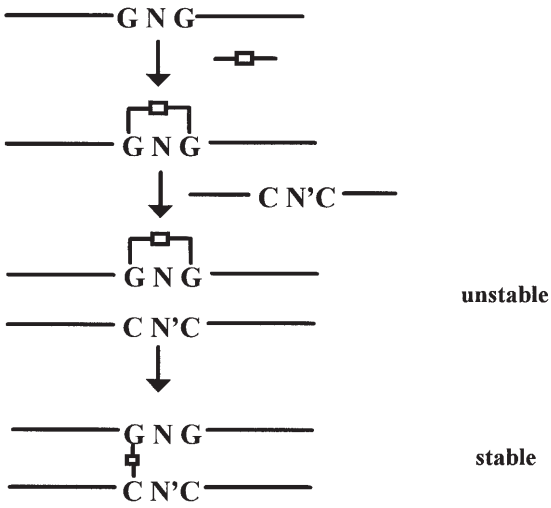

Fig. 6. *Rearrangement of the transplatin (–□–) (G1,G3)-intrastrand cross-link into an interstrand cross-link within a double helix.* N and N′ stand for nucleotide residues.

The strains due to the platinated macrocycle and the double helix are necessary to induce the linkage isomerization reaction. The DNA duplex containing the central sequence G*AG*·CTC (G* indicating the platinated guanine residue) in which the half-life of the intrastrand cross-link G*AG* is about 2 h, is taken as reference. The cleavage of the phosphodiester backbone at the level of the macrocycle decreases the rate of the rearrangement by at least a factor of ten. Over a period of 48 h, no rearrangement was detected when the 1,3-intrastrand cross-link is at the 3′- or 5′-end of the platinated strand in the duplex.

The intervening nucleoside residue between the two cross-linked guanine residues, the sugars, and the phosphate groups do not interfere directly in the reaction. The replacement of the intervening nucleoside by a propylene link, or of the phosphate groups by uncharged methylphosphonate groups, has no effect on the rate of the reaction. On the other hand the rate depends upon the nature of the base residues within the triplet complementary to the 1,3-intrastrand cross-link. The replacement of the thymine by a cytosine residue does not change the rate, while its replacement by a purine residue slows down the rate ($t_{1/2} \approx 15$–20 h).

The nature of the phosphodiester backbone of the sequences flanking the 1,3-intrastrand cross-link plays a role probably by acting on the local conformation of the hybrids, and subsequently on the relative position of the platinum and the attacking cytosine residue. The replacement of the complementary deoxy-strand by a ribo-strand results in a twenty-fold decrease of the rate ($t_{1/2} > 24$ h).

Fig. 7. *Stereoscopic view of a lowest-energy structure of the duplex d(TCTG*TG*TC)·d(GACACAGA) containing a single (G4,G6)-intrastrand cross-link.* The structure is represented pointing 5′ to 3′ upwards.

A major change in the rate is obtained by replacing the triplet complementary to the 1,3-intrastrand cross-link by the doublet 5′-UA within the hybrid platinated DNA-RNA. The rearrangement is faster ($t_{1/2} \approx 0.06$ h), and the interstrand cross-link is formed between the 5′ guanine and adenine residues. The reaction is even faster (complete in a few minutes) by pairing a platinated 2′-*O*-methyl-ribo-strand with the complementary ribo-strand containing the doublet 5′-UA opposite to the intrastrand cross-link. The doublet 5′-CA gives similar results whereas the doublets 5′-AA or 5′-GA are less efficient ($t_{1/2} \approx 0.5$ h). On the other hand, no rearrangement is detected over a period of 24 h when the unplatinated strand contains one of the following doublets: 5′-AU, 5′-AC, or 5′-GG. This drastic effect on the rate of the rearrangement confirms the crucial importance of the relative location of the two reactive species. It seems that the right location of these two species is favored by the A-form of the hybrids.

It is not yet known whether the non-leaving groups of transplatin interfere directly in the reaction. However, replacement of these groups by pyridine, iminoether, or dimethylamine (gifts from *N. Farrell*, *G. Natile*, and *B. Lippert*, respectively) prevents the rearrangement. In the case of methylamine the rearrangement is faster in the duplexes d(G*NG*)·d(CN′C), but unchanged in the hybrids d(G*NG*)·d(TA).

The specific and irreversible binding of the platinated oligonucleotides on large molecular weight targets and in various media has been demonstrated *in vitro* by the arrest of AMV reverse transcriptase and protein synthesis and in HBL 100ras1 cells [73].

Antisense and Antigene Strategies

Since the work of *Zamecnik* and *Stephenson* in 1978, showing that a synthetic 13-mer complementary to the 3′- and 5′-terminal sequences of the Rous Sarcoma Virus 35S was able to inhibit virus production in infected chick embryo fibroblast cells [77][78], numerous studies have been done to demonstrate that *in vivo* oligonucleotides can bind to their complementary sequences in mRNAs or DNA and subsequently act on the cellular machinery. The purpose of this section is to present briefly the potential interest of the platinated oligonucleotides in the context of the antisense and antigene strategies (for a comprehensive view of the antisense and antigene strategies, see [79–83] and references herein).

Oligonucleotides can form duplexes with single-stranded RNA or DNA through *Watson-Crick* hybridization or triplexes with double-stranded RNA or DNA through *Watson-Crick* and *Hoogsteen* hybridization [84]. The binding of oligonucleotides (the so-called antisense oligonucleotides) leads to

inhibition of translation or RNA metabolism by two general mechanisms, schematically represented in *Fig. 8*, which are either the degradation of the targeted mRNA through an RNase H-mediated cleavage, or the steric blocking of the cellular machinery. Up until now, the most promising approach seems to be the cleavage of mRNA by RNase H at the level of the hybrid mRNA-oligonucleotide. After the action of RNase H the oligonucleotide is released and is again available to bind to another mRNA molecule. One major impedement to this approach is that activation of RNase H requires the use of oligodeoxyribonucleotides, which are rapidly degraded by nucleases *in vivo*. Chemical modifications of the oligonucleotides can increase their resistance to nucleases, but, with the exception of phosphorothioate and phosphorodithioate analogues, the modified oligonucleotides are unable to activate RNase H. Although phosphorothioate oligodeoxyribonucleotides are currently being evaluated in clinical trials, it is known that, in the presence of cell extracts, more non-specific interactions are detected with the phosphorothioate than with the corresponding oligodeoxyribonucleotides and thus the oligonucleotides could be activated by a mechanism differing from the expected one [85][86]. More specific binding is achieved by the use of C(5)-propinylpyridine 2′-deoxyphosphorothioate oligonucleotides [87].

A major advantage of the steric blocking is that the oligonucleotides have chemically modified backbones which improve their resistance to nucleases. A serious constraint is that the oligonucleotide-RNA hybrids have to be stable enough to avoid dissociation caused by the cellular machinery. When directed to the coding region, oligonucleotides are dissociated from

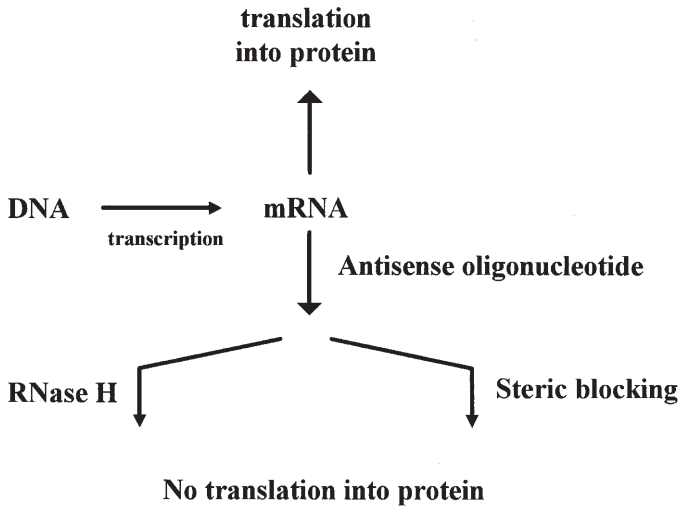

Fig. 8. *Schematic representation of the antisense strategy*

their targets by the translating ribosomes [88] even if the thermal stability of the hybrids containing a peptide nucleic acid [89] or a N(3′)–N(5′) phosphoramidate strand [90] is high [91]. The displacement of the oligonucleotides can be prevented by attaching to the oligonucleotides reagents, which can react with RNA after light activation or spontaneously [79][92]. Photochemical cross-linking of psoralen-derivatized oligonucleotides have been used with success, despite a modest yield of the photochemical reaction in hybrids [79][88][92]. However, irradiation of the samples in the *in vivo* experiments is difficult to realize. Non-specific reactions have been often observed for chemically induced cross-links, and the rates of the cross-linking reactions are generally slow as compared to the life-times of most mRNAs.

Pt^{II} complexes have been used to cross-link oligonucleotides to their complementary strands [93–96]. The platinated oligonucleotides contain a single monofunctional adduct. For the *in vivo* experiments, these platinated oligonucleotides present several disadvantages since the monofunctional adducts can react with residues within the oligonucleotides (suicide reaction) and with compounds in solution. In addition, the rate of closure into interstrand cross-links is rather slow. These pitfalls, also encountered with the other oligonucleotides bearing a reactive chemical group, explain why the second approach (steric blocking) has been less exploited than the first one (RNase H). Whether it is better to cleave or to block is still an open question. In this context, the use of the rearrangement of the transplatin 1,3-intrastrand cross-links into interstrand cross-links promoted by the formation of hybrids between the platinated oligonucleotides and their targets is promising.

In the antigene strategy, the oligonucleotides recognize specific sequences within double-stranded DNAs containing homopurine sequences via the formation of triple helices, the third strands (*Hoogsteen* strands) standing in the major groove of the double helix in a parallel or anti-parallel orientation with respect to the homopurine strand [83][84][97]. A major difficulty in this approach is that the stability of the triple helices is not sufficient to arrest the cellular machinery. As in the case of the antisense oligonucleotides, chemical modifications of the oligonucleotides including the covalent attachment of an intercalating agent give promising results [97][98]. Pt^{II} complexes have also been used to cross-link triplexes [95][96]. More recent work has been devoted to oligonucleotides containing a single *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ or *trans*-$[Pt(NH_3)_2(dC)Cl]^+$ adduct. It has been observed in nucleobases that replacement of a weakly acidic N-H proton in a hydrogen bond between two nucleobases by a metal species of suitable geometry generates metal-modified nucleobase pairs, and the linkage between the two nucleobase pairs becomes considerably stronger [101–103].

In the case of G · C *Hoogsteen* pairing and transplatin, the platinated pair fits almost exactly the requirement of normal DNA as far as interglycosyl distances are concerned. The platinated cytosine residue can be replaced by a platinated guanine residue but the cross-linking reaction requires a switch from the *anti-* to *syn*-conformation for this residue as shown on *Fig. 9*.

The cross-linking reaction occurs in triplexes containing a single *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ or *trans*-$[Pt(NH_3)_2(dC)Cl]^+$ adduct within the third strand. The rate of the reaction is faster with the *trans*-$[Pt(NH_3)_2(dC)Cl]^+$ adduct than with the *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct, but in both cases the cross-link is between the *Hoogsteen* and the purine strands. The reaction is specific, and no cross-link is formed with DNA which does not possess the targeted sequence. The formation of the cross-link stabilizes the triplex, but the presence of the monoadduct decreases the binding strength of the oligonucleotide to the duplex [99][100].

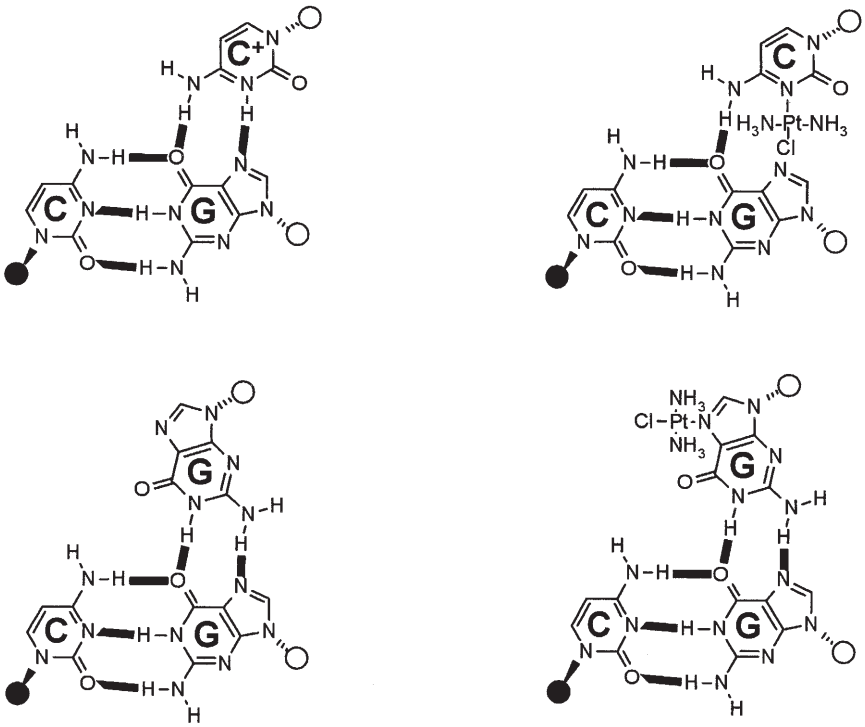

Fig. 9. *Base triplets formed between a* Watson-Crick *base pair G · C and a third base G or C* (*Hoogsteen*-pairing) (left) *or a transplatin-modified G or C* (right)

Conclusion

Although the major 1,2 intrastrand cross-links are considered as playing an important role in the anticancer activity of cisplatin, the contribution of the interstrand cross-links should also be taken into account. Cisplatin interstrand cross-links are generally believed to be cytotoxic by inhibition of DNA replication and/or transcription. Therefore, efficient repair of interstrand cross-links is significant for cell survival. Interstrand cross-links pose a unique challenge to the DNA repair machinery since both DNA strands are damaged. The general model for interstrand cross-link repair involves an incision step followed by a homologous recombination event [104]. Repair of interstrand cross-links in the nuclear genome of human cells has been demonstrated, although the precise mechanism is still unknown [31]. *In vitro*, eukaryotic repair of cisplatin interstrand cross-links has been recently studied [28][105]. In human cell extracts containing nucleotide excision repair activity, no cross-link-specific incision of a duplex with an unique cisplatin interstrand cross-link is observed [28], whereas an interstrand cross-link of a psoralen derivative is well excised with an unexpected pattern of incision [106]. It might be possible that the unusual structure of cisplatin interstrand cross-link is less or not recognized by proteins of nucleotide excision repair system, and that other proteins are necessary for more efficient repair. Further work is required to clarify the biological role of these adducts and to understand the possible relevance of the unusual structure of the interstrand cross-links in the specific binding of cellular proteins implicated in the process of this lesion.

In the reaction with DNA, transplatin forms monofunctional adducts which slowly close into interstrand cross-links. No intrastrand cross-links are detected in DNA platinated at a low level. It is tempting to relate the clinical inefficiency of transplatin to the long life-time of the monofunctional adducts combined with their high reactivity with glutathione. On the other hand, compounds having the *trans*-geometry, such as iminoether compounds, show *in vivo* antitumor activity towards murine tumors [38][39]. In the *in vitro* reaction with DNA, essentially monofunctional adducts are formed [107]. Due in large part, if not exclusively, to steric hindrance of the iminoether groups these adducts are much less reactive with thiourea than the transplatin monofunctional adducts [107][108]. Excision-repair deficient *xeroderma pigmentosum* group A cells are 4 times more sensitive to iminoether complexes than normal cells, suggesting cellular DNA as cytotoxic target. Although it is not yet demonstrated that the monofunctional adducts are related to the cytotoxicity of the iminoether complexes, these results and those obtained with Pt^{IV} open a field for the design of new antitumor drugs.

Another aspect of transplatin which might be indirectly related to cancer, is the rearrangement of the 1,3-intrastrand cross-links into interstrands. The adducts are stable within single-stranded oligonucleotides [73][74]. The formation of a duplex between the platinated oligonucleotide and its target triggers the rearrangement of the intrastrand cross-link into an interstrand cross-link. Conditions have been established in which the rate of the rearrangement is complete in a few minutes. In cells, the specific and irreversible binding of a platinated oligonucleotide to its targeted mRNA has been demonstrated, as well as inhibition of cell growth. It is likely that this inhibition is related to the arrest of the translation machinery by the cross-linked oligonucleotide. A systematic work is in progress to compare the efficiency and the relative advantages of the two approaches (steric blocking and RNase H cleavage) in the context of the antisense strategy. The platinated oligonucleotides can be also useful as a tool in biotechnology. They are used to trap the intermediate states in the folding of RNA molecules. For all these experiments, large quantities of oligonucleotides are needed, and the large-scale preparation of the platinated oligonucleotides is not yet available. The recent progress in the chemistry of protected platinated synthons [109][110] should make available in the near future the automated solid-phase synthesis of site-specific platinated oligonucleotides.

The rearrangement of the transplatin 1,3-intrastrand cross-links or the cisplatin monofunctional adducts of the form *cis*-$[Pt(NH_3)_2(dG)(Am)]^{(n+1)+}$ is not promoted by the formation of triple helices. Up to now, the third strand has been cross-linked to the purine strand of the duplex *via* the closure of transplatin monofunctional adducts. All the difficulties concerning the competitive reactions, as previously described in the context of the antisense strategy, have to be overcome in addition to the uptake of the oligonucleotides by cells and tissues and their transport to their targets. It is the hope that all these problems will be solved to allow a large use of the platinated oligonucleotides in both therapeutical and biotechnological fields.

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Part 4. Chemistry Relevant to Pt-Biomolecule Interactions

Platinum Complexes: Hydrolysis and Binding to N(7) and N(1) of Purines

R. Bruce Martin

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with Non-Sulfur-Containing Amino Acids and Peptides

Trevor G. Appleton

Platinum Complexes: Hydrolysis and Binding to N(7) and N(1) of Purines

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This article tabulates acidity constants for aqua complexes derived from cisplatin and recommends 'consensus' values. Combined with the equilibrium constants for water substitution for chloride ion, the predominant species in the blood plasma (104 mM Cl^-) are the dichloro and chloro-hydroxo complexes for both *cis*-$(NH_3)_2Pt^{II}$ and its *trans*-isomer. At equilibrium, in the cell nucleus (4 mM Cl^-) the chloro-hydroxo, aqua-hydroxo, and dihydroxo complexes appear in comparable amounts for both isomers. We now incorporate with the chloride equilibria the rate of reaction of each species with N(7) of inosine to find a weighted relative rate, to yield the order of decreasing weighted reactivity in the cell nucleus as *trans*-chloro-aqua > *cis*-hydroxo-aqua > *cis*-chloro-aqua > *cis*-diaqua. If we consider the chloride equilibria in the complexes to remain that of the plasma, the order of decreasing weighted reactivity is *trans*-chloro-aqua > *cis*-chloro-aqua > *trans*-dichloro > *cis*-dichloro. The main species of the drug reacting with nucleic bases upon entry into the cell nucleus is the chloro-aqua species, which, in about one hour, becomes superseded by the hydroxo-aqua species for the *cis*-isomer. These equilibria and rate considerations fail to account for physiological differences between *cis*- and *trans*-isomers. Features promoting binding by Pt^{II} and Pd^{II} at N(7), compared to the more basic N(1) site of purine derivatives, are reviewed. Considering together all nucleosides and nucleotides in neutral solutions, Pt^{II} and Pd^{II} favor binding at N(7) of guanosine. Further enhancement of N(7) binding occurs in 5′-nucleotides owing to a hydrogen bond from a coordinated amine to an uncoordinated phosphate group in a macrochelate. In solution, there is about 40% macrochelate with 5′-nucleotide monoanions including esters, and about 80% macrochelate with 5′-nucleoside monophosphate dianions and both ionic forms of 5′-nucleoside di- and triphosphates.

Introduction

The chapter title announces two different topics that are linked by a third. Since the aqua forms of Pt^{II} complexes are more reactive than the chloro species, the extent of hydrolysis of administered chloro complexes becomes a crucial issue. Although the slowness of Pt^{II} reactions permits them to reach

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their intracellular targets, it is often awkward in laboratory investigations where equilibrium may be difficult to achieve. Pd^{II} has the great merit of chemistry similar to Pt^{II} but a 10^4 to 10^5 times greater reactivity, permitting easily detailed equilibrium studies. Insights into both the hydrolysis on one hand and Pt^{II} binding to N(7) or N(1) of purines on the other have been gained by studies of analogous Pd^{II} complexes, that thus provide a linkage between the two subjects of the title. First, we discuss hydrolysis of cisplatin, then review some analogous Pd^{II} chemistry, and finally conclude with the competition between N(7) and N(1) in purines for these metal ions. The last section includes discussion of intramolecular hydrogen bonding from a coordinated amine to an uncoordinated phosphate group to form a macrochelate.

Hydrolysis of *cis*-Diamminedichloroplatinum(II)

Water Substitution for Chloride

Since antitumor *cis*-diamminedichloroplatinum(II) (*cis*-DDP) is administered as a relatively unreactive complex, it is necessary to consider the following equilibria. The first two reactions are aquations of the chloro groups to yield chloro-aqua and diaqua complexes.

$$cis\text{-}(NH_3)_2PtCl_2 + H_2O \rightleftharpoons Cl^- + cis\text{-}(NH_3)_2PtCl(H_2O)^+$$

$$cis\text{-}(NH_3)_2PtCl(H_2O)^+ + H_2O \rightleftharpoons Cl^- + cis\text{-}(NH_3)_2Pt(H_2O)_2{}^{2+}$$

The associated equilibrium constants are

$$K_1 = \frac{[Cl^-][cis\text{-}(NH_3)_2PtCl(H_2O)^+]}{[cis\text{-}(NH_3)_2PtCl_2]}$$

$$K_2 = \frac{[Cl^-][cis\text{-}(NH_3)_2Pt(H_2O)_2{}^{2+}]}{[cis\text{-}(NH_3)_2PtCl(H_2O)^+]}$$

These constants have been determined several times; for this article we will employ values in units of molar for 25 °C and 1M ionic strength with $NaClO_4$ background of log K_1= –2.19 and log K_2 = –3.53 [1]. For the corresponding *trans*-complexes the values in 0.1M $NaClO_4$ are log K_1 = –2.92 and log K_2 = –4.41. Thus, the aquation reactions are more favored in the *cis*-complexes. Values for all four constants are numerically greater by about 0.3 log units at 45 °C in 0.1M $NaClO_4$ [2][3], indicating that an increase in temperature modestly favors the chloro complexes.

Bound-Water Deprotonation

Both the monochloromonoaqua and diaqua complexes deprotonate in weakly acidic to neutral solutions.

$$cis\text{-}[(NH_3)_2PtCl(H_2O)]^+ \rightleftharpoons H^+ + cis\text{-}(NH_3)_2PtCl(OH)$$

The acidity constant that describes this reaction is

$$K_{a3} = \frac{[H^+][cis\text{-}(NH_3)_2PtCl(OH)]}{[cis\text{-}(NH_3)_2PtCl(H_2O)^+]}$$

The diaqua complex may undergo two successive deprotonations

$$cis\text{-}[(NH_3)_2Pt(H_2O)_2]^{2+} \rightleftharpoons H^+ + cis\text{-}[(NH_3)_2Pt(OH)(H_2O)]^+$$
$$cis\text{-}[(NH_3)_2Pt(OH)(H_2O)]^+ \rightleftharpoons H^+ + cis\text{-}(NH_3)_2Pt(OH)_2$$

with the associated acidity constants:

$$K_{a1} = \frac{[H^+][cis\text{-}(NH_3)_2Pt(OH)(H_2O)^+]}{[cis\text{-}(NH_3)_2Pt(H_2O)_2^{2+}]}$$

$$K_{a2} = \frac{[H^+][cis\text{-}(NH_3)_2Pt(OH)_2]}{[cis\text{-}(NH_3)_2Pt(OH)(H_2O)^+]}$$

All of the above reactions and accompanying equilibrium constants may also be written for the complexes derived from *trans*-DDP. We shall be comparing the equilibrium-constant values for aquation and deprotonation of the *cis*- and *trans*-complexes.

Table 1 tabulates literature values for acidity constants of seven amine-Pt^{II} complexes with notations on the temperature, ionic strength, total Pt^{II} concentration, method employed, conditions and other remarks, and the reference number. At least six factors enter into comparing determinations of a single complex. First is the purity of the complex under investigation. Because they rely on chemical shifts of an individual species, NMR methods are less dependent on purity than potentiometric titrations, which are interpreted on the basis of equivalents of added base. Rarely is the raw titration data published, but in one case it is evident from a plot of the data that the titration curve reveals up to about 10% impurity [7]. Without knowing whether the impurities are acidic, basic, inert, or even forming during

Table 1. *Acidity Constants of Platinum(II) Complexes*

pK_a[a])	°C	I[b]), M	[Pt], mM	Method	Conditions	Ref.
cis-$[(NH_3)_2Pt(H_2O)_2]^{2+}$						
5.37(9), 7.21(9)	26	0.02	5	^{1}H-NMR	No added salt	[4]
5.39(2), 7.23(2)	25	0.06	20	potentio[c])	NO_3^- salt of complex, no added salt	[5]
5.64, 7.40	25	0.1	< 0.4	kinetic	0.1M $NaClO_4$	[6]
5.24(3), 7.10(10)	22	0.06	20	potentio	$CF_3SO_3^-$ salt of complex, no added salt	[5]
5.24(5), 7.42(10)	22	0.06	20	potentio	ClO_4^- salt of complex, no added salt	[5]
5.56, 7.32	20	0.03	10	potentio	No added salt	[7]
5.93(10), 7.87(10)	5	0.3	100	^{15}N-NMR	No added salt	[8]
5.5, 7.3	25	0.16	low		'Consensus'	
$[(en)Pt(H_2O)_2]^{2+}$						
5.81(2), 7.62(2)	23	0.2	5.5	potentio	0.2M KNO_3, KNO_3 salt bridge	[9]
trans-$[(NH_3)_2Pt(H_2O)_2]^{2+}$						
4.48(2), 7.20(5)	25	0.1	5	potentio	0.1M $NaClO_4$	[10]
4.35, 7.40	25	0.3	100	^{15}N-NMR	No added salt	[11]
4.32, 7.38	20	0.03	10	potentio	No added salt	[7]
4.4, 7.3	25	0.16	low		'Consensus'	
cis-$[(NH_3)_2PtCl(H_2O)]^+$						
6.49(3)	25	0.01	4	potentio	No added salt	[d])
6.41(3)	26	0.02	5	^{1}H-NMR	No added salt	[4]
6.85(10)	25	0.3	100	^{15}N-NMR	No added salt	[8]
6.6	25	0.16	low		'Consensus'	
trans-$[(NH_3)_2PtCl(H_2O)]^+$						
5.94(2)	25	0.1	5	potentio	0.1M $NaClO_4$	[13]
5.63	25	0.3	100	^{15}N-NMR	No added salt	[11]
$[(NH_3)_3Pt(H_2O)]^{2+}$						
6.37(10)	25	0.4	131	^{15}N-NMR	No added salt	[8]
[(diethylenetriamine)$Pt(H_2O)]^{2+}$						
5.87(2)	35	0.2	50	half neut.	No added salt	[14]
6.0(2)	25	0.1	3	^{1}H-NMR	0.1M $NaClO_4$	[15]
6.13	25	0.1	1.8	potentio	0.1M $NaClO_4$	[16]
6.24	25	0.1	4	kinetic	0.1M $NaClO_4$	[17]

[a]) Number in parentheses is error limit in the last digit(s) provided in the reference.
[b]) Ionic strength.
[c]) Potentiometric titration.
[d]) From analysis of titration data in [12]; see text.

the course of the reaction, it is difficult to deal with them. An attempt to do so for the *cis*-diaqua complex suggested little reduction in pK_{a1} but a reduction in pK_{a2} of about 0.2 log units [18]. The latter result, if added to *Table 1*, would represent a second $pK_{a2} = 7.1$ value.

Interestingly, this volume celebrates 30 years of cisplatin, but work on both the *cis*- and *trans*-complexes harks back 60 years in [7] and in the Russian school of *A. A. Grinberg* [19].

Purity is a special problem with the important species *cis*-$[(NH_3)_2PtCl(H_2O)]^+$ (which has been purified [20]), and as they identify individual species, NMR methods are almost essential for evaluation of acidity constants. The titration curve of this complex, prepared in solution from *cis*-DDP by addition of one equivalent of $AgNO_3$ [12], is too spread out on the pH axis to represent one acidic group. However, it proves possible to resolve the potentiometric data by assuming that the 'impurity' is *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$. A non-linear least-squares analysis refines satisfactorily with 16(2)% 'impurity' (molar basis) and $pK_{a3} = 6.49(3)$. As *Table 1* indicates, the latter value agrees closely with one determined under similar conditions by ^{1}H-NMR (6.41). The amount of 'impurity' may be compared with that calculated independently from the disproportionation of the chloro-aqua complex to give diaqua and dichloro complexes. Use of the equilibrium constants for K_1 and K_2 leads to 15% diaqua complex at equilibrium, in agreement with the 16% figure. Thus, both conclusions from the potentiometric resolution are supported by different independent methods.

Decrease in temperature increases basicity and pK_a. The listing of determinations for each complex in *Table 1* is in order of decreasing temperature, which should produce an upward bias in pK_a values on reading down the table. An extensive temperature-dependent potentiometric titration study [5] of the two pK_a values for the first complex in *Table 1* suggests that a five-degree drop in temperature increases the pK_a values by about 0.13 log units, but there is considerable variation, including differences among $CF_3SO_3^-$, NO_3^-, and ClO_4^- salts of the complex that exceed any known binding strength of these anions. No background electrolyte was added in these experiments.

Some investigators suggest that supposedly inert anions interact with Pt^{II} complexes [5]. The differences in pK_a values for three anions in a single study were mentioned in the previous paragraph and appear in *Table 1*. The equilibrium constant for nitrate complexation to the *cis*-diaqua complex has been found by ^{195}Pt-NMR to be 0.17 M^{-1} [21]. With this constant, at 0.2M NO_3^- about 3% of the Pt is nitrate-bound and at 0.04M NO_3^- less than 1%. These fractions amount to only 0.01 log units or less in equilibrium constants considered. Perchlorate was found not to bind to the *cis*-diaqua complex [21]. Thus the differences in pK_a values for three salts of *cis*-diaqua complex cannot be ascribed to binding by the anions to Pt^{II}.

Increasing ionic strength also increases basicity and pK_a. For closely similar temperatures the listing for each complex in *Table 1* is in order of increasing ionic strength, again producing an upward bias in pK_a values on reading down the table. We might expect the pK_{a1} for a 2^+-ion to be up to 0.3 log units greater for the highest over the lowest ionic strengths in *Table 1*. The increase for a 1^+-ion should be 1/3 as great. If inert salt has not been added to control ionic strength, the ionic strength of a diaqua-complex solution with monoanions as counterions decreases by 1/3 upon titration with two equivalents of NaOH.

Because hydroxo complexes eventually form oligomers (see below under Pd^{II}) the total metal-ion concentration becomes important at high metal-ion concentrations if the investigation is not performed rapidly or the species measured individually as in NMR. Oligomer formation of the *cis*-diaqua complex was rapid enough at 100 mM total Pt^{II} to force the investigators to retreat to 5 °C for their pK_a determinations [8]. Oligomerization during the course of titrations was also suggested as a reason for variation of pK_{a2} values in the study at 20 mM Pt^{II} with three different complex anions [5].

Finally, chloride ions from an ordinary calomel electrode were found to interfere seriously with pH determinations, resulting in non-reproducible readings and end points [9]. Only in this one study was a special double-junction calomel reference electrode with KNO_3 in the outer tube employed to prevent interference by chloride ions. Use of this double-junction electrode is labeled as 'KNO_3 salt bridge' under *Conditions* in *Table 1*.

Undoubtedly, for the reasons just described, for a single complex, there is a greater spread of values in *Table 1* than is normally found between laboratories or even within one laboratory. For working purposes we need agree upon values for some of the complexes. For the *cis*-diaqua complex, its *trans*-analog, and the *cis*-chloro-aqua complex recommended 'consensus' values for room temperature and physiological ionic strength appear at the end of listing for the first, third, and fourth complexes. These 'consensus' values were used with the five equilibria and equilibrium constants to construct *Tables 2* and *3* and the distribution curves in *Figs. 1–3*.

Table 2. *Mole Fraction $(NH_3)_2Pt^{II}$ Species at pH 7.4, and Plasma and Nuclear Chloride-Ion Concentrations*

	cis		*trans*	
mM Chloride	104	4	104	4
Cl^-, Cl^-	0.67	0.03	0.68	0.05
Cl^-, OH^-	0.26	0.30	0.31	0.60
Cl^-, H_2O	0.04	0.05	0.008	0.02
H_2O, H_2O	1×10^{-4}	0.003	3×10^{-6}	1×10^{-4}
OH^-, H_2O	0.009	0.28	0.003	0.15
OH^-, OH^-	0.012	0.35	0.004	0.19

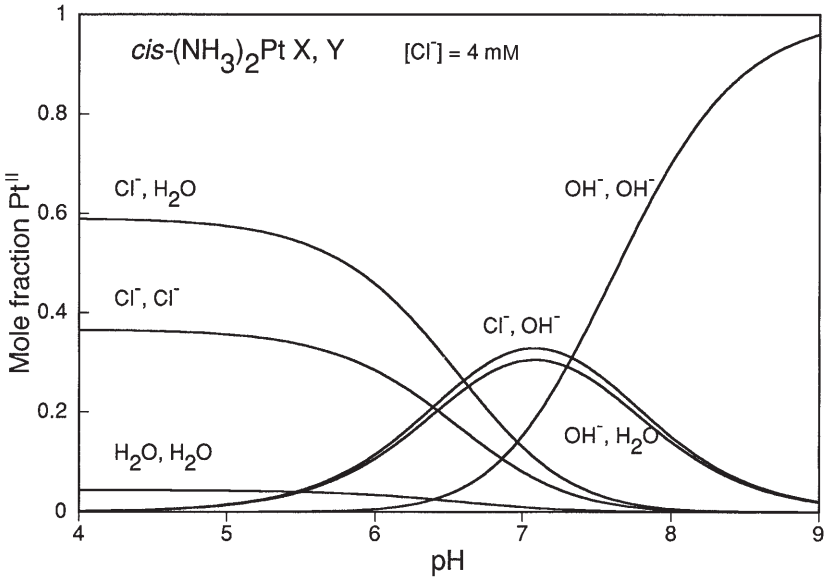

Fig. 1. *Mole fraction* cis-$(NH_3)_2Pt^{II}$ vs. *pH at 4 m*M *chloride-ion concentration*. The labels identify the groups in the remaining two positions.

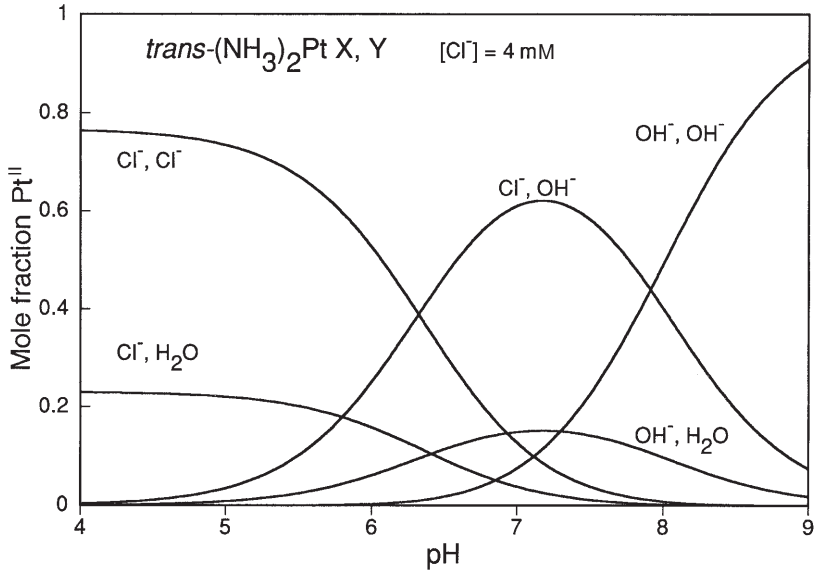

Fig. 2. *Mole fraction* trans-$(NH_3)_2Pt^{II}$ vs. *pH at 4 m*M *chloride-ion concentration*. The labels identify the groups in the remaining two positions. The diaqua complex curve is near the baseline.

Table 2 lists the mole fraction of six *cis*- and *trans*-$(NH_3)_2Pt^{II}$ species at pH 7.4 and at the ambient chloride concentrations of the blood plasma (104 mM) and of the cell nucleus (4 mM). Chloro complexes dominate in the plasma and hydroxo complexes in the nucleus.

At 104 mM chloride ion, typical of the plasma, for both *cis*-DDP and its *trans*-isomer, the species distribution as a function of pH is similar to that depicted earlier with the dichloro and chloro-hydroxo complexes being the dominant species at pH 7.4 [18]. Both dominant species are relatively inert kinetically. This reference also shows a plot of mole fraction *vs.* chloride-ion concentration at pH 7.0. Only at lower chloride-ion concentrations do the more reactive species containing an aqua ligand appear to a significant extent.

Fig. 1 shows the distribution curves for *cis*-$(NH_3)_2Pt^{II}$ at 4 mM chloride-ion concentration representative of that in the cell nucleus. *Fig. 2* shows the analogous plot for the *trans*-isomer. For both isomers the three dominant species at pH 7.4 are the chloro-hydroxo, dihydroxo, and reactive hydroxo-aqua complexes.

To this point we have considered only equilibria, not rates. Proton-transfer reactions onto a bound hydroxide and off a bound water are rapid. For the slower reactions involving bond breaking and making to Pt^{II} we consider two limiting situations. First, we assume that the Pt^{II} complexes are in equilibrium with 4 mM ambient chloride of the cell nucleus so that the equi-

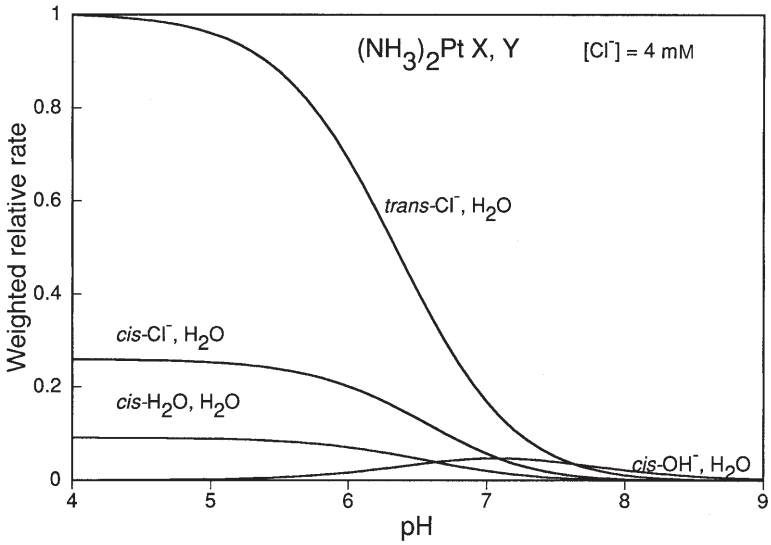

Fig. 3. *Weighted relative rate at 4 mM chloride based on distributions of* Figs. 1 *and* 2 *combined with rate of reaction at inosine N(7)* vs. *pH for species of both* cis- *and* trans-*$(NH_3)_2Pt^{II}$*. Curves for only one *trans*- and three *cis*-species are drawn. On this scale, curves for the other species only fatten the baseline.

libria displayed in *Figs. 1* and *2* have been attained. Second, we shall assume that the Pt^{II} complexes are in equilibrium with the 104 mM ambient chloride of the plasma; the complexes have not been in the cell nucleus long enough to achieve equilibrium with the lower chloride background before reacting with nucleic bases. We ignore oligomerization reactions on the grounds that the complex concentration in a physiological setting is too dilute for them to occur.

The pertinent question is, given the equilibrium distributions of *Figs. 1* and *2*, how fast do these species react with a nucleic base? Hydroxide is such a poor leaving group that it is virtually unreactive. There are four reactant complexes to consider: 1. dichloro, 2. chloro-aqua, 3. diaqua, and 4. hydroxo-aqua. Fortunately, studies have been made at 45 °C of the rate of each of the species with N(7) of inosine or 1-methylinosine. For *cis*-$(NH_3)_2Pt^{II}$ the relative rates of the four successive reactants with N(7) of inosine are 1.0, 74, 350, 26 [2][6], and for the *trans*-isomer on the same scale 3.4, 740, 35, and 4.2 [3][10]. From these values the order of decreasing leaving group capability is $H_2O > Cl^- >>> OH^-$. (The possibility that Pt^{II}-bound hydroxide may serve as a nucleophile affording an effective hydroxide concentration only attained at higher pH has received limited study [9].)

Fig. 3 shows a plot of weighted relative rates combining, on a single scale for both *cis*-$(NH_3)_2Pt^{II}$ and its *trans*-isomer, the equilibrium species distributions of *Figs. 1* and *2* with the relative rates of the previous paragraph. In *Fig. 3,* the reactivity decreases with increasing pH as inert hydroxo groups replace displaceable ligands. Throughout the entire pH range only four species make contributions significant enough to appear on the scale of *Fig. 3*. *Fig. 3* shows also that, in acidic solutions, the *trans*-chloro-aqua-complex is the most reactive species, with its contribution tailing off as the pH increases.

The results are shown quantitatively in *Table 3* for pH 7.4 in the columns headed 4 mM for the *cis*- and *trans*-species. *Table 3* shows the weighted relative rates scaled to the most effective species as 100. At equilibrium

Table 3. *Weighted Relative Rates for Reaction of Complexes $(NH_3)_2Pt^{II}$ with Inosine N(7) at pH 7.4 at Plasma and Nuclear Chloride-Ion Concentrations*

	cis		*trans*	
mM chloride	104	4	104	4
Cl^-, Cl^-	6	0.3	21	2
Cl^-, H_2O	28	31	51	100
H_2O, H_2O	0.4	11	0.001	0.05
OH^-, H_2O	2	65	0.1	6
Sum	36	107	72	108

in the cell nucleus at pH 7.4 and 4 mM chloride ion, for equal concentrations of *cis*-DDP and its *trans*-isomer the order of decreasing weighted reactivity is *trans*-chloro-aqua > *cis*-hydroxo-aqua > *cis*-chloro-aqua > *cis*-diaqua. All significant reactive species bear water as a ligand. As indicated in the last row of *Table 3* for 4 mM chloride at pH 7.4 the sum of the weighted relative rates of the four *cis*-species equals that of the four *trans*-species.

However, because of the slowness of Pt^{II} conversions, the various $(NH_3)_2Pt^{II}$ species may not be at equilibrium with ambient 4 mM chloride in the cell nucleus. The $(NH_3)_2Pt^{II}$ species may be more nearly in equilibrium with the ambient 104 mM chloride of the blood plasma, where the administered drug has circulated. For conversion from administered dichloro to diaqua complexes in acidic solutions the successive half lives at 45 °C are 1.0 and 0.8 h for *cis* and 0.18 and 48 h for *trans* isomers [3]. These times agree with the well-documented *trans*-activating order $Cl^- > NH_3 > H_2O$. Therefore, we have performed a similar analysis of the reaction rate with inosine N(7) assuming that the $(NH_3)_2Pt^{II}$ species are in equilibrium with the blood plasma and the results appear under the columns labeled *104 m*M in *Table 3*. At 104 mM Cl^-, the total reactivities of all *cis*-species are 1/3, and those of all *trans*-species 2/3 those at 4 mM.

Depending on the time elapsed since entering the low-chloride environment of the nucleus, for each isomer the real distribution in the cell nucleus appears somewhere between the columns headed *4 m*M and *104 m*M in *Table 3*. With the above times, the half-life for the Pt^{II} complexes to reach equilibrium upon passing from a 104 mM to 4 mM ambient chloride environment at 45 °C is about one hour for the *cis*- and 0.2 h for the *trans*-isomer. *Table 3* shows that except for a very significant contribution from the *cis*-diaqua complex in 4 mM chloride, the most reactive species for both isomers at both chloride concentrations is the chloro-aqua that appears in the second row. Therefore, the main species of the drug reacting with nucleic bases upon entry into the cell nucleus is the choro-aqua species, which in about one hour becomes superseded by the hydroxo-aqua species for the *cis*-isomer.

Fig. 3 and *Table 3* suggest that we are still unable to account for the significantly greater antitumor activity of *cis*-DDP over its *trans*-isomer. Joint consideration of the species distributions and relative rates of reaction reveals almost equal reactivity of the species from the two isomers at 4 mM chloride and a 2:1 advantage in favor of *trans* at 104 mM chloride. There must be an additional basis for the relatively greater efficacy of the *cis*-isomer. The remaining difference is the chelate-ring capability of the *cis*-isomer that is absent in the *trans*.

Palladium Analogs

Owing to the slowness of reactions of Pt^{II} complexes, it was proposed that investigation of reactions of the much more rapidly reacting Pd^{II} complexes would provide insights into Pt^{II} reactions at equilibrium [9][22]. Complexes of Pd^{II} react 10^4 to 10^5 times more rapidly than the corresponding complexes of Pt^{II}, while the equilibrium constants are only about ten times stronger for Pt^{II}. Both metal ions strongly prefer square-planar geometry and possess similar ionic radii. In the isostructural complexes (en)Pt(5′-GMP)$_2$ and (en)Pd(5′-GMP)$_2$, where GMP is bound at N(7), the metal-ion-to-nitrogen bond lengths differ by less than 0.5% [23].

Expectations for the usefulness of studying Pd^{II} complexes have been amply borne out. The important slow dimerization of *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$, which reduces the concentration of active species in neutral solutions, was uncovered in the ready dimerization of the Pd^{II} analog $[(en)Pd(H_2O)_2]^{2+}$. The equilibrium distribution of metal ion between the N(1) and N(7) sites of purine bases has been worked out for Pd^{II}, but is known for Pt^{II} only in a few cases. A coordinated-amine-to-uncoordinated-phosphate-group hydrogen bond was discovered in solution studies of Pd^{II}-complexed nucleoside monophosphates. We discuss each of these aspects in turn.

(Ethylenediamine)Pd^{II} and cis-*$(NH_3)_2Pt^{II}$*

To mimic *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$ with the more rapidly exchanging Pd^{II} and to prevent isomerization, it is necessary to employ the complex of ethylenediamine (en), $[(en)Pd(H_2O)_2]^{2+}$, that through chelation is necessarily *cis*. Upon titration with standard base an endpoint is reached after the addition of only one equivalent of base at pH 7.5, but the reversible titration curve is flattened on the pH axis and cannot be fitted with the equilibrium expression for a simple deprotonation. It was proposed that the monohydroxo complex dimerizes to a binuclear dihydroxo-bridged dimer [9]. The two reactions and their equilibrium constant expressions follow.

$$[(en)Pd(H_2O)_2]^{2+} \rightleftharpoons H^+ + [(en)Pd(OH)(H_2O)]^+$$

$$2\,[(en)Pd(OH)(H_2O)]^+ \rightleftharpoons 2\,H_2O + [(en)Pd(OH)_2Pd(en)]^{2+}$$

$$K_{a1} = \frac{[H^+][(en)Pd(OH)(H_2O)^+]}{[(en)Pd(H_2O)_2^{2+}]} \qquad K_d = \frac{[(en)Pd(OH)_2\,Pd(en)^{2+}]}{[(en)Pd(OH)(H_2O)^+]^2}$$

The equilibrium constant for the overall reaction is given by $K_{ov} = K_{a1}^2 \cdot K_d$. From analysis of titrations at two concentrations tenfold different it was found that $-\log K_{ov} = 8.4$ [9]. The dimer is so strong that minimum

amounts of mononuclear hydroxo complex occur in neutral solutions, and resolution of the two individual equilibrium constants is less certain.

Structures of crystals prepared from solutions containing cis-$[(NH_3)_2Pt(H_2O)_2]^{2+}$ revealed not only a dimer [24][25], but also a trihydroxo-bridged trimer [26–28]. We formulate trimer formation as follows.

$$3\ [(en)Pd(OH)(H_2O)]^+ \rightleftharpoons 3\ H_2O + [\{(en)Pd(OH)\}_3]^{3+}$$

$$K_t = \frac{[\{(en)Pd(OH)\}_3^{3+}]}{[(en)Pd(OH)(H_2O)^+]^3}$$

From an analysis of titration curves it was suggested that $pK_{a1} = 6.1$, $\log K_d = 3.7$ (M^{-1}), and $\log K_t = 6.5$ (M^{-2}) [18]. From these constants, it may be predicted that in a range of pH 6–9, a 0.37M solution containing originally $[(en)Pd(H_2O)_2]^{2+}$ would consist predominantly of trimers and dimers in a 2.9:1 trimer/dimer mole ratio on a Pd^{II} basis. More than ten years later, and by authors apparently innocent of a prediction from the titration analysis, in a 0.37M solution the trimer/dimer mole ratio was found to be 2.8:1 by ^{15}N-NMR [29]. Thus, there is striking agreement between the conclusions by two different methods determined wholly independently. (In contrast, some authors have evidently based their analysis on only one concentration and rejected trimer formation as unimportant [30].) It may be shown that the trimer/dimer mole ratio of 2.8:1 implies that $K_t = 8.6 \cdot K_d^{1.5}$, and we incorporate this relationship in all further analyses.

Above pH 8 the titration curve shows uptake of a second equivalent of base corresponding to production of $(en)Pd(OH)_2$. This second section of the titration curve is also flattened, owing to break up of oligomers by uptake of a second hydroxide at high pH. Though little reaction occurs directly by this route, we define the second acidity constant in the usual way.

$$[(en)Pd(OH)(H_2O)]^+ \rightleftharpoons H^+ + (en)Pd(OH)_2$$

$$K_{a2} = \frac{[H^+][(en)Pd(OH)_2]}{[(en)Pd(OH)(H_2O)^+]}$$

There is a symmetry to the first and second halves of the titration curve. Oligomerization flattens both halves by the same amount. Oligomerization also displaces the first half to lower pH by the same amount that the upper half is displaced to higher pH. Owing to this symmetry and introduction of a new constant, K_{a2}, consideration of the second half does not aid resolution of the equilibrium constants. Due to overlap of the deprotonations, the pK_a values may not be simply read from the midpoints of each half of the

whole titration curve. (From a published flattened titration curve, the midpoint pH of the first equivalent was incorrectly interpreted as the pK_{a1} value, yielding too low a value [31].) However, the sum of the midpoints gives the sum of (pK_{a1} + pK_{a2})/2 = 7.8, which is also the midpoint pH between the two halves of the titration curve.

We have four unknown equilibrium constants: the acidity constants K_{a1}, K_{a2}, and the oligomerization constants K_d and K_t. We know rather well the products $K_{ov} = K_{a1}^2 \cdot K_d$ and $K_{a1} \cdot K_{a2}$. By accepting the ^{15}N-NMR result we also have the relation $K_t = 8.6\ K_d^{1.5}$. We need one more item of information. The average value (p*K*a1 + pK_{a2})/2 is 7.8 for $[(en)Pd(H_2O)_2]^{2+}$ and 6.7 for the corresponding Pt^{II} complex in *Table 1*. Thus, we might project from the Pt^{II} results that for the Pd^{II} complex pK_{a1} = 6.9 and pK_{a2} = 8.7. However, careful non-linear least-squares analysis of the first equivalent in the titration curve at 1 mM concentration, where there is less oligomer formation, consistently refines to pK_{a1} = 6.1 (though greater values fit almost as well) and log K_d = 3.6. These results imply that pK_{a2} = 9.5 and log K_t = 6.3. Titration analysis of even more dilute systems should allow refinement of these conclusions. With this set of constants and considering Pd^{II}-based mole fractions, in neutral solutions dimer exceeds monomer at 0.3 mM total Pd^{II} and trimer exceeds dimer at 30 mM total Pd^{II}.

How may these results with $[(en)Pd(H_2O)_2]^{2+}$ apply to *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$? Early on it was recognized that the latter complex formed dimers and trimers in both crystals and solution [32]. Dimers and trimers appear to form to comparable extents in the two cases. Thus, the distribution of oligomers for the Pt^{II} complex may well be similar to that just described. Instead of nearly instantaneous, the time scale is slower with Pt^{II}, but still of the order of minutes in a 50 mM solution [32][33]. Thus, pK_a values in *Table 1* determined by potentiometry and reported for the more concentrated solutions may be compromised by oligomerization taking place during measurement. At the 20 mM total Pt^{II} employed in many of the determinations listed in *Table 1*, using the above constants, neutral solutions at equilibrium would contain only 8% of the Pt^{II} as monomers, and 50% in dimers and 42% in trimers. Reported instances of the dichloro species being more reactive than the diaqua are undoubtedly due to oligomerization of the latter [34].

(Diethylenetriamine)Pd^{II} and (dien)Pt^{II}

With only a single site available for substitution, the Pd^{II} complex of tridentate diethylenetriamine, $[(dien)Pd(H_2O)]^{2+}$, avoids complexities associated with a chelating metal ion and provides a facile intrinsic measure of binding at individual nucleic-base sites. Titration yields an endpoint after

the addition of one equivalent of base, but the curve appears too spread out on the pH axis to be represented by a simple ionization [9]. (Perhaps this fact accounts for 'complications' mentioned in a later article [35].) Titration curves for increasing complex concentrations pivot about the half equivalence point at a fixed pH, which is also the pK_a in this case. Data may be fitted precisely by including formation of a hydroxo-bridged dimer [36].

$$[(\text{dien})\text{Pd}(\text{H}_2\text{O})]^{2+} \rightleftharpoons \text{H}^+ + [(\text{dien})\text{Pd}(\text{OH})]^+$$

$$[(\text{dien})\text{Pd}(\text{OH})]^+ + [(\text{dien})\text{Pd}(\text{H}_2\text{O})]^{2+} \rightleftharpoons \text{H}_2\text{O} + [(\text{dien})\text{Pd}(\text{OH})\text{Pd}(\text{dien})]^{3+}$$

From a non-linear least-squares fit to the data at two concentrations near 21 °C and 0.5M ionic strength, for the first reaction, pK_a = 7.74(1), and for the dimerization reaction, K_d = 132(12) M^{-1}. This singly-bridged dimer is much weaker than the doubly-bridged one in the (en)Pd^{II} complex above. The maximum concentration of dimer occurs at the half-equivalence point, 0.57 mM for a solution that is 5 mM in total Pd^{II}. This weak dimerization does not affect the results with nucleic bases mentioned below [36].

The corresponding Pt^{II} complex, $[(\text{dien})\text{Pt}(\text{H}_2\text{O})]^{2+}$ dimerizes and reacts much more slowly. It is a 40 times stronger acid (*Table 1*) with a dimerization constant K_d = 108(15) M^{-1} at 35 °C [14]. This value is close to that of K_d = 132 M^{-1} near 21 °C just described for the analogous Pd^{II} complex. Since a higher temperature should favor decomposition, the values would be even closer if compared at the same temperature. Thus, dien complexes of Pt^{II} and Pd^{II} form comparable amounts of a weak dimer. This similarity supports the suggestion that at equilibrium the *cis*-$(NH_3)_2Pt^{II}$ and (en)Pd^{II} complexes form comparable amounts of dimer and trimer.

One may infer that *all the Pt^{II} complexes in* Table 1 *undergo time-dependent oligomerizations in weakly acidic to neutral solutions when there are comparable amounts of bound aqua and hydroxo groups.*

Metal-Ion Binding at N(7) *vs.* N(1) in Purines

While both metal ions form square-planar complexes, reactions of Pt^{II} are typically 10^5 times slower than those of Pd^{II}, so study of the latter suggests eventual equilibrium positions for the kinetically sluggish Pt^{II}. Both metal ions react primarily at the N(7) and N(1) sites of purines in nucleosides or nucleotides. Direct phosphate-metal-ion coordination occurs rarely and is not considered in this article. Direct but weak chelation with *cis*-$(NH_3)_2Pt^{II}$ bridging N(7) and a phosphate oxygen occurs with 5′-IMP and 5′-GMP [37].

Resolution of the N(7)-N(1) dichotomy is unequivocal in the case of Pd^{II} because the metal ion is both diamagnetic and, on the NMR time-scale, in slow exchange between the two sites. Thus, analysis of peak heights in ^{1}H-NMR reveals the relative populations of all species in solution [36]. Moreover, by employing the tightly chelated tridentate diethylenetriamine (= dien) only one binding site remains on the planar (dien)Pd^{II} to interact with other ligands. This avoids considerable complications from two *cis*-sites on the metal ion such as occur in (en)Pd^{II} [38–40].

Stability Sequences

We make use of a convenient tabulation of pK_a values for basic sites in nucleosides (and bases and nucleotides) [41][42] to order the sites in sequence of increasing basicities, with each nucleoside identified by its first letter (with I for inosine (= 2-deaminoguanosine)).

$$H^+\text{: A7, I7 < G7 << A1, C3 <<<<< I1, G1, U3, T3}$$

Each inequality sign represents about a tenfold increase in stability constant. The entire range for proton binding is slightly greater than eight log units. For the stability constants of (dien)Pd^{II} with nucleosides on the same basis the order is [36]

$$(\text{dien})Pd^{II}\text{: A7 < A1 < C3 < I7 < G7 < G1, I1, U3, T3}$$

The range is reduced to just less than five log units. Compared to the H^+-series we note enhancements for N(7) sites as A7 is now just one inequality sign from A1, and both I7 and G7 move up smartly. Compared to the proton, (dien)Pd^{II} strongly favors N(7) over N(1) sites in purine derivatives.

Owing to competition in neutral solutions by the proton at the four most basic sites, the stability-constant order is not that of binding. Utilizing conditional stability constants we find that the order of increasing binding strength of nucleosides in neutral solutions at pH 7.4 is given by the sequence

$$(\text{dien})Pd^{II}\text{, neutral: A7 < A1 < C3 < G1, T3, U3, I1, I7 < G7}$$

The range is further reduced to less than four log units, and the ordering of the N(7) and N(1) sites of both inosine and guanosine have crossed over. Below the ‘crossover pH’ the metal ion binds at N(7) and above this

pH at N(1). For inosine and (dien)Pd^{II} the crossover occurs at pH 6.1 [36]. (The concept of 'crossover pH' also applies to other metal ions [41-43].) The N(7) site of guanosine has risen to the top as the strongest (dien)Pd^{II} binder of nucleosides in neutral solutions. This result is consistent with the prevalent observation of *cis*-DDP binding at N(7) of guanosine groups.

The above three sequences apply to nucleosides. On the same basis the sequence of increasing stability constants for nucleoside 5′-monophosphates is the same as the first sequence above for the proton, H^+, but a new ordering pertains for (dien)Pd^{II} [34][36].

(dien)Pd^{II}: A1, A7 < C3 << I7 < G1, G7, I1, U3, T3

Each inequality sign signifies about a tenfold increase in binding strength.

Again we allow for competition between the proton and metal ion for the basic N(1) sites on purine and N(3) sites on pyrimidine 5′-nucleoside monophosphates to find for neutral solutions at pH 7.4 the sequence

(dien)Pd^{II}, neutral: A1, A7 < C3, G1, T3, U3, I1 < I7 < G7

Compared to the corresponding two sequences in the previous section we see N(7) sites moving up in binding strength upon addition of a 5′-phosphate group. Binding at A7 has become comparable to A1, and G7 has moved to the strongest binding group. These sequences are consistent with both equilibrium-constant values and the results obtained with mixtures of nucleoside 5′-monophosphates [34][36].

Though once commonly proposed, direct *N*(7)-*O*(6)-chelation by a metal ion in 6-oxopurines is now accepted to occur only in special cases [38][40][44]. Any chelation is indirect, through a metal-ion-bound water molecule, for example [45].

Steric hindrance by exocyclic amino groups reduces the equilibrium binding constant and the rate of complex formation. The 6-amino group in adenosine is sterically larger than the 6-oxo group in inosine and guanosine and hinders metal-ion binding at N(7) and especially N(1) [41][46–48]. It has been estimated that the 6-amino group reduces the stability constant of Cu^{II} binding to adenosine by 0.6 log units at N(7) and by 1.5 log units at N(1) [41]. Aquated (dien)Pt^{II} reacts more than ten times slower than expected at N(1) of 7-methylguanosine because of steric hindrance by the C(2) amino group [49]. Close comparision of equilibrium constants also reveals that (dien)Pd^{II} binds more strongly to N(1) of 5′-IMP than to the more basic 5′-GMP [36][50].

The sizable enhancement by 1.6 log units of (dien)Pd^{II} binding to N(7) of the 6-oxopurines, inosine and guanosine, compared to adenosine on a ba-

sicity adjusted basis remains unaccounted for [41][42][50]. To attribute this difference to steric hindrance in binding of adenosine and its nucleotides requires jettisoning of imidazole and benzimidazole as ligands setting the baseline, and an explanation of why a corresponding or greater difference does not appear for binding at N(1).

One expects Pt^{II} to behave similarly to Pd^{II}, but so slowly that the parallel may not always be evident for these equilibrium sequences. (dien)Pt^{II} does displace the proton from the very basic N(3) site in uridine ($pK_a = 9.2$) with a binding strength at least ten times greater than (dien)Pd^{II} [51]. There are other well-established examples of Pt^{II} interacting at the very basic N(3) sites of pyrimidines and N(1) sites of 6-oxopurines [44].

It is also possible for Pd^{II} and Pt^{II} to displace a proton from the exocyclic amino group of cytidine and adenosine to form a stable nitrogen-to-metal-ion bond [39][40][44]. However, this mode of interaction occurs very slowly with Pt^{II} and requires neutral to basic solutions. Unless deprotonated, the exocyclic amino group is not a metal-ion-binding site [45].

Metal-ion coordination at N(7) acidifies the proton at N(1) by up to two log units [36]. Metal-ion binding at N(7) weakens both the basicity and metal-ion binding capability at N(1). For inosine and guanosine and their nucleotides in neutral solutions, the decrease in metal-ion stability at N(1) is more than offset by the greater fraction of N(1) deprotonated species.

At pH 5–6 a migration of (dien)Pt^{II} from an equimolar amount of inosine N(7) to N(1) occurred *via* slow buildup and loss of a binuclear intermediate [18]. First the metal ion reacts at N(7)

$$M + BH_1 \rightarrow M_7BH_1$$

The proton at N(1) is more easily lost

$$M_7BH_1 \rightarrow H^+ + M_7B^-$$

rendering the N(1) site more accessible to metal ions

$$M + M_7B^- \rightarrow M_7BM_1$$

Finally, the strong binding of the metal ion at N(1) weakens the binding at N(7).

$$M_7BM_1 \rightarrow M + BM_1$$

Owing to the slowness of the reactions with Pt^{II}, displacement of the N(1) proton in inosine and guanosine and their nucleotides by Pt^{II} is diverted by prior coordination and kinetic fixation at N(7). There is often insufficient Pt^{II} to form significant amounts of the binuclear intermediate, and hence no convenient pathway to generate N(1)-metallated species.

Metal-ion coordination at N(7) in purines also promotes exchange of the H(8) hydrogen with solvent water [52].

N(7) vs. *N(1) Binding in Adenosine and Derivatives*

In the column for adenosine in *Table 4*, several Pt^{II} complexes including (dien)Pt^{II} bind with a significantly greater percentage at N(7) than does (dien)Pd^{II}. The three Pt^{II} complexes with others [48] distribute between the N(7) and N(1) sites in comparable amounts. Care must be taken in assuring equilibrium with Pt^{II}; heating favors the N(1) site [53]. Since the percentages in *Table 4* are the results of a competition between N(7) and N(1), the greater percentage for (dien)Pt^{II} over (dien)Pd^{II} indicates that, though it binds more strongly than Pd^{II} to both N(1) and N(7), the advantage for Pt^{II} is greater for N(7).

Owing to the difficulty of assuring equilibrium, more examples of the competition between purine N(1) and N(7) exist for Pd^{II} rather than Pt^{II} complexes. *Table 4* lists equilibrium percentages of metal ion at N(7) for adenosine and several of its nucleotides. In these comparisons the remaining metal ion to total 100% is at the N(1) site. The first entry shows that the percentage of (dien)Pd^{II} at N(7) increases upon phosphate deprotonation in 5′-AMP but not in 5′-ATP [34][36]. (Not included in *Table 4* are the results for tridentate dipeptide complexes where 57% of (dipeptide)Pd^{II} is at N(7) in 5′-AMP and 5′-ATP regardless whether the phosphate group is protonated [54].) As *Table 4* indicates, phosphate-group deprotonation does not change

Table 4. *Percentage Binding to N (7) of Adenosine and its Nucleotides* [a])

Complex	Adenosine	5′-AMP^{-}	5′-AMP^{2-}	5′-ATP[b])
(dien)Pd^{II}	20[c])	31[d])	55[d])	57[e])
(dien)Pt^{II}	≈50[f]), 60[g])	66[h])		
$(NH_3)_3Pt^{II}$	≈50[i])			
cis-$(NH_3)_2Pt^{II}$	55[j])			

[a]) As opposed to binding at N(1). AMP^{-} and AMP^{2-} differ by phosphate group protonation in the former with $pK_a = 6.2$.
[b]) For 5′-ATP both phosphate protonated and deprotonated forms yield the same percentages.
[c]) [36] [51].
[d]) [36] [34], which also reports 20% for both ionic forms of 2′/3′-AMP and cyclic 3′,5′-AMP.
[e]) [34], which also reports 57% for both ionic forms of 5′-ADP.
[f]) [51]. Solution was heated, which favors binding at N(1).
[g]) [53].
[h]) [18].
[i]) K. Inagaki, M. Kuwayama, and Y. Kidani, *J. Inorg. Biochem.* **1982**, *16*, 59.
[j]) Value in [48] adjusted for about 1/4 N(1)-protonated form

the percentage of (dien)Pd^{II} at N(7) for 2′/3′-AMP, cyclic 3′,5′-AMP, ADP, or ATP, but increases the percentage only for 5′-AMP. We now discuss the reason for this interesting contrast.

Intramolecular Hydrogen Bonding from Coordinated Amine to Uncoordinated Phosphate Group

Intramolecular hydrogen bonding from a coordinated amine-hydrogen to an uncoordinated phosphate group was first proposed upon observation of stronger metal-ion binding and acidification of the phosphate group in (dien)Pd(5′-AMP) [34]. The N(7)/N(1) molar ratio of (dien)Pd^{II} binding to adenosine, and both ionic forms of both 2′/3′-AMP, and cyclic 3′,5′-AMP is about 0.2, while for 5′-AMP it is 0.5 for the anion and 1.2 for the dianion. The last increase in ratio is coupled quantitatively to acidification of the phosphate group by 0.4 to 0.5 log units. Only metal-ion binding at N(7) results in acidification of the phosphate group. Only for N(7)-bound 5′-AMP is the intramolecular hydrogen bond possible.

The resulting macrochelate became established by comparing stabilities of (dien)Pd^{II} and (pentamethyldien)Pd^{II} complexes of N(7)-bound 5′-nucleotides of AMP, GMP, and IMP [50]. Two arguments support the hydrogen-bonding proposal. In pentamethyldien, all five nitrogen-bound hydrogens of dien are replaced by methyl groups. Only for (dien)Pd^{II} binding at N(7) (not at N(1)) are the stabilities of the nucleotides enhanced by 0.5 to 0.7 log units over those for the nucleosides. Also only in the (dien)Pd^{II} complexes is the phosphate group acidified by 0.4 to 0.5 log units. Neither of these augmentations occur with (pentamethyldien)Pd^{II}. These results strongly suggested an intramolecular hydrogen bond from a coordinated amine-hydrogen to the uncoordinated phosphate group to form a macrochelate [42][50]. The coordinated-amine-to-uncoordinated-phosphate intramolecular hydrogen bond was proposed from solution studies before any crystal-structure determinations.

Later, a crystal structure showed an intramolecular hydrogen bond from a coordinated ammonia to the dinucleotide terminal 5′-phosphate group in *cis*-[$(NH_3)_2$Pt{d(pGpG)}] [55]. An intramolecular hydrogen bond from coordinated amine to uncoordinated phosphate group has also been found in crystal structures of N(7)-bound 5′-nucleotides in (en)Pt(5′-GMP)$_2$ and the isostructural Pd^{II} complex [23], and in (en)Pd(5′-IMP)$_2$ [56]. The former study supported retention of the intramolecular hydrogen bond in solution with NMR evidence. Other NMR results supporting intramolecular hydrogen bonding from coordinated amine to N(7)-bound 5′-phosphates include: (en)Pt(5′-dAMP)$_2$ [47], *cis*-$(NH_3)_2$Pt[d(CpGpG)] [57], *cis*-$(NH_3)_2$Pt(5′-

$GMP)_2$ [58], (en)Pt(5′-$AMP)_2$ and (en)Pt(5′-$GMP)_2$ [59], (en)Pt[d(pGpG)] [60], and (dien)Pt(5′-GMP) [61].

From potentiometric titration comparisons of the phosphate acidification, it is estimated that in solution 40% of *cis*-$(NH_3)_2$Pt(5′-$dGMP)_2$ complexes participate in intramolecular hydrogen bonding [62]. However, in all the crystal structures cited in the previous paragraph, the hydrogen bond is to a protonated phosphate. Since some of the phosphate-protonated species might also be intramoleculary hydrogen-bonded in the solution study, the 40% amount represents the percentage difference of intramolecularly hydrogen-bonded complexes in the unprotonated and phosphate-protonated forms. We avoid this uncertainty by using a different approach and calculating separately the percentage of intramolecularly hydrogen-bonded species in each ionic form.

To find the percentage of intramolecularly hydrogen-bonded species, we calculate the excess metal-ion binding to the N(7) site in cases where hydrogen bonding may occur compared to those related complexes where such hydrogen bonding cannot occur. From *Table 4* we note that in five cases where hydrogen bonding cannot occur – adenosine, and both ionic forms of both 2′/3′-AMP and cyclic 3′,5′-AMP – the percentage of (dien)Pd^{II} at N(7) is 20% for an N(7)/N(1) mole ratio of 0.25. We label this normalized ratio R_n. In an intramolecularly hydrogen-bonded species, there is an additional isomeric equilibrium constant [63], I = [closed]/[open]. With hydrogen bonding we designate the [N(7)]/[N(1)] ratio as R_H and obtain $R_H = R_n(1+I)$. The fraction of intramolecularly hydrogen-bonded or macrochelate species is given by $f = I/(1+I)$, from which we conclude, $f = 1 - R_n/R_H$. From *Table 4*, for (dien)Pd^{II} and monoanionic AMP^- we have f = 1 – 0.25/0.44 = 0.43 or 43% macrochelate. Similarly for (dien)Pt^{II} and monoanionic AMP^- from *Table 4* we have f = 1 – 1.2/1.9 = 0.37 or 37% macrochelate. The calculated percentages are sensitive to small changes in the ratios. There is good agreement between the two dien-metal-ion complexes with 40% macrochelate with phosphate-protonated monoanionic AMP^-.

For the phosphate-deprotonated dianionic AMP^{2-} from *Table 4*, we have for (dien)Pd^{II}, f = 1 – 0.25/1.2 = 0.79 or 79% macrochelate. We may compare this value with that for dianionic IMP^{2-} normalized to the [N(7)]/[N(1)] ratio for inosine, where an intramolecularly phosphate-hydrogen-bonded species is impossible [36]. In this case, binding of (dien)Pd^{II} at N(1) is much stronger, and we have a very different set of ratios to give f = 1 – 0.030/0.18 = 0.83 or 83% macrochelate. Thus, despite two very different sets of [N(7)]/[N(1)] ratios for (dien)Pd^{II} with the two phosphate deprotonated dianionic nucleotides, we find about 80% of the complexes are intramolecularly hydrogen-bonded in both cases. The difference in percentage macrochelate between the dianionic and monoanionic nucleotides of 80 – 40 =

40% corresponds to the percentage estimated from acidification of the phosphate deprotonation mentioned two paragraphs above. Therefore, there is a self-consistent picture of the percentage of complexes intramolecularly hydrogen-bonded from coordinated amine to both protonated and deprotonated phosphate in a variety of complexes.

The degree of intramolecular hydrogen bonding from coordinated amine to a protonated and deprotonated phosphate group is reflected in the extent of its acidification. We designate the isomeric equilibrium-constant mentioned above as I_P for the deprotonated and as I_{PH} for the protonated phosphate group. From the equilibria involved, it may be shown that the experimental pK_{a7P} is given by $pK_{a7P} = pk_{a7P} + \log[(1 + I_{PH})/(1 + I_P)]$, where the low-case pk_{a7P} represents the value in the absence of any intramolecular hydrogen bonding. We calculate for 80% hydrogen-bonded complexes, $I_P = 4.0$, and for 40% hydrogen-bonded complexes, $I_{PH} = 0.67$. Substitution of these values into the last equation yields as the difference between hydrogen-bonded and non-hydrogen-bonded complexes, for the phosphate group $pK_{a7P} - pk_{a7P} = -0.48$. This difference agrees with consistent observation of a 0.4 to 0.5 log-unit acidification of the phosphate group in complexes with coordinated amines [34][36][47][50][59-62]. Acidification of the phosphate group is minimal when metal-ion binding is at N(1), or at N(7) when hydrogen bonding cannot occur as in 2′/3′-AMP and cyclic 3′,5′-AMP.

From the results recorded in *Table 4*, the percentage macrochelate with (dien)Pd^{II} and both anionic forms of both ADP and ATP is also about 80%. The conclusions of about 80% macrochelate with both ionic forms of 5′-nucleoside di- and triphosphates and 5′-nucleoside-monophosphate dianions, and 40% macrochelate with 5′-nucleotide monoanions including esters with amine complexes of either Pt^{II} or Pd^{II} should be generally applicable. Intramolecular coordinated-amine-to-phosphate-ester hydrogen bonding is then expected in polynucleotides and may furnish an intermediate for the metal ion *en route* to stronger binding with two nitrogen donors. Geometrical requirements for this pathway may furnish a distinction between *cis*- and *trans*-DDP.

Applications to Antitumor *cis*-$(NH_3)_2Pt^{II}$

Antitumor *cis*-$(NH_3)_2Pt^{II}$ favors binding at guanine N(7) in DNA [64][65]. The following six factors contribute to the preference for Pt^{II} binding at G7. *1*) In the neutral nucleosides G7 is 0.8 log units more basic than A7 [41]. However, this contribution is small compared to the next. *2*) There is a 1.6 log-unit enhancement for (dien)Pd^{II} binding at N(7) of 6-oxopurines

[41][42][50], and it is likely that this N(7) binding enhancement also occurs with Pt^{II} complexes. That the enhancement also occurs with (pentamethyldien)Pd^{II} argues against an often suggested Pt^{II}-bound ammonia-to-6-oxo-group hydrogen bond as the source of the G7 favoritism in the antitumor complexes. *3*) *N*(1)-protonation of 6-oxopurines and *N*(3)-protonation of 4-oxopyrimidines greatly reduces the availability of these sites in neutral and acidic solutions. *4*) Steric hindrance by the amino group at C(2) slows reaction of Pt^{II} at guanosine N(1). *5*) Pt^{II} complexes react so slowly that in most cases they never reach equilibrium with the protonated sites of points 3 and 4. *6*) In DNA interstrand hydrogen bonding between purine N(1) and pyrimidine N(3) leaves only purine N(7) accessible to metal ions. Acidification of the proton at N(1) by metal-ion binding at N(7) may strengthen the purine-N(1)-to-pyrimidine-N(3) hydrogen bond in polynucleotides. The culmination of these six contributions leaves G7 as the preeminent Pt^{II}-binding site.

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Reactivity and Inertness of Pt-Nucleobase Complexes

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This chapter focuses on the properties and reactions of various Pt-nucleobase complexes. After short description of various binding modes, attention will be paid to the effects of coordinated platinum. Coordination of electrophilic platinum to nucleobases modifies the electron density of the heterocyclic ring atoms. This may result in changes in acid-base properties, hydrogen-bonding abilities, and hydrolytic stability of the coordinated nucleobase-derivative, and may facilitate migration of Pt from one binding site to another within the base moiety. Binding of bifunctional Pt^{II} compounds to DNA is a two-step process. The initial step involves the formation of Pt-nucleobase monoadducts of the type PtN_3X, the lifetime of which largely depends on the remaining leaving group X. Usually, hydrolysis of X is considered as the rate-limiting step in the conversion of monadducts to bisadducts. However, this may be an oversimplification, since the lability of the coordinated water molecule drastically decreases with increasing pH upon its conversion to the OH group. On the other hand, Pt-nucleobase bis(complexes) and analogous model compounds with a PtN_4 coordination sphere are quite inert to substitution reactions. Strong nucleophiles (CN^- and sulfur-containing molecules) can displace N-donors from Pt unless steric obstacles make the nucleophilic attack difficult. In addition, a nitrogen atom can act as a powerful nucleophile toward Pt if spatially in a correct position.

Introduction

The ability of Pt^{II} to form covalent adducts with the base residues in DNA is crucial for the biological activity of various anticancer Pt drugs [1][2]. A common factor to cisplatin-related compounds (*cis*-[$PtCl_2$-$(NH_3)_2$], the parent platinum drug [3]) is the coordination sphere of Pt consisting of two tightly bound am(m)ine ligands and two labile leaving groups. These labile ligands (usually Cl^- ions or oxygen donor groups) are replaced by donor atoms of the nucleobases when the Pt compound binds to DNA [1][4]. Coordination of bifunctional Pt^{II} compounds to the base residues of

Cisplatin. Edited by Bernhard Lippert

nucleic acids is a two-step process. The first step involves the formation of monofunctional adducts, primarily at the N(7) atoms of guanine or adenine moieties. These monoadducts then react further to form intrastrand and interstrand cross-links [1][4][5]. Because of the inertness of Pt^{II}, factors affecting the lifetime of different monoadducts become important, also by taking possible side-reactions with other biomolecules into account [2]. In contrast, various bifunctional adducts are considered stable under physiological conditions and will be decomposed only in the presence of strong nucleophiles that have a high affinity for Pt [5][6]. However, in a few cases relatively easy migration of coordinated Pt^{II} from one nucleobase to another has been reported for both single-stranded [1][5] and double-stranded [5][7] oligonucleotides. Unfortunately, the exact mechanism of the migration reactions is largely unknown [5].

The purpose of this chapter is to explore the properties and reactions of various Pt-nucleobase complexes. After a short description of various binding modes, attention will be paid on the effects of coordinated platinum. Topics include, *e.g.*, isomerization, thermodynamic stability, and solvolytic reactions of Pt-nucleobase complexes. Finally, factors affecting the mechanism and kinetics of substitution reactions by various nucleophiles will be discussed.

Binding Sites

Heteroaromatic purine and pyrimidine nucleobases and their model compounds exhibit a wide variety of potential binding sites for metal ions [6]. The distribution of metal ions between various donor atoms depends on the basicity of the donor atom, steric factors, interligand interactions, and on the nature of the metal. Under appropriate reaction conditions most of the heteroatoms in purine and pyrimidine moieties are capable to coordinate Pt^{II} [6]. In addition, platinum-binding also to the carbon atoms (*e.g.*, to C(5) in 1,3-dimethyluracil) has been established [8].

With 9-substituted 6-oxopurines (*Fig. 1*), the predominant Pt^{II}-binding site is the N(7) atom of the base, because the prevailing keto tautomer requires proton at N(1) even in mildly acidic or neutral conditions that efficiently prevents platinum binding to this site [6]. Although deprotonation of N(1)H under basic conditions results in competition between the N(1) and N(7) sites for Pt^{II}, the latter remains as the preferred binding mode [9]. With 9-substituted adenine derivatives, selective *N*(7)-platination occurs only in acidic solution. Above pH 2, Pt-binding to both N(1) and N(7) sites is usually observed. The dichotomy of *N*(1)- *vs.* *N*(7)-platination in purine nucleobases has been discussed elsewhere in this book. The major binding site

in 1-substituted pyrimidines is the N(3) atom (*Fig. 1*). In the case of uracil and thymine derivatives, proton abstraction from N(3)H is required, and hence, Pt-binding to this site usually occurs only at high pH. The strong preference of platinum coordination to the N(7) site in purine bases may be attributed to the negative molecular electrostatic potential [10] and to minor steric hindrances [11] associated with this site.

Complexes of the Type PtN_3X and PtN_4: General Aspects

Bifunctional platinum compounds of the type $[PtA_2X_2]$, where A is an amine and X denotes a labile leaving group, form monoadducts of the type $[PtA_2(L)X]$ (charges omitted) upon displacement of X with incoming nucleobase derivative L. Usually, the substitution reactions follow the solvent-path mechanism, where the rate-limiting step is the replacement of X by the solvent molecule (S) followed by rapid displacement of S by the nucleobase [12][13]. Similarly, the conversion of monoadducts to bisadducts occurs by solvent path, unless the concentration of the incoming nucleobase is very high [14]. For example, with isomeric Pt^{II}-inosine complexes *cis*- and *trans*-$[PtCl(NH_3)_2(Ino\text{-}N(7))]^+$, the direct substitution of Cl^- with inosine becomes

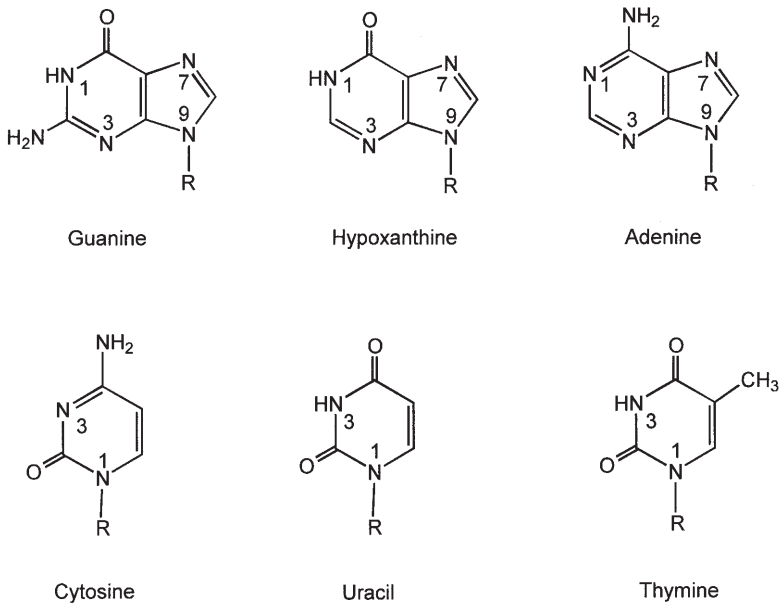


Fig. 1. *Structures of common purine and pyrimidine nucleobases*

comparable to the hydrolysis step when [L] > 0.25M and > 0.1M at 45 °C [14].

Although the aqua ligand in the Pt^{II} coordination sphere is very labile, its lability can be controlled by the pH of the solution, which renders the water molecule to the less reactive OH group. Several kinetic studies have shown that the OH group bound to Pt^{II} is substitution inert relative to the aqua ligand [13][15]. The pK_a values of the aqua ligand in different Pt^{II}-nucleobase complexes are given in *Table 1*. Comparison of the kinetic and equilibrium data reported for isomeric inosine complexes *cis*- and *trans*-$[Pt(NH_3)_2(Ino\text{-}N(7))X]^{n+}$ suggests similar reactivities for the corresponding chloro/aqua derivatives at about pH 8.5 [14][16]. With 6-oxopurine derivatives, however, proton transfer formally from N(1)H to the deprotonated OH group bound to Pt^{II} gives substitution labile aqua ligand [9]. This improves the complexation ability of the aqua species at high pH, since the reactivity of this aqua ligand is comparable to that of the dicationic species.

Coordination of electrophilic platinum to the ring atoms of the nucleobases withdraws electron density from the ring. As a result, the heteroatoms of neutral nucleobases capable of deprotonation become more acidic and those capable to accept a proton become less basic. For example, the N(1)H proton of 9-substituted 6-oxopurines is acidified by 1.2 – 2.0 log units upon platination of the N(7) site, depending predominantly on the charge of the platinum compounds [6][19][20]. With 9-substituted adenines, the pK_a of the N(1) site and that of the exocyclic NH_2 group is lowered about 2 and 4 log units due to *N(7)*-platination, respectively [6]. An even more dramatic change has been observed for *N(1)*,*N(7)*-diplatinated 9-methyladenine, where the pK_a of the NH_2 group is lowered by about 6 log units [6]. On the other hand, an increase in basicity is observed when Pt displaces a proton upon coordination to nucleobases. In the case of inosine, *N*(1)-platination makes the N(7) site about 1.1 log units more basic [21]. An increase of about 1.8 log units has been observed for the pK_a of the N(7) site in 9-ethylguanine when Pt displaces the proton at N(1) [6]. With 1-substituted uracil and

Table 1. *Acidity Constants of the Aqua Ligand in Various Pt^{II}-Nucleobase Complexes*

Compound	pK_a	Ref.
cis-$[Pt(NH_3)_2(1\text{-}MeIno)(H_2O)]^{2+}$	5.79	[20]
cis-$[Pt(NH_3)_2(Ino)(H_2O)]^{2+}$	5.78	[20]
trans-$[Pt(NH_3)_2(1\text{-}MeIno)(H_2O)]^{2+}$	5.27	[9]
trans-$[Pt(NH_3)_2(Ino)(H_2O)]^{2+}$	5.4	[9]
cis-$[Pt(NH_3)_2(1\text{-}MeCyt)(H_2O)]^{2+}$	5.9	[17]
cis-$[Pt(NH_3)_2(3'\text{-}GMP)(H_2O)]^{n+}$	5.26	[18]
cis-$[Pt(NH_3)_2(5'\text{-}GMP)(H_2O)]^{n+}$	5.22	[18]

thymine derivatives, an increase of 4–5 log units in the pK_a of the exocyclic oxygens results from Pt-binding to the N(3) site [6].

Accordingly, the formation of different multiply platinated complexes becomes feasible, as seen in the formation of triplatinated 9-ethylguanine complex bearing Pt^{II} at the N(1), N(3), and N(7) positions, for example [6]. In this case, the triplatinum species is formed simultaneously with the *N(1),N(7)*-diplatinated complex, in line with opposite electronic effects of *N(7)*- and *N(1)*-bound Pt. With adenosine and 2′-deoxyadenosine, simultaneous binding of 4-picoline(2,2′:6′2″-terpyridine)platinum(II) to the N(1) and N(6) sites has been reported [22]. After initial Pt-binding to the N(1) site, a loss of a proton from the C(6)–NH_2 group leads to subsequent rapid platination of the deprotonated N(6). There was no evidence of monoplatinated intermediates [22]. The second platination step may be further facilitated by stacking of the terpyridine moieties. Coordination of Pt^{II} to nucleobases may also affect the hydrogen-bonding properties of the bases. A very recent review focuses on the effects of metal-ion binding on nucleobase pairing through H-bonding [23].

In the conversion of monoadducts into bisadducts, aquated *cis*-Pt^{II} diamines show a clear preference for 6-oxopurine derivatives. With nucleosides and dinucleotides, this has been attributed to favourable hydrogen-bonding interaction between the aqua ligand and the 6-oxo group [24][25]. On the other hand, a H-bonding network involving coordinated am(m)ine and/or aqua ligand and the 6-oxo and/or 5′-phosphate oxygen may affect Pt-binding to 6-oxopurine derivatives with 5′-mononucleotides [26][27][28]. Recently, this type of interaction has been confirmed in solid state [29].

Isomerization Reactions

The isomerization reactions of Pt-nucleobase adducts are expected to be difficult owing to the inertness and thermodynamic stability of the Pt–N bond [30]. For example, a half-life of about 23 years has been estimated for the direct NH_3 exchange in $[Pt(NH_3)_4]^{2+}$ in aqueous NH_3 solution at 25 °C [31]. Unfortunately, data on thermodynamic stability constants for Pt–N complexes is very limited because of their inertness (*vide infra*). Nevertheless, a few studies have reported Pt^{II} isomerization reactions in nucleobase complexes.

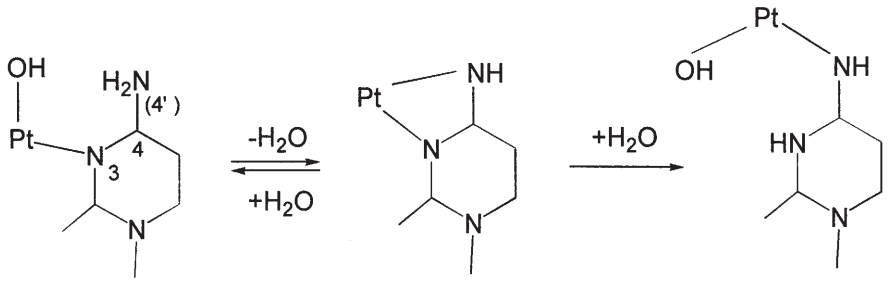
Considering the greater basicity of the N(1) site over the N(7) site in purine bases, the N(7) → N(1) migration of Pt may be anticipated. In fact, this type of isomerization has been observed in Pt^{II}(dien) (dien = diethylenetriamine) complexes of inosine [32] and adenosine [33]. Both isomerization reactions have been proposed to follow similar mechanism, *i.e.*, the change

of the Pt^{II}-binding mode proceeds *via N(1),N(7)*-diplatinated species. With inosine, initial Pt-binding to the N(7) site facilitates the second platination step at N(1) upon displacement of the proton at pH 5–6. An equimolar mixture of inosine and aquated Pt^{II}(dien) produced a complete reaction to give *N(7)*-bound 1:1 complex, followed by appearance of the binuclear *N(1),N(7)*-diplatinated species and reappearance of free nucleoside. Finally, release of Pt at N(7) gives the *N(1)*-platinated species. The overall reaction is slow, even in the analogous system with the corresponding Pd^{II} compound [32]. An alternative method to prepare *N(1)*-platinated 6-oxopurine derivatives utilizes nucleophilic attack on the *N(1),N(7)*-diplatinated nucleobase complex. For example, the CN^- ion is able to remove quite selectively the N(7)-bound Pt^{II}(dien) entity from the *N(1),N(7)*-diplatined 9-ethylguanine, which gives the N(1)-bound complex in a reasonable yield [34]. In the case of adenosine, the reaction with Pt^{II}(dien) at pH 4 initially gives a mixture of *N(1)*- and *N(7)*-bound 1:1 complexes. In excess of the nucleoside, the initial *N(1)/N(7)*-binding ratio of about 2:3 slowly increases to *ca.* 3:1 during 5 days at 85 °C. In an equimolar mixture of Pt^{II} and adenosine, HPLC traces revealed a third (minor) product which was assigned to the *N(1),N(7)*-diplatinated species [33].

Although these findings suggest greater thermodynamic strength for the *N(1)*-platinated complexes over the *N(7)*-bound species, migration of Pt also in the opposite direction seems to be possible. With 9-ethylguanine, both mono- and bifunctional Pt^{II} have been reported to migrate from N(1) to the N(7) site [35]. In the case of Pt^{II}(dien), the process is significantly faster than those mentioned above (complete reaction in 2 h at 80 °C), but it occurs only in acidic solution (pH 2.8). Under neutral and basic conditions no isomerization was observed. In addition, the *N(1),N(7)*-diplatinated complex is perfectly stable also at pH 2.8. Therefore, it was concluded that protonation of the unplatinated N(7)-site is necessary for the migration reaction. Very interestingly, this N(1) → N(7) migration was found to occur intramolecularly, since addition of excess of Cl^- (a good inactivator for Pt^{II}) caused no significant difference in the overall process [35]. It was suggested that Pt^{II} remains hydrogen bonded to the C(6)–O group during migration which could explain the rapid and efficient conversion to the *N(7)*-bound species, since after breaking the Pt–N(1) bond, very fast deprotonation (N(7)) and protonation (N(1)) take place.

In 1-substituted pyrimidine complexes, migration of platinum from endocyclic to exocyclic nitrogen has been observed, *i.e.*, migration of Pt^{IV} from N(3) to the exocyclic C(4)-NH_2 group in 1-MeCyt (1-MeCyt = 1-methylcytosine) [36]. In the initial complex *trans,trans,trans*-$[Pt(OH)_2(NH_3)_2$(1-MeCyt-N(3))$_2]^{2+}$, the *N*(3),*N*(4′)-chelate is formed with the elimination of H_2O from the complex (*Scheme 1*). Addition of H_2O to the chelated com-

Scheme 1



plex results in reformation of Pt–OH and opening of the Pt–N(3) bond. The overall isomerizarion process is quite slow (40–50 h at 70–80 °C, pH 4.3) [36]. A similar N(3) → N(4′) migration seems to occur also in Pt^{II} species, as exemplified by conversion of *trans*-$[Pt(NH_3)_2(1\text{-MeCyt-N(3)})_2]^{2+}$ into *trans*-$[Pt(NH_3)_2(1\text{-MeCyt-N(4)})_2]^{2+}$ [37]. However, in this case the first reaction step involves oxidation of Pt^{II} to Pt^{IV}, followed by migration as above, and finally Pt^{IV} is reduced to Pt^{II}.

The corresponding isomerization appears to be possible also in 9-substituted adenines, *i.e.*, migration of Pt^{II} from the N(1) site to the exocyclic C(6)–NH_2 group. Most probably, the Pt^{II}–N bond rearrangement in the adenine moiety is mechanistically different from that in pyrimide complexes, since the latter occurs in acidic solution *via* Pt^{IV}, while the adenine N(1) → N(6) migration proceeds in strongly basic solution without any detectable redox reaction. It has been found that treatment of the complex $[Pt(dien)(9\text{-MeAde-N(1)})]^{2+}$ (9-MeAde = 9–methyladenine) with base (pH 13, 3 h at 65 °C) yields almost quantitatively the *N*(6)-coordinated complex $[Pt(dien)(9\text{-MeAde-N(6)})]^{+}$ [38]. Isolation of this species at pH 6.2 afforded crystals of the dicationic complex, the X-ray structure of which confirms Pt-binding to the deprotonated exocyclic amino group (*Fig. 2*). Most probably, either N(1) or N(7) acts as the protonation site under these conditions, although unambiguous assignment of the protonation site was not possible from the X-ray data. Preliminary NMR-data suggest that both ring nitrogens N(1) and N(7) may accept the proton necessary for the dicationic species [38].

Two mechanistic explanations may be given for the adenine N(1)→N(6) isomerization, both of which require deprotonation of the C(6)–NH_2 group. First, migration of Pt may be analogous to the *Dimroth* rearrangement, in which an alkyl group migrates from a heterocyclic nitrogen to an α-amino or α-imino group [39]. This type of reaction involves hydrolytic cleavage of the N(1)–C(2) bond, followed by rotation and recyclization. In this process, verified by ^{15}N-labelling, the endocyclic nitrogen N(1) bearing the al-

kyl group becomes the exocyclic N(6) atom [40]. Alternatively, the imino group at C(6) may directly attack Pt^{II} in the starting compound resulting in a pentacoordinate intermediate, where cleavage of the proposedly weaker Pt–N(1) bond gives the *N(6)*-bound species. It is worth noting that in the corresponding adenosine complex, showing two crystallographically different cations of $[Pt(dien)(Ado–N(1))]^{2+}$ (*Fig. 3*), the distance between N(6) and Pt^{II} is 3.25 Å (unit *A*) and 3.16 Å (unit *B*) [33]. At this stage, both explanations are feasible, although simultaneous Pt-binding to the endocyclic

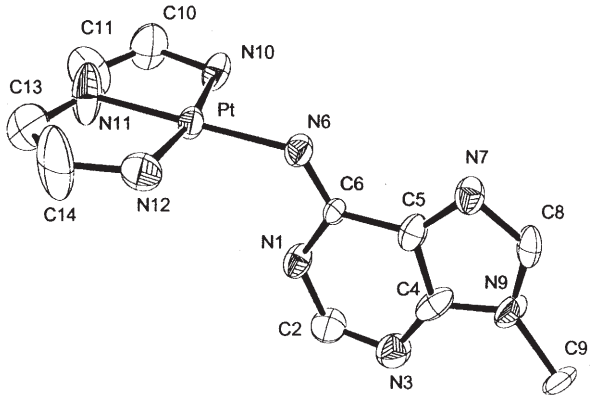


Fig. 2. *Molecular structure of the cation* $[Pt(dien)(9\text{-}MeAde\text{-}N(6))]^{2+}$. The protonation site is ambiguous.

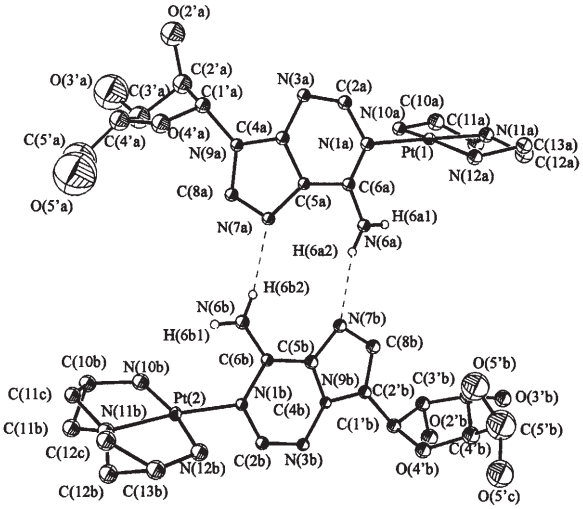


Fig. 3. *The two crystallographically different cations of* $[Pt(dien)(Ado\text{-}N(1))]^{2+}$. The dashed lines represent proposed hydrogen bonds (reproduced with permission from [33]).

and exocyclic N-atoms proposed in the pyrimidine complexes lends support to the latter explanation in the N(1)→N(6) migration of Pt^{II} in the adenine ring.

Thermodynamic Stability

In an early study, almost equal formation constants (log $K = 3.6 \pm 0.1$) were reported for the 1:1 complexes of adenosine, cytidine, and guanosine with aquated *cis*-$(NH_3)_2Pt^{II}$ at pH 6.5 [41]. However, this lack of thermodynamic selectivity was subsequently questioned [42]. More recently, the log K values of 7.5 ± 0.1 and 6.1 ± 0.2 have been given for the N(7)-bound Pt^{II}(dien) complexes of guanosine and and adenosine at 25 °C, respectively [43]. For comparison, a value of log $K > 6.6$ has been estimated for Pt^{II}-binding to guanosine based on data found for the faster-reacting Pd^{II} analog [42]. With deprotonated uridine, aquated *cis*-$(NH_3)_2Pt^{II}$ forms a N(3)-bound 1:1 complex, for which a logarithmic stability constant of *ca*. 9.6 has been estimated [44]. This study also estimates a log K value of 2.9 for the similar complex with cytidine at pH 3, though it was considered as too small. Accordingly, the thermodynamic stability of the most common Pt^{II}-binding modes seems to follow the order Cyt-N(3) ≤ Ado-N(7) < Ado-N(1) < Guo-N(7) < Urd-N(3) by taking the migration reaction discussed above into account.

Hydrolytic Reactions

Coordination of Pt^{II} to the ring nitrogens of the base moiety may influence the hydrolytic stability of nucleobase derivative by blocking of the proton-binding site(s) combined with the electron withdrawing effect of the coordinated Pt^{II}. In particular, the stability the *N*-glycosidic bond in 2′-deoxyribonucleosides seems to be altered. Binding of Pt^{II}(dien) to the N(7) site of dIno and dGuo has been found to enhance spontaneous cleavage of the *N*-glycosidic bond, whereas the acid-catalyzed depurination is retarded by two orders of magnitude [45]. Thus, rate acceleration takes place at pH > 4 and rate retardation at pH < 4. With *N(7)*-platinated dAdo, hydrolysis is retarded only at pH < 2. Under these conditions, the hydrolysis of dAdo occurs *via N(1),N(7)*-diprotonated species in the absence of metal ions. It has been proposed, therefore, that binding of Pt^{II} to the N(7) site in the adenine moiety retards the hydrolysis *via* the substrate dication rather than *via* the monocation [45]. By contrast, platination of the N(1) site in purine nucleosides does not significantly alter their acid-catalyzed depurination. The

N(1),N(7)-diplatinated species exhibit a significant pH-independent depurination. With dIno and dAdo, these reactions become faster than the acid-catalyzed hydrolysis of the uncomplexed nucleoside at pH > 5 and at pH > 3, respectively [45]. Addition of aquated Pd^{II}(dien) to the solutions of *N(1),N(7)*-diplatinated dIno and dAdo markedly accelerates depurination reactions. It has been proposed that this rate enhancement is due to the binding of Pd^{II}(dien) to the N(3) site of the purine moiety, which destabilizes the *N*-glycosidic bond [45].

Substitution Reactions

In the presence of competing ligands, such as the water molecule or another nucleobase, that exhibit only weak or moderate nucleophilic power, the substitution reactions of Pt-nucleobase complexes are extremely slow. For example, reactions of a series of model Pt-nucleobase complexes toward a 40-fold excess of 5′-GMP (GMP = guanosine monophosphate) or inosine showed no reaction within 100 h (37 °C, pH 4.7–7) [46]. However, the displacement of nucleobases from Pt can be facilitated by the attack of strong nucleophiles, *e.g.*, CN^-, I^- and sulfur ligands. Reactions with sulfur-containing (bio)molecules are of particular interest owing to their important roles in biological processing of anticarcinogenic Pt-drugs [4]. In addition, they are used as trapping agents in studying platinum binding to nucleic-acid fragments [1]. It is noteworthy that certain, yet unknown, Pt-DNA adducts may be highly resistant even toward CN^-, since not all Pt bound to DNA can be removed with CN^- treatment [6].

In general, substitution reactions of Pt^{II} overwhelmingly follow an associative mechanism [12][30]. Although steric retardations may slow down the substitution rate by several orders of magnitude, they seem not to cause changeover from an associative to a dissociative mechanism [15]. In excess of the nucleophile, stepwise dissociation may be anticipated for Pt-bis(nucleobase) complexes exhibiting *cis*-geometry. Because of the highly inert nature of the NH_3 ligand, the dissociation of the nucleobases may be regarded as the rate-limiting step in the overall reaction. Substitution of the nucleobase with the nucleophile Y renders the NH_3 group in *trans*-position more labile (*trans*-effect Y > N) resulting in fast substitution of NH_3 by Y. Accordingly, the overall reaction may be depicted by *Scheme 2*, where charges are omitted for clarity. Rate constants for the substitution reactions of various Pt^{II}-nucleobase complexes are listed in *Table 2*.

Early qualitative studies already indicated that the Pt–N(3) bond in 1-subsituted thymine and uracil complexes is particularly inert toward the attack of CN^-, which has been attributed to the remarkable protective effect

Scheme 2

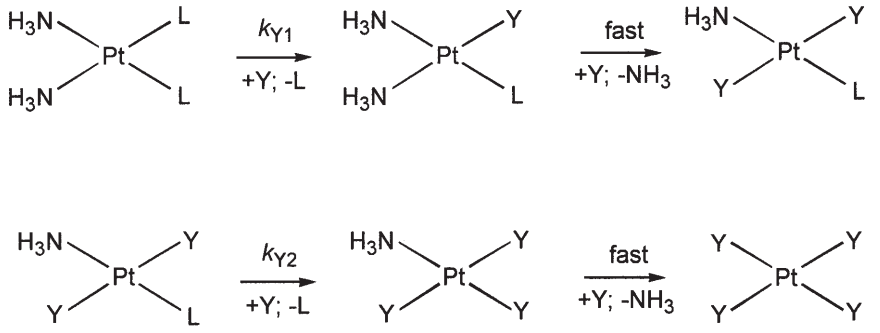


Table 2. *Rate Constants* (k_i/(10^{-5} M^{-1} s^{-1}) *for Substitution Reactions of Various* Pt^{II}*-Nucleobase Complexes with Different Nucleophiles* (*Y*) *in Aqueous Solution*[a])

Complex	Y[b])	k_{Y1}	k_{Y2}	*T*/K	Ref.
cis-$[Pt(NH_3)_2(G\text{-}N(7))_2]^{2+}$	tu	5.9 ± 0.6		316	[47]
	tu	7.96 ± 0.05	3.70 ± 0.05	318.2	[48]
	CN^-	25 ± 4		303	[49]
cis-$[Pt(NH_3)_2(G\text{-}N(7))(A\text{-}N(7))]^{2+}$	tu	4.7 ± 0.1[c])	10 ± 1[c])	318.2	[48]
		10 ± 0.1[d])	3.3 ± 0.1[d])		
cis-$[Pt(NH_3)_2(G\text{-}N(7))(A\text{-}N(1))]^{2+}$	tu	2.7 ± 0.2[c])	7.8 ± 0.1[c])	318.2	[48]
		3.5 ± 0.2[d])	4.1 ± 0.1[d])		
$[Pt(dien)(G\text{-}N(7))]^{2+}$	tu	82.5 ± 0.7[e])		318.2	[43]
	tu	175 ± 1[f])	49.9 ± 0.7[g])	318.2	
	I^-	48.0 ± 0.3		318.2	
	H_2O	0.021 ± 0.007[h])		318.2	
$[Pt(dien)(A\text{-}N(7))]^{2+}$	tu	24.5 ± 0.2[e])		318.2	[43]
	tu	111 ± 2[f])	88 ± 2[g])	318.2	
	I^-	31.8 ± 0.4		318.2	
	H_2O	0.023 ± 0.002[h])		318.2	
cis-$[Pt(NH_3)_2(1\text{-}MeU)(H_2O)]^+$	tu	37000 ± 2000[i])		298.2	[15]
	I^-	6010 ± 40[i])			
cis-$[Pt(NH_3)_2(1\text{-}MeU)_2]$	I^-	no reaction[j])		333.2	[15]
	H_2O	0.078 ± 0.001[k])		298.2	
cis-$[Pt(NH_3)_2(5'\text{-}GMP\text{-}N(7))_2]$	tu	1.6[l])		310.2	[50]
trans-$[Pt(NH_3)_2(5'\text{-}GMP\text{-}N(7))_2]$	tu	7.7[l])		310.2	[50]

[a]) For the notation of the rate constants, see *Scheme 2*.[b]) tu = thiourea. [c]) For the dissociation of adenosine. [d]) For the dissociation of guanosine. [e]) pH ≈ 6.5. [f]) For the disappearance of the starting material at pH ≈ 3. [g]) For the disappearance of $[Pt(dienH)(L\text{-}N(7))(tu)]^{3+}$ at pH ≈ 3. [h]) First-order rate constant for the solvolysis reaction at pH ≈ 6.5. [i]) For the substitution of H_2O, pH 3.0. [j]) pH range 4–7. [k]) First-order rate constant for the solvolysis reaction at pH 3.0. [l]) Approximate values.

of the exocyclic oxygens [51] [52]. More quantitatively, the bis(1-methyluracilato) complex of *cis*-$(NH_3)_2Pt^{II}$ is inert to substitution by thiourea and I^-, unless the exocyclic O(4)-atom is protonated [15]. The reactivity of the 1-methyluracilato 1:1 complexes bearing Cl^- or H_2O as the fourth ligand is controlled by the lability of the aqua ligand, which drastically decreases with increasing pH. With dicationic Pt^{II} complexes, the reactivity of different nucleophiles (as shown by the k_Y values in *Table 2*) follows the order CN^- > thiourea ≈ I^-, different from the standard n°_{Pt} values (nucleophilic reactivity constant) [12] [30].

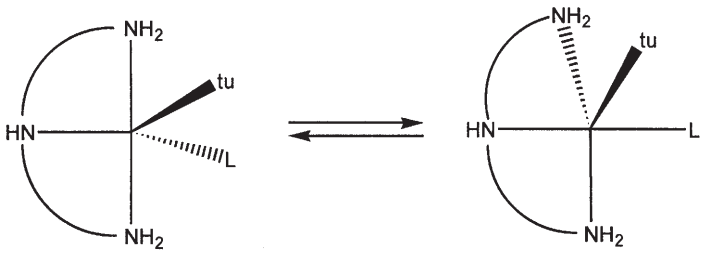
In spite of the expected inertness of the Pt–NH_3 bond, dissociation of the ammine ligand has been found in *cis*-$[PtCl(NH_3)_2(1\text{-MeCyt})]^+$ in the presence of excess of Cl^- [53]. The substitution of NH_3 by Cl^- proceeds in surprisingly mild conditions (three weeks at room temperature in aqueous soln.), which treatment yields 24–31% of *trans*-$[PtCl_2(NH_3)(1\text{-MeCyt})] \cdot ½\,H_2O$. Although substitution of NH_3 by Cl^- is in line with the *trans*-effects of these groups utilized, *e.g.*, in the preparation of *trans*-$[PtCl_2(NH_3)_2]$ [54], the release of NH_3 may also be due to the *trans*-labilizing effect of the pyrimidine followed by isomerization and uptake of Cl^- [53]. However, the reaction of *cis*-$[PtCl(NH_3)_2(1\text{-MeCyt})]Cl$ with 1-methylcytosine gave only minor quantities of the *trans*-species, while the major product was bis(1-methylcytosine) complex [53]. Although this product distribution may result from kinetic factors, it clearly indicates that the *trans*-effect of the N(3)-bound pyrimidine is not very strong. Nevertheless, this type of NH_3 substitution offers new synthetic pathways, since in the presence of excess of various nucleobases this dichloro species can be converted into tris(nucleobase) complexes [55] [56].

Substitution reactions of $[Pt(dien)(L\text{–}N(7))]^{2+}$ (L = Ado or Guo) by thiourea (tu) have been shown to proceed mechanistically in a different manner in neutral compared to slightly acidic aqueous solution [43]. Under neutral conditions dissociation of both complexes gives, expectedly, only free nucleoside and $[Pt(dien)(tu)]^{2+}$. By contrast, in acidic solution (pH 3) the end products are free nucleoside and $[Pt(tu)_4]^{2+}$. Kinetic analyses have shown that, in the latter case, the end products are formed *via* two parallel routes, *i.e.*, directly from the starting material as under neutral conditions and *via* a tetracoordinate dien ring-opened species $[Pt(dienH)(L\text{–}N(7))(tu)]^{3+}$. NMR Data for isolated $[Pt(dienH)(L\text{–}N(7))(tu)]^{3+}$ are consistent with tetracoordinate Pt^{II} compounds, in which the dien ligand acts a bidentate group and one of the dien amino groups has been trapped by a proton [57]. These ring-opened species appear to be stable in cold acidic solution. However, when the pH is raised they decompose to the starting material and free ligand in a ratio of about 10:1, and the rate constant for this decomposition reaction linearly increases with increasing pH; already at pH

5.5 the reaction is relatively fast ($t_{1/2}$ < 3 min at 65 °C) [43]. Interestingly, a closely related ring-opened species [Pt(dienH)(L-Met–S,N)]$^{2+}$ (L-MetH = L-methionine) has a very long lifetime (days) in 0.55M $NH_4H_2PO_4$ at pH 4.0 [58].

It has been suggested that the substitution reactions of [Pt(dien)(L–N(7))]$^{2+}$ in acidic solution follow associative mechanism *via* pseudorotation of the pentacoordinate intermediates IM1 and IM2 depicted in *Scheme 3*. The intermediate IM1 leads to replacement of the nucleoside by thiourea, while IM2 yields the ring-opened species upon dissociation of dien–NH_2. The reversal of the ring-opening step is very important for two reasons. First, simultaneous formation of starting material and free nucleoside gives strong support the pseudorotation mechanism. And second, the facile displacement of sulfur-bound thiourea from Pt^{II} by a nitrogen donor demonstrates the nucleophilic power of a group which is spatially in a favorable position. The replacement of a sulfur ligand by a nitrogen donor has been found also in other cases. For example, 5′-GMP is able to remove S-bound L-methionine from the complex [Pt(dien)(Met–S)]$^{2+}$ [4] [59]. In addition, a slow intramolecular S → N replacement has been reported in *S*-guanosyl-L-homocysteine (sgh), where the initially formed ($t_{1/2} \approx 2$ h) [Pt(dien)(sgh–S)]$^{2+}$ slowly isomerizes ($t_{1/2} \approx 10$ h) to [Pt(dien)(sgh–N(7))]$^{2+}$ [60].

Scheme 3



IM1 IM2

Concluding Remarks

Binding of bifunctional Pt^{II} compounds to DNA is a two-step process. The initial step involves the formation of Pt-nucleobase monoadducts, the lifetime of which largely depends on the remaining leaving group X. Usually, hydrolysis of X is considered as the rate-limiting step in the conversion of monoadducts to bisadducts. However, this may be an oversimplification, since the lability of the coordinated water molecule drastically de-

creases with increasing pH upon its conversion to the OH group. Coordination of electrophilic platinum to nucleobases modifies the electron density of the heterocyclic ring atoms. This may result in changes in acid-base properties, hydrogen-bonding abilities and hydrolytic stability of the coordinated nucleobase derivative, and facilitates migration of Pt from one binding site to another within a base moiety. Pt-nucleobase bis(complexes) and analogous model compounds with a PtN_4 coordination sphere are quite inert to substitution reactions. Strong nucleophiles (CN^- and sulfur-containing molecules) can displace N-donors from Pt unless steric obstacles make the nucleophilic attack difficult. However, a nitrogen atom can also act as a powerful nucleophile toward Pt if spatially in a correct position, which makes S→N substitution reactions feasible. In addition, direct substitution of a coordinated nucleobase by a neighboring base residue may explain the isomerization reactions of certain platinum bisadducts in double-stranded oligonucleotides [61].

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Kinetics and Selectivity of DNA-Platination

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This contribution deals with the following aspects of DNA-platination: *i*) The nature of the actual platinating species with a discussion of the local environments of the various coordination steps, *ii*) the nature of the adducts formed, monoadducts, intra- and interstrand adducts, with a discussion of the influence of the geometry of the complex and of the role of the DNA-duplex structure, and *iii*) the kinetic data obtained on isolated DNA and their comparison with the results of detailed studies on model oligonucleotides. The DNA-model works have given a clear picture of the binding steps of the complexes to the nucleic acids. However, a better insight is needed about the very first interaction between the complex and DNA in the nucleus. Much has also to be uncovered about DNA as a 'reactive local environment'. With the present knowledge, it is yet possible to design new platinum drugs which should selectively bind to DNA, to give adducts able to induce the cascade of events leading to apoptosis.

DNA having been established as the major target of cisplatin and its analogues, at the origin of their antitumor activity, this chapter will address the following questions:

– What are the actual platinating species *in vitro* and *in vivo*?
– As a target, how does isolated DNA compare with DNA in the nucleus?
– How do the target sequences and the adducts identified depend on the nature of the complexes?
– What do the kinetic studies of DNA-platination indicate about the various steps of adduct formation?
– What do the kinetic studies of oligonucleotide platination indicate about the factors which control each step of the formation of the final diadducts?

The discussion will focus on:

– the understanding of DNA-platination and the remaining questions,

Cisplatin. Edited by Bernhard Lippert

– the possible use of the present knowledge to design new complexes for selective DNA-platination, to use long-lived monoadducts in the cell to cross-link proteins.

Many reviews covering several aspects of these topics have appeared in the recent years [1–7]. The purpose of this contribution is to focus on the major common features of the DNA-binding reactions of a now wide variety of complexes. The aim is to identify the specific parameters of each step of the overall platination reactions in order to design sequence-selective drugs.

The Actual Platinating Species

It was soon realized that chloride aquation, as well as acid-base equilibria of the aqua ligands, had to be taken into account to determine the actual platinating species [8] (see the contribution of *R. B. Martin* in this *Part*).

The aquation-anation and acid-base equilibria of cisplatin, as well as its dimerization reaction, are presented in *Scheme 1*. It is also noteworthy that proton exchange occurs on the ammine ligands [9].

Similar reactions occur for monochloroplatinum complexes, for the *cis*-dichloro analogues of cisplatin, as well as for transplatin. *Table 1* gives the aquation-anation rate constants and equilibrium constants for cis- and transplatin. *Table 2* compares the same data, together with the corresponding activation parameters for $PtCl_2$(en) and *rac*-$PtCl_2(R_1$-en) in which R_1-en is (1*R*,2*R*,4*S*)-*exo*-2-amino-2-(aminomethyl)-7-oxabicyclo[2.2.1]heptane. The results suggest that the presence of substituents on the amine ligands does not significantly alter the aquation rates of the chloride ligands which occurs *via* an associative activation mode [14].

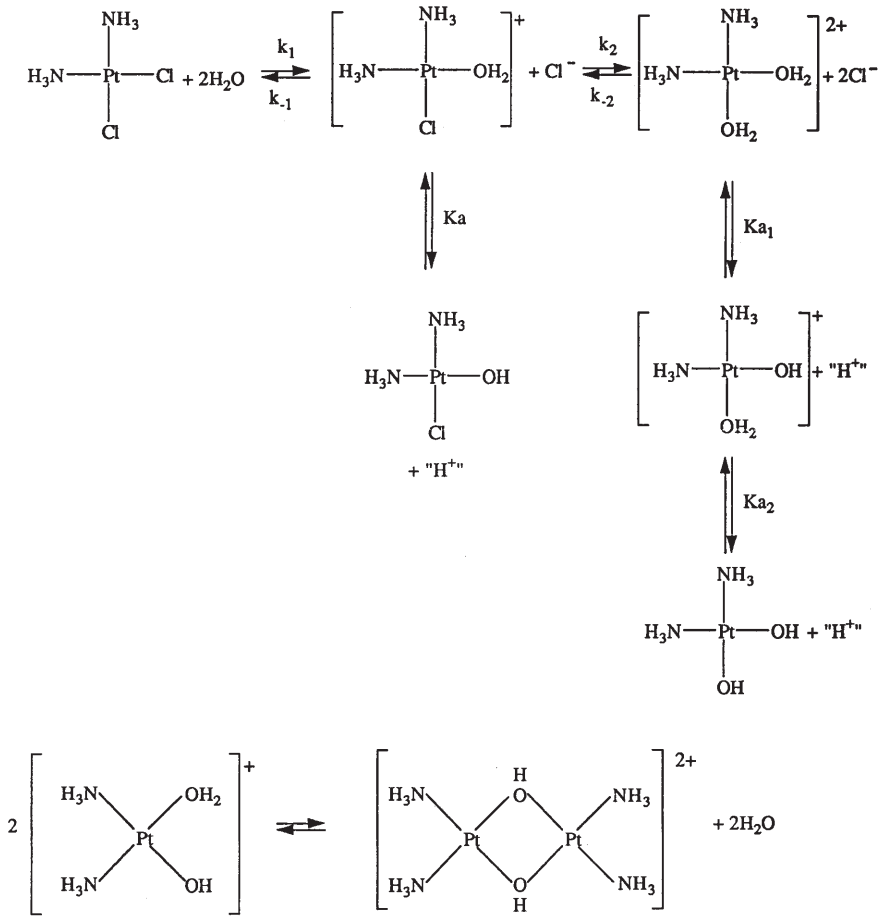
The pK_a's of several platinum aqua complexes are given in *Table 3*. From the data of *Tables 1–3*, all *cis*-dichloro compounds appear to have a

Table 1. *Aquation-Anation Rate Constants and Equilibrium Constants for Cis-and Transplatin* (nomenclature as in *Fig.*) [a])

	Cisplatin [b])	Transplatin [c])
k_1 [s^{-1}]	$(1.9 \pm 0.2) \times 10^{-4}$	$(1.05 \pm 0.03) \times 10^{-3}$
k_{-1} [M^{-1} s^{-1}]	$(6.0 \pm 1.5) \times 10^{-2}$	2.2 ± 0.4
K_1	3.2×10^{-3}	4.8 ± 10^{-4}
k_2 [s^{-1}]	$(2.3 \pm 0.3) \times 10^{-4}$	$(4 \pm 2) \times 10^{-6}$
k_{-2} [M^{-1} s^{-1}]	$(9.8 \pm 1.4) \times 10^{-1}$	$(2 \pm 0.2) \times 10^{-1}$
K_2	2.3×10^{-4}	2×10^{-5}

[a]) 318.2 K , $NaClO_4$ 0.1M, pH 2.8–3.4. [b]) From [10]. [c]) From [11].

Scheme 1. *Aquation-Anation, Acid-Base Equilibria and Dimerization Reaction of Cisplatin in Aqueous Solution*



similar behavior in aqueous solution. However, the mono- and diaqua species (*Scheme 1*) are more thermodynamically favored for cisplatin than for the *trans*-isomer. Even if the first hydrolysis constant k_1 is higher for transplatin than for the *cis*-isomer (*Table 1*), due to the higher *trans*-effect of Cl^- compared to NH_3 [22], the anation-rate constant k_{-1} is also much higher. Moreover, the second hydrolysis is about two orders of magnitude slower for the monoaqua derivative of transplatin compared to that of the *cis*-isomer. If one adds the lower pK_a and pK_{a1} values for the aquated forms of transplatin (*Table 3*), it appears that this isomer should give much lower concentrations of the $[PtCl(NH_3)_2(H_2O)]^+$, $[Pt(OH)(NH_3)_2(H_2O)]^+$ and $[Pt(NH_3)_2(H_2O)_2]^{2+}$ cationic species having a labile aqua ligand.

Table 2. *Aquation-Anation Kinetic and Thermodynamic Parameters for [PtCl$_2$(en)]* [a] [b] *and [PtCl$_2$(R_1-en)]* [c] *at 298 K*

	Complex	Step			
		(1)	(–1)	(2)	(–2)
		[10^{-5} s^{-1}]	[10^{-2} M^{-1} s^{-1}]	[10^{-5} s^{-1}]	[10^{-1} M^{-1} s^{-1}]
k	en	3.4 ± 0.4 [e]	1.54 ± 0.03	4.4 ± 0.6	3.1 ± 0.4
	R_1-en	3.2 ± 0.2	4.4 ± 0.2	7.8 ± 1.1	6.7 ± 0.1
		K_1 [10^{-4} M]		K_2 [10^{-4} M]	
K	en	22 ± 1 [e]		1.4 ± 0.1	
	R_1-en	7.2 ± 0.3		1.2 ± 0.1	
		[kJ · mol^{-1}]	[kJ · mol^{-1}]	[kJ · mol^{-1}]	[kJ · mol^{-1}]
$\Delta H^{\ddagger}$	en	85 ± 10 [e]	73 ± 3	34 ± 12	37 ± 12
	R_1-en	86.0 ± 2.8	75.0 ± 2.6	91.1 ± 0.9	74.3 ± 1.4
		[J · K^{-1} · mol^{-1}]	[J · K^{-1} · mol^{-1}]	[J · K^{-1} · mol^{-1}]	[J · K^{-1} · mol^{-1}]
$\Delta S^{\ddagger}$	en	–42 ± 33 [e]	–33 ± 8	–210 ± 42	–130 ± 42
	R_1-en	–42.8 ± 9	–19.5 ± 9	–18 ± 31	+0.9 ± 5
		[cm^3 · mol^{-1}]	[cm^3 · mol^{-1}]	[cm^3 · mol^{-1}]	[cm^3 · mol^{-1}]
$\Delta V^{\ddagger}$ [f]	en	–9.2 ± 1.0			
	R_1-en	–9.4 ± 0.7	–4.0 ± 0.4	–6.6 ± 1.7	–4.4 ± 0.5

[a], [b]) Work from [12] [13]. [c]) Work from [14], R_1-en = *rac*-(1*R*, 2*R*, 4*S*)-*exo*-2-amino-2-(aminomethyl)-7-oxabicyclo[2.2.1]heptane. [d]) In the pH range 2.2–5.8, k_1 and k_{-1} are independent of pH; various temperatures and ionic strengths were studied. [e]) See also [15]. [f]) Activation volume [14].

Table 3. *p*K_a *Values for Various Acid-Base Equilibria of Aquaplatinum Complexes* (nomenclature according to *Scheme 1*, Y = Cl or H_2O)

	pK_a (Y = Cl)	pK_{a1} (Y = H_2O)	pK_{a2} (Y = H_2O)	T [K], Reference
$[Pt(dien)(H_2O)]^{2+}$	5.87			298 [16]
	6.13			308 [16]
cis-$[Pt(NH_3)_2(H_2O)Y]$	6.85 ± 0.1	5.93 ± 0.1	7.9 ± 0.1	278 [17]
	6.41 ± 0.02	5.37 ± 0.09	7.21 ± 0.09	300 [18]
		5.24 ± 0.05	7.42 ± 0.10	295 [19]
$[Pt(en)(H_2O)Y]$	7.4	5.8	7.6	293 [8]
$[Pt(dach)(H_2O)_2]^{2+}$		6.14	7.56	298 [20]
trans-$[Pt(NH_3)_2(H_2O)Y]$	5.63	4.35	7.40	298 [21]

Taking into account the chloride-ion concentrations in human plasma (103×10^{-3} M) and in the cell (4×10^{-3} M, an actually calculated value) and assuming a pH of 7.4 at 37 °C, the percentages of $PtCl_2(en)$ and its aquation derivatives were calculated at equilibrium. From 94.7% of dichloro complex in the plasma (90% of which get actually bound to plasma proteins [23]), the cell proportions become 25.3% $PtCl_2(en)$, 17.5% $[PtCl(H_2O)(en)]^+$, 17.5% PtCl(OH)(en), 0.6% $[Pt(H_2O)_2(en)]^{2+}$, 24.1% $[Pt(OH)(H_2O)(en)]^+$, 15% $Pt(OH)_2(en)$. From these data, the monochloromonoaqua and monohydroxomonoaqua complexes appear as the most abundant cationic species being electrophilic, with a good aqua leaving group. If these proportions are expected to be similar for the various *cis*-compounds, we can expect much lower concentrations of the corresponding species derived from *trans*-platinum complexes (*vide supra*).

Actually, a number of studies have reported intracellular chloride-ion concentrations, between 10 and 76×10^{-3} M for various cell types including ovarian carcinoma cells (23×10^{-3} M), much larger than 4×10^{-3} M [31].

For the *cis*-complexes, a dimerization reaction to μ-hydroxo species can occur (*Scheme 1*). Such a reaction becomes significant at high platinum concentrations [20], when the pH is in the 5–7 range [24] or when the aqua species remain in solution for long periods [17]. It can be also observed with triamineaqua complexes [16].

DNA as a Target *in Vitro vs.* in the Nucleus

It was early determined that in the cell, the electrophilic species derived from cisplatin bind to three major targets: RNA (which is present at high concentration in cytoplasm, together with nucleoproteins) (50%), DNA (40%) and proteins (10%) [25]. It also binds to cysteine and methionine residues of proteins, to metallothioneins, glutathione, methionine, and glutamate which are good traps for chloro and/or aqua platinum electrophiles [26].

Transplatin reacts 360 times faster than cisplatin with glutathione [27], a reaction likely to remove platinum from the cell, through ATP-dependent efflux [28]. It has been shown that a thioether ligand on a platinum triamine complex can be slowly replaced by a guanine-N(7), suggesting a possible platinum transporter role for such thioether compounds towards DNA [6]. (This topic is discussed in the contribution by *J. Reedijk* and *J. M. Teuben*.)

In the nucleus, DNA is packed in chromatin. In this compact structure, most DNA-sequences are structurally inaccessible and functionally inactive. The nucleosomes are the fundamental subunits of chromatin, they consist of a core of histones with two turns of DNA coiled around it. Despite

this packing, no difference was found between the binding pattern of cis- or transplatin to nucleosomes compared to that of isolated DNA [29].

Chloride ions slow down DNA-platination *in vitro*, as expected from *Scheme 1*. This is shown by the use of *Tris*-HCl buffer, which requires a 10-fold higher concentration of cisplatin to get a similar damage as that observed in *Hepes* buffer [30]. However, tumor cells having less than 8% of the basal chloride level, after incubation in a nitrate medium, incorporated the same amount of cisplatin as in normal medium, but revealed no change in cytotoxicity and no difference in platination of cellular DNA [31]. Such a result suggests that either the chloride ion depletion did not affect the nucleus or that the actual DNA-platinating species might be formed in very close proximity to DNA (*vide infra*). Very few data are available about the local pH and chloride ion concentration in the parts of the nucleus where DNA could be accessible [32]. In control cytosolic solution, isolated nuclei exhibit an intranuclear electrical potential of –6.5 ± 0.5 mV. It is assigned to an excess of intranuclear negative charges associated with DNA-phosphate residues not neutralized by positively charged histones [33]. Such a potential should favor the reaction with platinating cationic species. The water-accessible surface of the DNA double helix is 45% charged, 17% polar and 39% nonpolar [32]. The polyelectrolyte behavior of nucleic acids leads to a large accumulation of cations and to an exclusion of anions, at their highly charged surface. For double helix DNA, at a bulk salt concentration of 10^{-3} M, the local concentration of a univalent cation was calculated to exceed 1M, whereas that of a univalent anion is less than 10^{-6} M [34]. Such local concentration gradients, formed spontaneously, are as large as many transmembrane solute concentration gradients established at a significant free-energy cost [35][36].

Adducts and Target Sequences as a Function of the Complexes

The aquated forms of the platination complexes behave as electrophilic reagents and as such they first bind to guanine-N(7) as do the nitrogen mustard-drugs [37]. Guanine has long been known as the most nucleophilic base with the decreasing order of reactivity: guanine-N(7) (G-N(7)) ≫ adenine-N(7) (A-N(7)) > cytosine-N(3) (C-N(3)) [38] [39]. This has been related to the calculated electrostatic potentials of the bases [40]. Only guanine- and adenine-N(7) provide directly available binding sites in the major groove of B-DNA [41]. Whatever the platinum complex is, it first binds to a guanine on DNA and when the complex contains a second exchangeable ligand it gives all the possible crosslinks, intra- or interstrand, with the available binding sites (G-N(7), A-N(7), A-N(1), C-N(3)) according to *i*)

the *cis*- or *trans*-geometry of the complex, *ii*) the single- or double-stranded structure of the nucleic acid, and *iii*) the locally perturbed DNA structure, due either to the initial monoadduct or to a first formed unstable intrastrand diadduct which rearranges into an interstrand crosslink. (This type of rearrangement is presented by *J.-M. Malinge* and *M. Leng* in *Part 3* of this book).

The DNA-adducts are presented in brief as they have been discussed in many reviews, particularly for cis- and transplatin [1–7].

The *monoadducts* result from the substitution of one chloride ligand by a guanine-N(7). They have been detected and trapped by several nucleophiles including guanosine [42], ammonia from ammonium bicarbonate [46] (see also the comparison with thiourea), thiourea [43–45], cyanide [48], and glutathione [47].

The chloromonoadducts are unstable in aqueous solution and lead to intra and/or interstrand diadducts after aquation of the chloride ligand (*vide infra*). They can also cross-link DNA to proteins either directly or after aquation [25]. The half-life of the monoadducts formed from cisplatin and $PtCl(R_2\text{-en})$ (with R_2-en = *rac*-(1*S*,2*S*,4*S*)-*exo*-2-amino-2-(aminomethyl)-7-oxabicyclo[2.2.1]heptane) on DNA at 310K are 2 h 40 ± 30 min and 8 h 20 ± 20 min [49], respectively, a rather long time on the scale of cellular 'processing'.

The 'monofunctional' complexes $[PtCl(dien)]^+$, $[PtCl(NH_3)_3]^+$, and the active *cis*-compounds *cis*-$[PtCl(NH_3)_2\ (Am)]^+$, where Am is an heterocyclic or aromatic amine ligand like pyridine, pyrimidine, purine, or aniline, only form stable monoadducts with DNA [50][51]. However, when Am = *N*-methyl-2,7-diazapyrenium (a strong intercalator), the monoadduct is stable only on single-stranded DNA. On double-stranded DNA it is hydrolyzed with release of *cis*-$[Pt(NH_3)_2(Am)(H_2O)]^{3+}$ or of Am generating the aqua monoadduct of cisplatin [52].

The *diadducts* differ for cis- and transplatin and their analogues.

Cisplatin and Analogues. For Salmon sperm DNA, typically treated with cisplatin (for $PtCl_2$(en) at $r_b = 0.002$ (r_b = number of platinum atoms bound per nucleotide)) for 16 hours, 65% of the platinum bound to DNA appears as the *cis*-$[Pt(NH_3)_2$ d(GpG)] or [Pt(en)d(GpG)] chelate (GG-*cisPt*), 20 to 25% as *cis*-$[Pt(NH_3)_2$d(ApG)] or [Pt(en)d(ApG)] chelate (AG-*cisPt*). The 65% proportion of GG-adduct exceeds the 37% probability of having a guanine adjacent to another guanine, whereas the amount of AG adduct agrees with the 23% A neighboring probability [43]. Diadducts G-Pt-G account for 4 to 8% of the platinum bound and could not be specifically assigned to intrastrand chelates between non-adjacent guanines or to inter-

strand cross-links (because the analytical method based on enzymatic digestion gives the same bis-dGMP final complex) [44] [46]. It has been demonstrated, by using immunochemical methods, that similar patterns of adducts to those found on isolated DNA were obtained with DNA from platinated cells in culture and from blood cells and tumor tissue of cancer patients receiving chemotherapy: 55–76% GG-*cisPt*, 10–21% AG-*cisPt*, 5–24% G-Pt-G, 2–17% monoadducts G-Pt [53–55].

Five remarks can be made about this set of results. *i*) No GA adduct has ever been mentioned in the quantitative studies reported. *ii*) It is likely that the interstrand crosslinks have been underestimated [46][56][57] (see *J.-M. Malinge* and *M. Leng*, *Part 3*). They are preferentially formed between two guanines at d(GC)-d(GC) sites [58]. *iii*) It has been shown that the formation of interstrand crosslinks is favored in supercoiled DNA [59][60]. *iv*) There has been no systematic evaluation of the intermolecular adducts formed between platinated DNA and cellular nucleophiles. In the case of chinese hamster ovary cells, treated with platinum compounds, they were estimated to 0.15% of the total amount of adducts [61] [62]. *v*) It is known that intrastrand adducts, in a particular environment, can rearrange into other intra- or interstrand crosslinks [63], and the reverse is also known for interstrand adducts giving intrastrand chelates *via* monoadduct intermediates [64][65].

Enzymatic methods, using restriction enzymes, exonuclease III, T_4 DNA- or *Taq* DNA-polymerases, have revealed a marked preference for the binding of cisplatin and its analogues to $(dG)_n$ sequences with $n \geq 2$ [66–68]. They also detected minor adducts, on GA, GC, and TCAT sequences. For the sake of comparison, it must be recalled that nitrogen mustards also exhibit a preference for $(dG)_n$ sequences with $n = 2$–5 [69]. It has also been found, using T_4 DNA polymerase mapping assay on a 184 bp DNA fragment, that different d(GpG) sequences might exhibit up to a five-fold difference in reactivity *vs.* cisplatin [71]. However, this result could not only reflect different levels of platination but also other factors interfering with replication blockage [70]. In the case of $PtCl_2$(dach), the diamine ligand appeared to favor platination of the $(dG)_3$ sites [45].

With the very efficient tool based on *Taq* DNA-polymerase and a linear expression system, it became possible to compare the DNA-platination sites and intensity of binding of 13 analogues of cisplatin including carboplatin and the Pt^{IV}-complex tetraplatin. It is not the purpose of this contribution to discuss cisplatin analogues. But it is relevant to DNA-platination that, for the compounds studied in human cells and with purified DNA, the sequence specific positions and relative intensities of damage were similar [72]; for the complexes used on plasmid pUC19, the sequence specificity was similar in position and relative intensity of damage, the only difference lying in the efficiency to obtain similar platination damage in a 5 to 100 mM

concentration range [30]. These data clearly point to a similar platination mechanism for cisplatin and all the *cis*-dichloro complexes.

Transplatin. Because of its *trans*-geometry, transplatin gives different adducts on single-stranded and double-stranded DNA (*in vitro*). The following percentages were determined after removal of the monofunctional adducts by glutathione, followed by enzymatic digestion; respectively for single- and double-stranded DNA (r_b = 0.01): *trans*-Pt(dG)(dC) 5 and 50%, *trans*-Pt(dG)$_2$ 60 and 40%, *trans*-Pt(dG)(dA) 35 and 10% (with A-N(1) or A-N(7) binding) [47][73]. It was claimed that on both single- and double-stranded DNA 1,3*G*N*G*, and *A*N*G* adducts were formed, and also that no more than 2% of all bifunctional adducts on the duplex form represented interstrand crosslinks. More recent work on duplex DNA has modified this picture. *i*) The *trans*-Pt(dG)(dC) adducts essentially correspond to interstrand crosslinks [74]. *ii*) Platination of a restriction fragment with transplatin showed that after 24 h, 80% of the adducts were monofunctional and the diadducts were essentially interstrand cross-links [75].

Transplatin gives 6 times more DNA-protein cross-links than cisplatin [76], despite similar rates of closure of mono- to bifunctional adducts for both the *cis*- and *trans*-isomers [80].

Kinetics of DNA-Platination

The rate of formation of cisplatin-DNA adducts was found to be independent of superhelicity [59] and appears to be unaffected by the presence of histones in nucleosomes [29] and in chromatin [77]. Therefore, isolated DNA in aqueous solution appears to be a relevant model for kinetic and mechanistic studies of cellular DNA-platination. It was early checked that the cisplatin-DNA adducts were stable for a least three days at 37 °C after their formation [78]. There are now a few cases reported of unstable platinum adducts (*vide supra*): *i*) monoadducts with the diazapyrenium ligand [52], *ii*) a cisplatin intrastrand GG chelate rearranging into a GG interstrand crosslink [63], *iii*) cisplatin GG interstrand diadducts, slowly rearranging into intrastrand ones [65], *iv*) transplatin intrastrand *G*N*G* diadducts rearranging into interstrand crosslinks (*J.-M. Malinge* and *M. Leng*, *Part 3*).

Apart from the related case *i*), there is no evidence of reversible binding of the $[PtCl(NH_3)_2(H_2O)]^+$, $[Pt(OH)(NH_3)_2(H_2O)]^+$, or $[Pt(NH_3)_2(H_2O)_2]^{2+}$ complexes to DNA, either of *cis*- or *trans*-geometry. Therefore, the first binding step to DNA can be considered under kinetic control.

The mechanism of formation of cisplatin-DNA adducts is presented in *Scheme 2*. In the experimental conditions of the kinetic studies, the anation

reactions (–1), (–2) and (–3) are negligible compared to the platination (P_1) (P_2) and chelation (C) reactions. It is noteworthy that, *in vivo*, with all the competing nucleophiles present in the cell, none of the aquation equilibra are likely to be established.

Table 4 gives a summary of the kinetic data for the formation of cisplatin (and one analogue)-DNA adducts. One can make the following comments:

Platination Step. *i*) Starting from the dichloro complex, the first aquation (1) (*Scheme 2*) is rate-determining, but the kinetic data do not tell which is/are the actual DNA-platinating species in the various conditions. *ii*) The k_{p1} rate constants differ by one order of magnitude from 0.3 to 2.5 M^{-1} s^{-1} [48][78] [82], but the value of 2.08 ± 0.07 M^{-1} s^{-1} that we have also deter-

Scheme 2. *Mechanism of Formation of Cisplatin-DNA Adducts*. k_1, k_2, k_3 are aquation-rate constants (k_1 and k_2 are in *Scheme 1*). k_{p1} and k_{p2} are platination-rate constants, k_c is the chelation-rate constant.

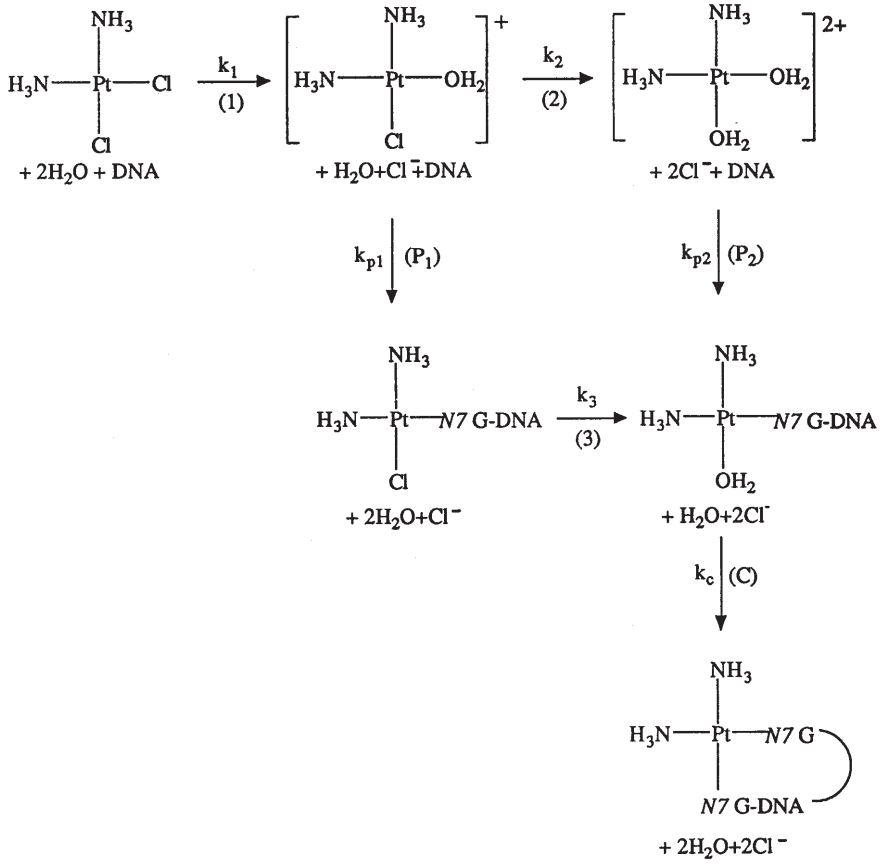


Table 4. *Rate Constants* (as defined in *Scheme 2*) *for the Formation of Cisplatin(and one Analogue)-DNA Adducts*

Complex	Technique	Experimental conditions	Kinetic constants
$[PtCl_2(NH_3)_2]$	Intercalator fluorescence [79]	Pseudo-1st-order *vs*. Pt; 1.6 mM DNA[a]) 40 mM *cis*-DDP; 10 mM $NaClO_4$; pH 5.5; 37 °C	$k_1 = 1.6 \times 10^{-4}\ s^{-1}$ $k_2 = 0.8 \times 10^{-4}\ s^{-1}$ $k_3 = 0.5 \times 10^{-4}\ s^{-1}$
$[PtCl_2(NH_3)_2]$	^{195}Pt-NMR [80]	Pseudo-1st-order *vs*. N [b]) 0.23 M DNA [c]); 10 mM *cis*-DDP [d]); 3 mM NaCl; 1 mM NaH_2PO_4;	$k_1 = 1.0 \times 10^{-4}\ s^{-1}$ $k_3 = 0.9 \times 10^{-4}\ s^{-1}$
$[PtCl(NH_3)_2(N(7)G\text{-DNA})]$		pH 6.5; 37 °C	$k_c > 8.3 \times 10^{-3}\ s^{-1}$
$[PtCl(NH_3)_2(H_2O)]^+$ 47.6% and 45.2% $[Pt(NH_3)_2(H_2O)_2]^{2+}$	Filter binding assay [78]	Pseudo-1st-order *vs*. N [b]) 0.1–1 mM DNA [a]); 9 μM *cis*-DDP; 5 mM $NaClO_4$; pH 5.5; 25 °C	$k_2 = 0.2 \times 10^{-4}\ s^{-1}$ $k_{p1} = 0.3\ M^{-1}\ s^{-1}$ $k_{p2} = 140\ M^{-1}\ s^{-1}$
$[PtCl(NH_3)_2(H_2O)]^+$	Atomic absorption [81]	Pseudo-1st-order *vs*. N [b]) 4.8 μM *cis*-DDP; 0.8 mM DNA [e]) 0.8 mM DNA [f]); 10 mM $NaClO_4$; pH 5.7; 37 °C	$k_{p1} = 0.34\ M^{-1}\ s^{-1}$ $k_{p1} = 0.2\ M^{-1}\ s^{-1}$
$[PtCl(NH_3)_2(H_2O)]^+$ $[Pt(NH_3)_2(H_2O)_2]^{2+}$	Inhibition of DNA synthesis [48] [82]	Pseudo-1st-order *vs*. G 0.5–3 mM DNA [g]); 0.02 mM *cis*-DDP; 10 mM KNO_3; pH 5.5 ; 37 °C	$k_{p1} = 2.5\ M^{-1}\ s^{-1}$ $k_{p2} = 42\ M^{-1}\ s^{-1}$ $k_3 = 0.7 \times 10^{-4}\ s^{-1}$ $k_{c1} = 3.7 \times 10^{-3}\ s^{-1}$ [h]) $k_{c2} = 3.7 \times 10^{-4}\ s^{-1}$ [h])
$[PtCl(NH_3)_2(H_2O)]^+$	Enzymatic digestion HPLC [83]	Pseudo-1st-order *vs*. N [b]) 0.05–1 mM DNA [g]); < 0.1 mM *cis*-DDP; 1 mM MES buffer [i]); pH 5.5; 37 °C	$k_3 = 1 \times 10^{-4}\ s^{-1}$ $k_{c1} = 3.7 \times 10^{-3}\ s^{-1}$ [h]) $k_{c2} = 1.6–3.7 \times 10^{-4}\ s^{-1}$ [h])
$[Pt(NH_3)_2(H_2O)$ $(N(7)G\text{-DNA})]$	^{14}C-G trapping [42]	2.9×10^{-4} M ^{14}C-G; 3.3×10^{-4} M DNA [g]); 10 mM $NaClO_4$; 37 °C	$k_c = 0.13 \times 10^{-4}\ s^{-1}$
$[PtCl(NH_3)_2(N(7)G\text{-DNA})]$ $[Pt(NH_3)_2(H_2O)$ $(N(7)G\text{-DNA})]$	thiourea trapping [84]	poly(dG-dC); 10 mM $NaClO_4$; 37 °C	$k_c = 0.5 \times 10^{-4}\ s^{-1}$ $k_c = 3.5 \times 10^{-4}\ s^{-1}$
$[PtCl_2(NH_3)_2]$ [b]) or $[PtCl_2(R_2\text{-en})]$ [j])	Atomic absorption [85]	Pseudo-1st-order *vs*. GG 0.2–0.92 mM DNA [g]) [Pt] = 1μM; I = 0.01M pH 5.5; 38 °C	$k_1 = 1.1 \times 10^{-4}\ s^{-1}$ $k_{p1} = 2.08\ M^{-1}\ s^{-1}$ $k_1 = 1.7 \times 10^{-4}\ s^{-1}$ $k_{p1} = 3.9\ M^{-1}\ s^{-1}$

[a]) Calf thymus DNA; [b]) Nucleotide; [c]) Chicken erythrocyte DNA; [d]) Due to the high DNA concentration, the solution must have been viscous; the pseudo-first-order condition was not met for GG or even G; [e]) *Micrococcus lysodeikticus* DNA (35% G; 11.2%.GG); [f]) *Clostridium perfringens* DNA (15.8% G ; 0.26% GG ; [g]) Salmon sperm DNA; [h]) 2 phases were observed for the cyclization step; [i]) 2-[*N*-morpholino] ethanesulfonic acid; [j]) R_2-en = *rac*-(1*S*,2*S*,4*S*)-*exo*-2-amino-2-(aminomethyl)-7-oxabicyclo[2.2.1]heptane.

mined with salmon sperm DNA, in strictly pseudo-first-order conditions (GG concentration larger than ten times that of platinum) agrees well with the second one [85]. *iii*) Also the k_{p2} values 140 and 42 M^{-1} s^{-1} [48][78][82] differ slightly, but here again the second one is closer to the values determined with oligonucleotide models (*vide infra*). (iv) The k_{p1} rate constants differ by a factor less than 2 (0.34 *vs*. 0.2 M^{-1} s^{-1}) for two DNAs with the following respective G and GG compositions: 35 and 15.8% G and 11.2 and 0.26% GG [81]; such a difference is smaller than expected on the basis of the (dG)n reactivity (*vide infra*).

Chelation Step. This step implies the preliminary aquation of the chloromonoadduct. The reported k_c value 1.3×10^{-5} s^{-1} [42] is actually very similar to the k_2 values 2.0×10^{-5} s^{-1} for *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ [78] and 4.4×10^{-5} s^{-1} for $[PtCl(en)(H_2O)]^+$ [12][13] and is identical to that of 1.35×10^{-5} s^{-1} that we have recently determined for *cis*-$[PtCl(NH_3)_2(dGuo)]$ [86]. *Holler* and coworkers found that two exponentials were needed to account for the disappearance of the monoadducts, and that the corresponding chelation constants differed by a factor of 10 [48][82][83]. This clearly shows that different monoadducts have different lifetimes (3 to 75 min here at 37 °C). The same authors reported a 3 times faster chelation for the DNA aquamonoadduct of *meso*-[1,2-bis(2,6-dichloro-4-hydroxyphenyl)dien]diaquaplatin(II) than for that of cisplatin [83]. Looking at the relative rates of interstrand G-Pt-G cross-linking, from DNA *cis*-$[PtCl(NH_3)_2(dG)]$ monoadducts, the following half-lives were found: $t_{1/2} = 1.6, 8, > 20$ h respectively for AG, GA, and GTG sequences [87]. The latter results involve the aquation step (*Scheme 2*). The chelation $t_{1/2}$ was also raised to 8 h 20 min by the presence of a bulky norbornyl group on the en ligand (*vide supra*) [49].

We can now identify the major factors which play a role in the DNA-platination and chelation steps: *i*) The nature and charge of the actual platinum species, *ii*) the bases to be platinated and their neighboring sequences, and *iii*) the nature of the nonleaving platinum ligands and their eventual interaction with the nucleic acid. To get a deeper insight into these parameters, in order to achieve selective DNA-platination, the study of oligonucleotide models is an appropriate approach.

Kinetics and Selectivity of Oligonucleotide Platination

Evidence for Outer-Sphere Association between Cationic Platinum Complexes and DNA

The platination-rate constants k_p in *Table 4* correspond to a bimolecular one-step coordination of an N(7)-guanine to platinum *via* an associative

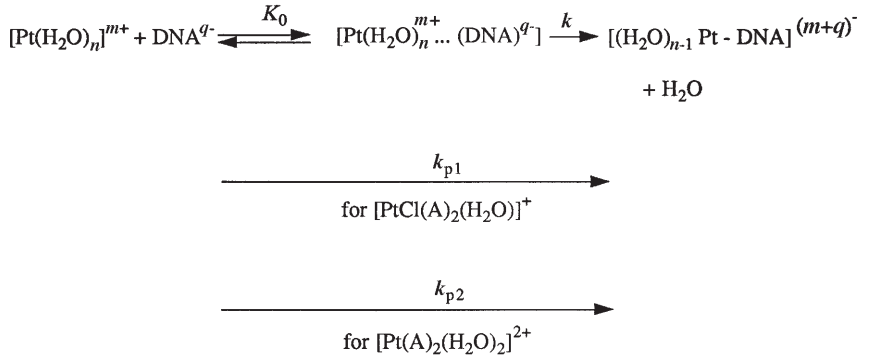
process [22]. However, the platinating species which do react with DNA are monocationic and/or dicationic (*Scheme 2*) and first interact with polyanionic DNA [32][33]. The local concentration gradient expelling chloride ions from the vicinity of polyanionic DNA should favor the diaqua complex (*Scheme 2*) [34][35].

The association between DNA or oligonucleotides with the aqua ions Mg^{II}, Ni^{II}, Co^{II} has been studied recently and the equilibrium constants K_0 were determined for various oligonucleotides in different conditions [88–90]. The equilibrium constants K_0 range from 40 to 10^5 M^{-1}: *i*) K_0 increases upon decreasing ionic strength, *e.g.*, for Mg^{II}/poly(dG-dC), K_0 increases from 870 to 76×10^3 M^{-1} with [NaCl] decreasing from 10^{-1} to 10^{-3} M; *ii*) K_0 depends on the length and on the composition of the nucleotide sequence, *e.g.*, for Mg^{II}/d(pA)$_8$-d(pT)$_8$, $K_0 = 120$ M^{-1}, and for poly(dA)-poly(dT), $K_0 = 10^3$ M^{-1}, whereas for Ni^{2+}/poly(dG)-poly(dC), $K_0 = 2 \times 10^5$ M^{-1}. These results partly reflect the influence of the molecular electrostatic potentials calculated for B-DNA-regions of GC and AT base-pairs [91][92]. The more negative sites were found on the N(7) positions of guanines in the major groove and on the N(3) of adenines in the minor groove. *iii*) K_0 depends on the charge of the complex and the hydrogen-bond acceptor ability of the ligands: $[Mg(H_2O)_6]^{2+}$, 12800 M^{-1}; $[Co(NH_3)_6]^{3+}$, 14800 M^{-1}; $[Co(NH_3)_5(NO_2)]^{2+}$, 1500 M^{-1}; $[Co(NH_3)_4(NO_2)_2]^{+}$, 20 M^{-1} [93], *iv*) K_0 depends on the configuration of the complex: for $[Co(en)_3]^{3+}$/d(CAATCCGGATTG)$_2$ in 10^{-1} M NaCl and 10^{-2} M phosphate buffer, $K_0 = 1000 \pm 500$ for the Δ-isomer and 100 ± 50 for the Λ-isomer [94].

Studies with aqua species of Ni^{II} showed a marked preference for G-rich oligonucleotides ($K_0 \approx 2 \times 10^5$ M^{-1} for poly(dG)-poly(dC)), an association that is actually followed by the reversible binding of nickel to a G-N(7) [88]. This case can be considered as intermediate between that of Mg^{II} and that of the aqua platinum(II) complexes which will irreversibly bind to a guanine after outer-sphere association. The corresponding kinetic equation can be written as in *Scheme 3*. The formation of the outer-sphere association is likely to be diffusion controlled in solution, *in vitro*. The coordination step (k) should be rate-determining [95].

A study of the reaction of cisplatin with several single-stranded oligonucleotides of varying length and with two hexadecanucleotides single- and double-stranded, all bearing a central phosphorothioate and in one case a GG sequence, has revealed an increasing reactivity of the platinating species with oligonucleotide length. It is noteworthy that the same reactivity was found for the single- and double-stranded oligonucleotides. An outer-sphere association was proposed to account for these results, involving a diffusion of the platinum species along the nucleic-acid chain [96][97].

Scheme 3. *Kinetic Scheme for DNA-Platination Involving the Preliminary Formation of an Outer-Sphere Association*. Pt stands for Pt^{II} bound to amino and eventually chloro ligands.



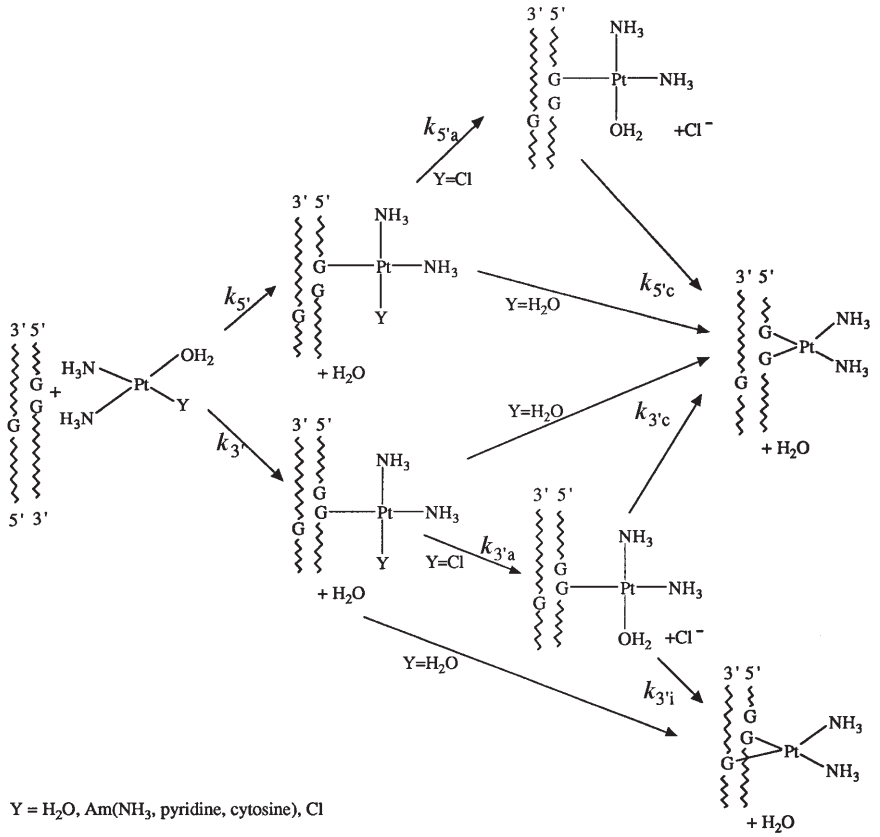
In an attempt to get evidence for an outer-sphere association, we studied the platination of $[poly(dG\text{-}dC)]_2$ by $[Pt(NH_3)_3(H_2O)]^{2+}$ in pseudo-first-order conditions. In our experimental conditions ($[DNA] < 5 \times 10^{-5}$ M ; $[Pt(NH_3)_3(H_2O)]^{2+} < 5 \times 10^{-6}$ M ; $[NaClO_4] = 10^{-3}$ M; pH = 5.5; 38 °C), we checked by UV-visible spectroscopy the presence of B-DNA. A good fit of the kinetic data was only obtained according to *Scheme 3* with $K_0 = (1.5 \pm 1.6) \times 10^5$ M^{-1} and $k = (2.7 \pm 0.6)\ 10^{-3}$ s^{-1} [98]. Due to experimental constraints, we did not improve the precision on K_0, yet, but its value is of the order of magnitude of those given above for dications interacting with $[poly(dG\text{-}dC)]_2$ [88].

More work is needed to clearly establish the role of outer-sphere association in DNA-platination. We can infer its influence on the rate of platination according to the relation $k_p = kK_0\,[N]/(1 + K_0\,[N])$ (with N = nucleotide-binding sites of Pt, *i.e.*, N ≠ G, [N] >> [Pt]) (*Scheme 3*). It could also influence the selectivity of platination *via* selective association between the cationic species and the sites of higher negative electrostatic potential. To test this hypothesis one will have to analyze the influence of various sequences, of different types of platinum ligands, and of the ionic status of the DNA medium.

Oligonucleotide Studies to Understand Platination and Chelation Selectivities

Provided that $(dG)_n$ sequences are the target of the first platination step (*vide supra*), the following questions are addressed (refering to *Scheme 4*): *i*) Do the different guanines of a $(dG)_n$ sequence exhibit the same reactivity

Scheme 4. *General Scheme for the Oligonucleotide Kinetic Studies*. Because of neutral or anionic Y ligand, the charge is omitted on the platinum complexes. Y = Am: monoadduct formation; Y = H_2O: platination directly followed by chelation; Y = Cl: platination, aquation of the chloro ligand on the monoadduct, followed by chelation.



($k_{5'}$ *vs.* $k_{3'}$)? *ii*) Are the 5′-G and 3′-G monoadducts chelated at the same rate by their adjacent guanine ($k_{5'c}$ *vs.* $k_{3'c}$)? *iii*) Are the 5′-G and 3′-G chloroadducts transformed into aqua intermediates at the same rate ($k_{5'a}$ *vs.* $k_{3'a}$)? *iv*) What is the influence of the single- or double-stranded structures of DNA on these reactions? *v*) What is the influence of the neighboring nucleotides and local groove environment on these reactions and on the competition between intra- and interstrand crosslinking?

To answer these questions, a series of studies have been carried out with the oligonucleotides presented in the *Figure* and the platinum complexes *cis*-[PtY(NH_3)$_2$(H_2O)].

In the following, any discussion of GG, XG, and GX sequences will imply that the neighboring nucleotides are pyrimidines which have no

Fig. *Oligonucleotides used to study the first platination, the monoadduct aquation, and the chelation steps involved in DNA-platination.* The theoretical molecular electrostatic potentials, in kcal mol^{-1}, of the 5′- and 3′-purines respectively, for each duplex structure are as follows: II (T*GG*C) –249.5, –248.7; IV (T*GG*T) –249.5, –251.4; V (C*GG*T) –245.7, –251.4; VII (T*GG*T) –249.5, –251.4; VIII (T*AG*T) –230.5, –245 [40][91][92].

marked orienting effect on the platination (at least as far as electrostatics are concerned).

First Platination Step. The data are presented in *Tables 5* and *6*. The following conclusions can be drawn: *i*) All the complexes react faster with double-stranded than single-stranded oligonucleotides. *ii*) The reactivity of the complexes is greater the higher their cationic charge and the better the ability of their ligands to participate in hydrogen bonding; for *cis*-$[PtY(NH_3)_2(H_2O)]^{n+}$ the reactivity decreases as a function of Y: $H_2O \gg NH_3 \geq$ pyridine $\gg$ cytosine $\approx Cl^-$. *iii*) All complexes, except Y = Cl, exhibit a selectivity of binding to the 5′-guanine. It is enhanced by the presence of hydrogen-bonding ligands. Whereas the ratio $k_{5'}/k_{3'}$ varies between 1 and 12 with Y = NH_3, H_2O , it varies between 1 and 0.2 with Y = Cl. *iv*) Duplex II appears peculiar compared to IV and V with an overall higher re-

activity resulting from higher $k_{5'}$ (× 3–4) and a smaller $k_{3'}$ (× 1/3–1/2) rate constant. The stability of II in the platinating conditions was ascertained [99][100], and work is under way to assess any particular features of the duplex GGC-GCC or GGCC-GGCC blocks. *v*) The theoretical electrostatic potentials of the different guanines (*Fig.*) cannot account for the preferred 5′-G-platination in IV, V, VII, even if the electrostatic potentials do not contradict the platination pattern.

Table 5. *First Platination Rate Constants.* Influence of the nature of Y in *cis*-[$PtY(NH_3)_2(H_2O)$] and of the single- or double-stranded structure of the oligonucleotide (*Scheme 4*, *Fig.*).

Complex	Oligonucleotide Platination rate constants (M^{-1} s^{-1}) [a] [99–101]					
Y	I			II [b]		
	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$
H_2O [a]	4.2(7)	2.0(2)	2.1	54(7)	4.4(7)	12
H_2O [c]				21(1)	1.7(3)	12
NH_3	1.1(1)	0.49(5)	2.2	4.5(5)	0.9(1)	5
pyridine	n.d. [d]	n.d.	1.7	3.7(2)	0.55(4)	6.7
cytosine-N(3)	n.d.	n.d.	1.6	0.26(4)	0.15(2)	1.7
Cl				0.19(2)	0.20(2)	1

[a]) 20 °C, $NaClO_4$ 0.1M, pH 4.4. [b]) The value of the rate constants corresponds to two GG sequences per duplex molecule. [c]) pH 6.0. [d]) Not determined.

Table 6. *First Platination Rate Constants*. Influence of the nature of the oligonucleotide on the platination with *cis*-[$PtY(NH_3)_2(H_2O)$] (Y= H_2O, NH_3, Cl) (*Scheme 4*, *Fig.*) [99–103].

Oligo-nucleotide	Complex platination rate constants [M^{-1} s^{-1}] [a]								
	Y = H_2O			Y = NH_3			Y = Cl		
	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$
II [b]	54(7)	4.4(7)	12	4.5(5)	0.9(1)	5	0.19(2)	0.20(2)	1
III	6.7(9)	4.7(7)	1.4	1.8(1)	0.8(1)	2.1			
IV	18(2)	15(1)	1.2	1.7(2)	0.8(1)	2.1	0.12(1)	0.28(1)	0.4
V [b]	14(2)	10(1)	1.4	2.8(2)	1.2(1)	2.3			
VI [c]	4.5(9)	5(1)	0.9				0.05(3)	0.28(5)	0.2
VII [c]	n.d.	n.d.	2				0.15(3)	0.47(8)	0.3

[a]) 20 °C, $NaClO_4$ 0.1M, pH 4.4. [b]) The rate constants correspond to two GG sequences per duplex molecule. [c]) 15 °C, $NaClO_4$ 0.1M, pH 4.8 [102].

Aquation of the Chloro Monoadducts and Chelation Steps. The data are presented in *Table 7*. The chelation reactions were easily studied starting from the diaqua complex (Y = H_2O, *Scheme 4*) [99–102]. Fewer experiments have been done starting from *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ [103].

The following conclusions can be drawn. *i*) The chelation reaction is considerably retarded when going from single-stranded to double-stranded monoadducts. *ii*) This slowing down is larger for the 5′-monoadducts than for the 3′-monoadducts, and this is at the origin of the factor of 10 found for all the duplexes in favor of the chelation of the 3′-monoadduct by its 5′-adjacent guanine. *iii*) The difference between $k_{3'c}$ and $k_{5'c}$ can be assigned to the relative rigidity of the B-DNA structure and to the theoretical distances of 3.9 and 5.5 Å between the platinum atom and the N(7) of the 5′- and 3′-adjacent guanines respectively [104]. *iv*) The rate constant of the interstrand crosslinking of the 3′-monoadduct of duplex II is only one half that of its chelation by the 5′-guanine. This was unexpected in view of the theoretical distances in B-DNA between the platinum atom and the N(7) of the 5′-guanine (3.9 Å), respectively, and the complementary strand guanine (7.5 Å). It will be interesting to know whether this rather fast interstrand reaction reflects a particular structure or conformational mobility of the (GGC)(GCC) or (GGCC)(GGCC) boxes. *v*) It is noteworthy that the aquation reaction of the chloro ligand is 10 times faster for the monoadduct on the 3′-guanine than on the 5′-one; this difference of one order of magnitude had been found for the overall chelation of chloro monoadducts [105] confirming that the aquation is the rate-determining step; a similar difference between chelation rates due to the relative position of the chelating purine has been observed for the d(A*G*TC) and d(C*G*AT) sequences with respective $t_{1/2}$ of 1.6 and 8 h [87]. *vi*) On GG sequences, there are two types of monoadducts, the 3′- and 5′-ones. The former are formed faster but undergo a slower aquation

Table 7. *Chelation-Rate Constants of Aqua-Monoadducts and Aquation-Rate Constants of Chloro Monoadducts* (*cis*-oligoG-$Pt(NH_3)_2Y$, Y = H_2O, Cl) *of Various Oligonucleotides* (*Scheme 4*, *Fig.*) [99–103]

Oligo-nucleotide	Chelation, Y = H_2O (10^{-3} s^{-1}) [a]				Aquation, Y = Cl (10^{-5} s^{-1}) [a]		
	$k_{5'}$	$k_{3'c}$	$k_{3'c}/k_{5'c}$	$k_{3'i}$	$k_{5'a}$	$k_{3'a}$	$k_{3'a}/k_{5'a}$
I	1.0(3)	3.3(4)	3.3				
II	0.06(4)	0.8(2)	13	0.4(1)			
III	1.0(1)	4.1(2)	4.1				
IV	0.18(5)	1.9(1)	10.5		0.19(1)	1.7(1)	9
V	0.41(1)	4(1)	10				
VII					0.7(2)	4.6(3)	13 [b]

[a]) 20 °C, $NaClO_4$ 0.1M, pH 4.4. [b]) 25 °C, NMR monitoring of the overall chelation [105].

and a slower chelation than the latter. This might correspond to the two different k_c values reported in *Table 4* [48].

The GG and AG Chelates, the Two Major DNA-Adducts of Cisplatin. It is interesting to compare the platination and chelation data of *cis*-$[Pt(NH_3)_2(H_2O)_2)]^{2+}$ reacting with the two hairpins IV (GG) and VIII (AG) (*Fig.*). It comes respectively for the platination step (k [M^{-1} s^{-1}]) $k_{5'}$ 18(2) *vs.* 1.5(3), $k_{3'}$ 15(1) *vs.* 9(1), $k_{5'}/k_{3'}$ 1.2 *vs.* 0.2; for the chelation step (k_c [10^{-3} s^{-1}]) $k_{5'c}$ 0.18(5) *vs.* 0.3(1), $k_{3'c}$ 1.9(1) *vs.* 0.08(1), $k_{3'c}/k_{5'c}$ 10.5 *vs.* 0.3 [106].

The following conclusions can be drawn: *i*) The platination-rate constants as well as the low AG $k_{3'c}$ value agree with the lower nucleophilicity of A compared to G. *ii*) Considering the GA case (under study) one can expect a lower $k_{5'c}$ for the less nucleophilic A in the geometrically unfavored 3′-position to the platinum atom of the monoadduct. This could account for the absence of GA adduct.

Discussion

All the work that has now been done on DNA and oligonucleotide platination gives a clear picture of the various steps of the reaction. There are still several questions to be answered for a complete understanding: *i*) What is the role of DNA-(platinum complex) outer-sphere association? In which actual 'medium' does it occur? Could the monocationic chloro aqua complex, major species in the cytoplasm, be transformed into the dicationic diaqua species in this outer-sphere association and be the actual platinating species? It is noteworthy that the *in vitro* and *in vivo* relative proportions of GG- and AG-*cis*Pt adducts are the same as those we found with the model study using the diaqua complex. The same work is underway with the chloroaqua complex [103]. *ii*) Could an outer-sphere association occur with the dicarboxylato complexes, as carboplatin and oxaliplatin, which give GG and AG adducts similar to those of cisplatin [107]? *iii*) DNA is not a 'passive' polyanionic substrate but rather facilitates and catalyses the transformation of some platinum mono- and diadducts [52][63][65] (*J.-M. Malinge* and *M. Leng* in *Part 3*). Preliminary results, in collaboration with Prof. *Sadler*'s group, have shown that *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$, reacting with $d(TTGGCCAA)_2$, gives a slower reaction than the dicationic diaqua complex but with exactly the same selectivity, whereas the monocationic chloroaqua complex gives a very much slower reaction and no selectivity (*Table 5*). This, together with other data comparing NH_3 to H_2O and to other amine ligands, suggests a major role for hydrogen bonding in the major groove in addition to that earlier assigned to the ammine-phosphate inter-

action [1–4]. *iv*) DNA also plays a role in the aquation step of the chloro monoadducts, which may be important when the chloroaqua complex is the platinating species; this controls the next chelation step. *v*) Geometric factors can influence the orientation of platination. Accordingly, *cis*-[$PtCl_2$-$(NH_3)(C_6H_{11}NH_2)$] gives 54% of GG but only 8% of AG intrastrand adducts [108], an interesting result considering the higher mutagenicity of the AG-compared to GG-*cis*Pt adduct [109][110]. The [$PtCl_2$(hpip)] complex (hpip = 1,4-diazacycloheptane), designed to form interstrand adducts, actually gives the same level of them as that obtained with cisplatin, but forms less than half the amount of intrastrand adducts [111]. These results suggest that molecular modelling should be of help to design selective complexes. *vi*) The control of the formation of long-lived monoadducts, still bearing a labile ligand, favors the crosslinking of DNA with repair or recognition proteins [49] and might be a tool to study protein binding to platinated DNA.

Conclusion

After thirty years of research, cisplatin has led to the discovery of several new generation anticancer drugs discussed in this book. Cisplatin has also become an outstanding tool to study all aspects of DNA properties, from reaction with small cationic species to interaction with proteins which induce biological signals triggering apoptosis.

This chapter has shown that we now understand correctly the various steps of the binding of platinum complexes to DNA. We are in a position to use this knowledge to design new complexes which should preferentially bind to sequences selected for the ability of their adducts to induce the desired cellular lethal cascade.

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Structure and Dynamics of Pt Anticancer Drug Adducts from Nucleotides to Oligonucleotides As Revealed by NMR Methods

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We review pioneering NMR studies of Pt anticancer drug adducts with DNA fragments, drawing attention to the widely overlooked 'dynamic motion problem'. Specifically, NMR spectroscopy has not distinguished between relatively static and very dynamic oligonucleotide adducts. The rapid dynamic motion of simple *cis*-$Pt(NH_3)_2G_2$ models (G = guanine derivatives) can be understood by both the small size and the turnstile rotation of NH_3 allowing the guanine base O(6) to pass by the amine unhindered. This motion could explain why similarities in NMR-spectral features of several *cis*-$Pt(NH_3)_2$(dGpG) duplexes have led to differing published structural models, all failing to account for some of the data. New cisplatin analogs containing chirality-controlling chelate (CCC) diamine ligands with bulk near the PtN_4 plane decrease motion and allow large steps to be taken in understanding the NMR and CD spectra, and hence properties, of *cis*-PtA_2G_2 complexes. Our unprecedented recent findings include the following: *a*) G O(6) amine hydrogen bonding, by tilting the bases, decreases favorable base-base dipole interactions; *b*) *head-to-head* (*HH*) and *head-to-tail* (*HT*) adducts are kinetically and thermodynamically favored, respectively; and *c*) GpG adducts exist in unusual conformations, including *HT* forms and an abnormal *HH* form with the opposite direction of propagation of the sugar-phosphate backbone. Some adducts exhibit unusual NMR-spectral features found in larger adducts such as a hairpin adduct. We conclude that future discoveries will reveal important novel aspects of the oligonucleotide adduct structures.

Introduction

Pt-DNA Adducts are widely believed to be responsible for the antitumor activity of cisplatin (*cis*-$PtCl_2(NH_3)_2$) (*Fig. 1*) and have been studied for many years. In competition reactions at low Pt/nucleotide concentrations, cisplatin was shown to bind to the N(7) position of guanine (G* = *N(7)*-platinated G or G derivative) and, to a lesser extent, of adenine (A)

Cisplatin. Edited by Bernhard Lippert

(*Fig. 2*) [1]. Under neutral conditions, cisplatin can also bind to the N(3) position of cytosine (C) and the N(1) position of A, although these positions are less accessible in a duplex because of base-pairing interactions (*Fig. 2*) [2]. In the reaction of cisplatin or $PtCl_2(en)$ (en = ethylenediamine) with salmon sperm DNA, the most abundant adduct was determined to be a 1,2-intrastrand d(G*pG*) crosslink (60–65%), while the next most prevalent adduct was a 1,2-intrastrand d(A*pG*) crosslink (20–25%) [3][4]. Because

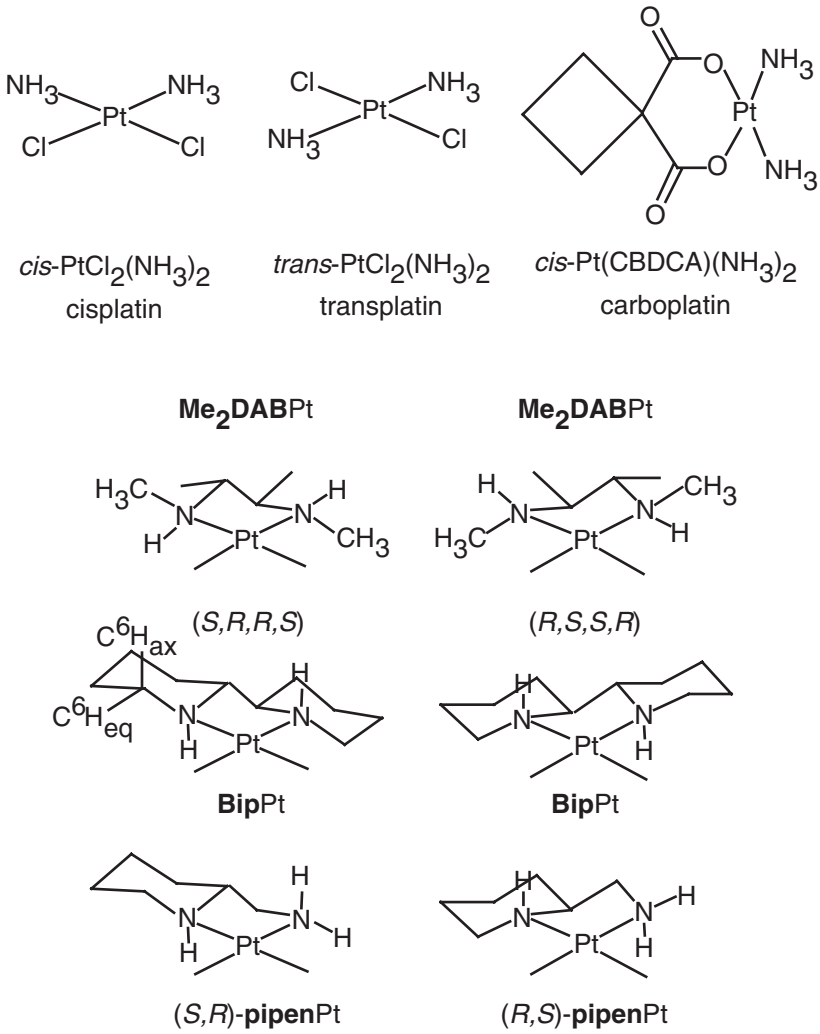

Fig. 1. *Sketches of the antitumor-active Pt compounds cisplatin and carboplatin, and the inactive transplatin.* Also shown are CCC or CCC-like Pt compounds described in the text, drawn with the G-coordination sites toward the front.

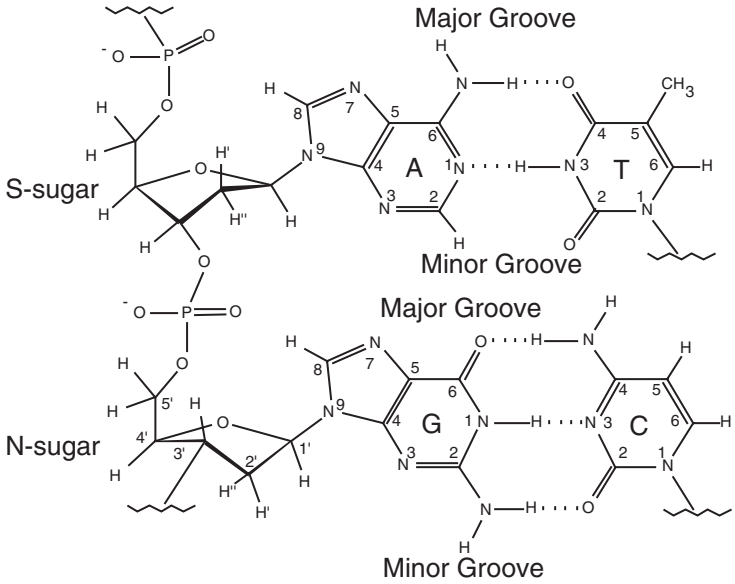

Fig. 2. *Schematic representation of the DNA base pairs showing base and sugar numbering schemes, sugar puckers, and the location of the major and minor grooves*

the percentage of the d(G*pG*) adduct formed was larger than statistically expected, this crosslink has generally been assumed to be the important adduct for anticancer activity. Further support for this conclusion came from transplatin's inability to form 1,2-intrastrand crosslinks and its low antitumor activity [5][6]. Both isomers can form interstrand crosslinks, and there is still support for interstrand crosslinks as the key lesion [7][8].

It should be noted that Zn^{II} and Pt^{II} complexes bind at sites that are very nucleophilic, and GG sites fall into this category [9–11]. However, Zn^{II} and other metal centers generally do not have the ability to form an intrastrand crosslink [11]. Formation of such a crosslink requires disruption of the DNA conformation, and most metals are not able to induce such a disruption. Because such adducts have distorted structures, the DNA has altered biochemistry. These features lie at the heart of anticancer activity and will influence repair and protein binding of DNA. For example, proteins containing high-mobility group (HMG) binding domains are known to recognize the structural perturbations induced by Pt binding [12–15]. Platination of oligonucleotides (oligomers) can lead to distorted intrastrand (single-stranded species, hairpins, and duplexes) or interstrand crosslinks. The role of the major intrastrand d(G*pG*) adducts is not fully understood; however, structural perturbations in DNA may play a key role in the antitumor activity of cisplatin [16]. In fact, cisplatin-modified DNA has been shown to be recognized by certain proteins; structure-specific recognition protein 1 (SSRP1)

[15][17–20] and homologous high-mobility group protein 1 (HMG1) are cisplatin-modified DNA-binding proteins [15][20].

Because of the importance of the d(G*pG*) crosslink, previous studies in which 1,2-intrastrand d(G*pG*) crosslinks have been modeled and investigated using NMR spectroscopy are reviewed. Models for interstrand crosslinks are also reviewed, although fewer studies are available [21][22]. A number of techniques can be utilized to study the conformation of Pt-DNA adducts, but NMR spectroscopy is the focus of this chapter. There are three types of models for Pt-DNA interactions that contribute to our understanding of the interpretation of NMR results: *a*) Pt complexes with nucleobases, nucleosides, or nucleotides (abbreviated in this work as *cis*-PtA_2G_2; A_2 = a diamine or two amines, and the G derivative is bound *via* N(7) in all cases described here); *b*) Pt adducts with single-stranded DNA (Pt-ssDNA); and *c*) Pt complexes with double-stranded DNA (Pt-dsDNA).

NMR spectroscopy is used to examine Pt-DNA adducts in solution, more accurately reflecting the biological environment of Pt-DNA adducts. ^{1}H-NMR spectroscopy is particularly useful for studying DNA because of the well-resolved base and H(1′) regions of the spectrum. Additional information can be gathered from studies with nuclei such as ^{31}P, ^{13}C, ^{15}N, and ^{195}Pt. Because DNA is conformationally fluxional, often the isolated crystallized form is A-form DNA, whereas a variety of physical methods show that DNA is normally B-form in aqueous solution. Thus, solution studies are particularly important for DNA.

NMR Methods can be used to obtain dynamic, structural, and thermodynamic information on solutions. Until recently, all evidence has pointed to the intrastrand crosslink having a *head-to-head* (*HH*) form with the guanine bases oriented in the same direction (*Fig. 3*). We shall discuss the fea-

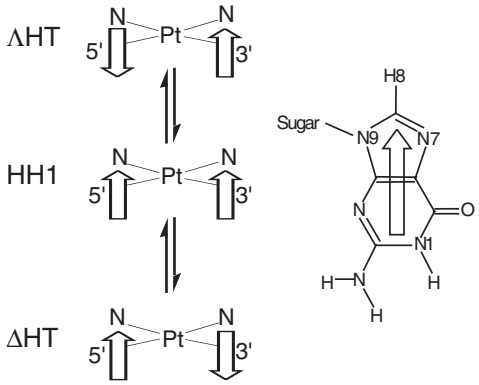

Fig. 3. *Schematic representation of the possible atropisomers and their interconversion in a* C_2*-symmetrical* cis-PtA_2G_2 *complex.* Arrows represents an *N(7)*-bound G as shown on the right side of the figure.

tures of this form in detail below, but we call it *HH*1 here. If the bases are oriented in opposite directions, the crosslink is a *head-to-tail* (*HT*) form (*Fig. 3*). In principle, NMR methods can distinguish among these forms. We note that since the symmetry of a DNA chain is low and each atom is unique, NMR methods cannot easily differentiate between the case of one conformer in a relatively fixed state and the case of a mixture of conformers in rapid dynamic motion. More amenable to NMR study are simple models with unconnected nucleotides, which are highly fluxional and interconvert rapidly between forms in which the bases rotate through ~180° (see below) [23]. Conformations of adducts with the *cis*-$(NH_3)_2Pt^{II}$ moiety itself are especially difficult to elucidate. Attachment of the NH_3 ligands to Pt by single bonds permits the NH_3 ligands to adopt independently numerous orientations that allow the NH groups to form hydrogen bonds to the nucleic acid target or to avoid steric interactions with the target. As a result, multiple similar conformations probably co-exist, and the barriers between the conformers are probably shallow, making *cis*-$(NH_3)_2Pt^{II}$ adducts especially fluxional. The unsymmetrical nature of the d(G*pG*) crosslinks and the dynamic nature of the *cis*-PtA_2G_2 adducts make an understanding of solution conformation and dynamics difficult when A_2 is $(NH_3)_2$ or has two primary amine donors. Such species make the most active drugs. Collectively, we call these complications the dynamic motion problem. Despite this situation, it has generally been agreed that for the simple *cis*-PtA_2G_2 adducts the sugar phosphate backbone linkage between the bases has two interrelated effects: *i*) it makes rotation about the Pt–N(7) bond very slow or very unfavorable and *ii*) it stabilizes the *HH*1 form. Implicit in this analysis has been the assumption that if *HT* forms were present, the dynamic processes leading to the *HH*1 form would be slow, and the *HT* form would be detected.

Nucleobase and Nucleotide Models

Nucleotide complexes have been used to model Pt-DNA interactions because they are simple yet provide information relevant to DNA adducts. In typical *cis*-PtA_2G_2 adducts, the appearance of one time-averaged set of signals in the ^{1}H-NMR spectrum indicates that rotation about the Pt–N(7) bond has been shown to be fast on the NMR time scale [24][25]. However, a bulky A_2 can slow the rotation about the Pt–N(7) bond, permitting the observation of rotamers [23][25–34]. Restricted rotation in *cis*-PtA_2G_2 complexes is evident from the appearance of multiple sets of ^{1}H-NMR signals corresponding to *HH* and *HT* conformers [26]. For *cis*-PtA_2G_2 complexes with C_2-symmetrical PtA_2 moieties, one *HH* and Δ and Λ*HT* conformers are

possible (*Fig. 3*). When viewing the *cis*-PtA_2G_2 complex from the G-coordination side, a line connecting the O(6) atoms will be rotated (by an angle < 90°) clockwise (*ΛHT*) or counterclockwise (*ΔHT*) in order to be aligned with the perpendicular to the coordination plane [35]. Each *HT* atropisomer is C_2-symmetrical and has one H(8) signal, but the *HH* atropisomer with two nonequivalent H(8)'s has two H(8) signals; thus, four H(8) signals are expected for the three atropisomers. When the *cis*-PtA_2 moiety is not C_2 symmetric, there are two *HH* forms, HH_1 and HH_2.

The first NMR evidence for restricted Pt–N(7) bond rotation came with $[Me_4enPt(Guo)_2]^{2+}$ (Guo = guanosine; Me_4en = *N,N,N′,N′*-tetramethylethylenediamine), in which the two observed H(8) signals were interpreted as indicating the presence of two *HT* rotamers [26]. Even with non-bulky amine ligands, restricted rotation has been demonstrated when Pt binds to A or C [32]. This condition arises because of the greater bulk of the NH_2 groups near the platination site (compared to O(6) for G). However, alternative explanations have been advanced [36][37]. Facile rotation about the Pt–N(7) bond has been proposed to be critical for the formation of the d(G*pG*) intrastrand crosslink [23][25–32].

In addition to providing information useful for analyzing rotation rates and the number of atropisomers, the shifts of the H(8) ^{1}H-NMR signals are diagnostic of coordination by G at N(7) [38][40]; when N(7) is coordinated, the G* H(8) signal does not shift downfield when the solution is made strongly acidic. The H(8) shift can be used with care to assess other features. The H(8) signal of [*cis*-PtA_2(5′-GMP)(H_2O)] complexes (~9.1 ppm) is downfield of the H(8) signal of dynamic *cis*-PtA_2(5′-GMP)$_2$ complexes (~8.5 ppm), both of which are normally downfield from the H(8) signal of free 5′-GMP (~8.2 ppm) [24][29–31][38]. This same relationship is found for *cis*-PtA_2G(Cl) complexes where G = Guo or 1-MeGuo, although the downfield shift is only ~0.1 ppm in these instances [31]. However, the situation is more complicated when more than one nucleobase is present and dynamic motion is decreased by a bulky A_2 group or by a backbone linkage. Mutual anisotropic effects of a *cis*-G* moiety, anisotropic effects of the nearby bases in longer sequences, and Pt anisotropy can influence the shifts. Thus, it is useful to have other NMR information such as NOE data.

Inherent purine 'stacking' forces (dipole-dipole interactions) drive nonbridged G* moieties to orient in an *HT* arrangement, an established phenomenon both in solution-state [26][30][33][34][39] and solid-state studies [27][40–44]. For the $[Me_4enPt(Guo)_2]^{2+}$ complex, where the bulky tertiary amine groups cannot form hydrogen bonds [26], the only atropisomers observed have *HT* conformations; perhaps in this case *HH* conformers are sterically disfavored. The two *HT* conformers are equally favored in solution, but the *ΔHT* crystallized and was characterized by X-ray crystallography

[27]. In the less hindered systems, it is not possible to assess the atropisomer distribution.

In the solid state, the *ΔHT* form of nucleoside and nucleotide complexes are observed almost exclusively [26][40–44]. However, this 'delta preference' for the solid-state *HT* conformer is not restricted to Pt or to square-planar complexes, and its origin is unknown [41]. In contrast, the *HH* form appears to be dominant for Re and Ru benzimidazole complexes [45-47]. These systems have a ligated imidazole ring, such as that in purine nucleobases; the conformation is clearly dictated to a large extent by an electrostatic attraction of the partially positively charged N_2CH proton to the *cis* negative ligands [47]. The analogous N_2CH proton, H(8), will also have a significant partial positive charge. However, in the square planar *cis*-type Pt drugs there are no *cis* negative ligands, and the factors dictating conformation are not so clear as for the octahedral Re and Ru complexes. Part of our goal is to understand such factors.

For *cis*-PtA_2G_2 complexes, the *HH* form typically has one H(8) signal upfield and one downfield from the two *HT* H(8) signals [33]. In systems in which dynamic motion is slow, we are beginning to understand many features of crosslink models. The upfield shift of one *HH* H(8) signal can be attributed to canting of the base and the influence of the *cis*-nucleobase anisotropy (*Fig. 4*). The H(8) of the more canted base is in the upfield-shifting region of the less canted base. Such canting/anisotropy has proved useful in detecting *HH* rotamers in Re and Ru benzimidazole complexes in solution, where the presence of 'probe' nuclei in the six-membered ring provides very compelling evidence also for downfield shifting by the more canted base [45–47].

For *cis*-PtA_2G_2 complexes, the solid-state *ΔHT* adducts cluster into two groups differing in the degree and direction of the tilt [48]; *i.e.*, the bases can have either a left-handed (L) or a right-handed (R) tilt, illustrated for (*S,R,R,S*)- and (*R,S,S,R*)-$Me_2DABPtG_2$ (Me_2DAB = *N,N′*-dimethyl-2,3-diaminobutane) (*Fig. 4*). Relative to the average H(8) signal, a lesser tilt gives less shielding and hence a deshielded (d) H(8) signal, and the greater tilt gives a shielded (s) H(8) signal. In theory, three sets of two variables lead to eight (2^3) possible forms. However, due to the dynamic nature of adducts in solution, neither the tilt nor the absolute conformation in solution was known for typical *cis*-PtA_2G_2 adducts. Our results now indicate that there are only four stable *HT* forms, as follows: *ΔHT*Ls, *ΔHT*Rd, *ΛHT*Ld, and *ΛHT*Rs.

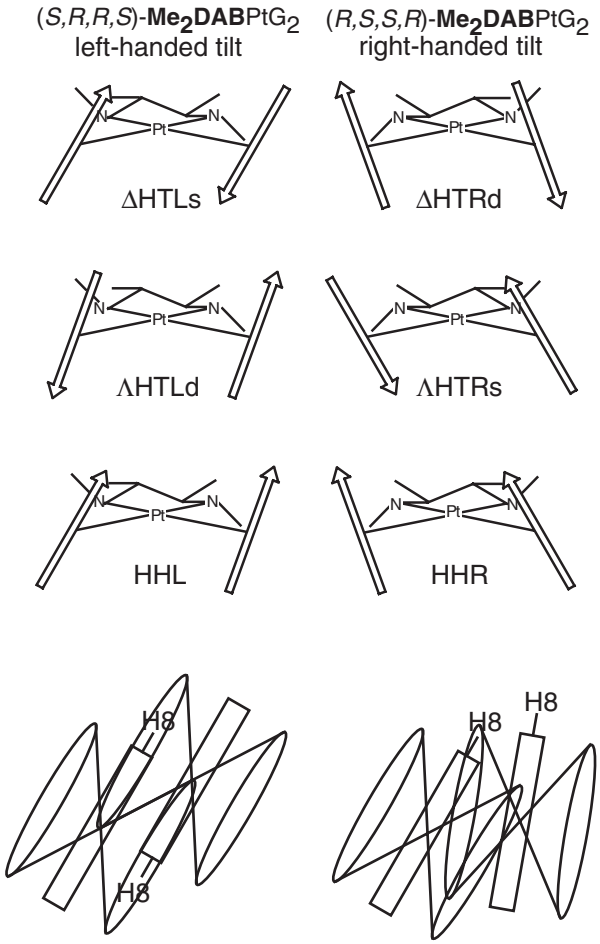

Fig. 4. *Sketches of (*S,R,R,S*)- and (*R,S,S,R*)-Me$_2$DABPtG$_2$ rotamers with left-handed and right-handed tilt, respectively*. At the bottom, we show the effects of base anisotropy. At the left bottom, we show a *ΔHT* left-handed, more tilted form indicating that both H(8)'s are in shielding regions. Less tilted forms would be similar, but the H(8)'s are away from the center of the shielding cone. On the bottom right, we show a left-handed *HH* form. One H(8) is in the shielding region and one is outside this region. Bases are shown schematically from the edge. However, the planes are not parallel since the N(7)–Pt–N(7) angle is close to 90°.

Less Dynamic Models

We began a cooperative program several years ago with the *Natile* laboratory in Bari to design chirality-controlling chelate (CCC) ligands that could both slow the dynamic motions and favor particular conformers in solution. In our early studies, we investigated bis 5′-GMP-Pt complexes with CCC = the C_2-symmetrical isomers of Me_2DAB (*Fig. 1*). This CCC ligand

was designed with fixed *C*-methyl groups to influence the stereochemistry at the nitrogen centers [33][49][50]. Both (*R,S,S,R*)-Me_2DABPt(5′-GMP)$_2$ and (*S,R,R,S*)-Me_2DABPt(5′-GMP)$_2$ (in which the configuration at the four asymmetric chelate ring atoms is *R, S, S, R* or *S, R, R, S* at N, C, C, and N, respectively) existed as mixtures of all three possible atropisomers; this was the first report of a *HH* atropisomer in solution [33].

Like all other C_2-symmetrical $Me_2DABPtG_2$ complexes, which are excellent representative systems for interpretation of chemical shifts, [Me_2-DABPt(9-EtG)$_2$]$^{2+}$ has a major *HT* atropisomer with a downfield H(8) signal and a minor *HT* atropisomer with an upfield H(8) signal [50]. Thus, even with its very simple N(9) substituent, this complex is representative. Several factors could influence this shift relationship. However, a likely interpretation is that the major atropisomer has a lesser tilt, and the minor atropisomer has a greater tilt, since a shift difference of ~0.3 ppm was predicted for these differently tilted forms [48]. From our analysis of conformations, the minor forms could form G* O(6)–NH hydrogen bonds. Such hydrogen bonding will increase the degree of tilt. The less tilted conformer had favorable base-base dipole-dipole interactions. From similar reasoning, the *HH* form has the same tilt direction as the major and minor forms, but one G* is less tilted and one G* more tilted. Such tilting not only minimizes steric interactions, but also provides an explanation for the dispersion of the H(8) signals. It is likely that the G* that cannot form a G* O(6)–NH(Me_2DAB) hydrogen bond and will have a lesser tilt (possibly even in the other direction) than in the respective major *HT* form, whereas the other G* will have as great, if not greater, tilt than in the respective minor *HT* form.

These Me_2DAB and related less symmetrical Me_2DAB [34][51] systems have limitations since the rotamers were highly fluxional, interconverting rapidly *via* rotation about the Pt–N(7) bond even below room temperature as evidenced by broad signals and EXSY cross-peaks in the NOESY spectra even at 5 °C [33][34][50]. The search for less dynamic systems led to the (*S,R*)- and (*R,S*)-pipenPtG_2 complexes (pipen = 2-aminomethylpiperidine, with (*S,R*)- or (*R,S*)-configurations at the two asymmetric centers, N, and C, respectively, *Fig. 1*) [52][53]. One half of the pipen compound closely resembles clinically used drugs, and information relevant to clinically used drugs can be obtained by studying these pipenPtG_2 complexes since an environment very similar to that in clinically used drugs is created. In the adduct, (*S,R*)-pipenPt(5′-GMP)$_2$, restricted rotation of the two nonequivalent 5′-GMP's about the Pt–N(7) bonds potentially could lead to *ΛHT*, *ΔHT*, and to HH_1 and HH_2 atropisomers. However, 1D- and 2D-NOESY-NMR data at pH ~3 indicated the dominance of the two *HT* atropisomers in a *ΛHT* / *ΔHT* ratio of 2 : 1 [53]. Deprotonation of the phosphate group (pH 7) further stabilized the *ΛHT* form. However, at pH 9.5, where the 5′-GMP

H(1) was largely deprotonated, the NMR spectrum revealed that the *ΛHT* form had decreased. When the pH was jumped down to 6.9, the *ΛHT* form increased with a half-time of ~3 min. Thus, the pip ring lengthens the atropisomerization time from seconds for Pt(en)(5′-GMP)$_2$ to minutes for (*S,R*)-pipenPt(5′-GMP)$_2$.

The (*S,R*)-pipenPt(5′-GMP)$_2$ H(8) signals also shifted as a function of pH for reasons easily understood using the *ΛHT* atropisomer as an example (*Fig. 5*). One G* H(8) (tip of arrow), G^*_S (the G* next to the secondary amine), is always away from the other G*, G^*_P (next to the primary amine), regardless of any tilt changes in G^*_P. Thus, G^*_S H(8) is downfield and affected little by pH changes. The H(8) of G^*_P, on the other hand, is affected by G^*_S anisotropy at low pH because of its tilt caused by the suspected G^*_P O(6)-NH(pi-

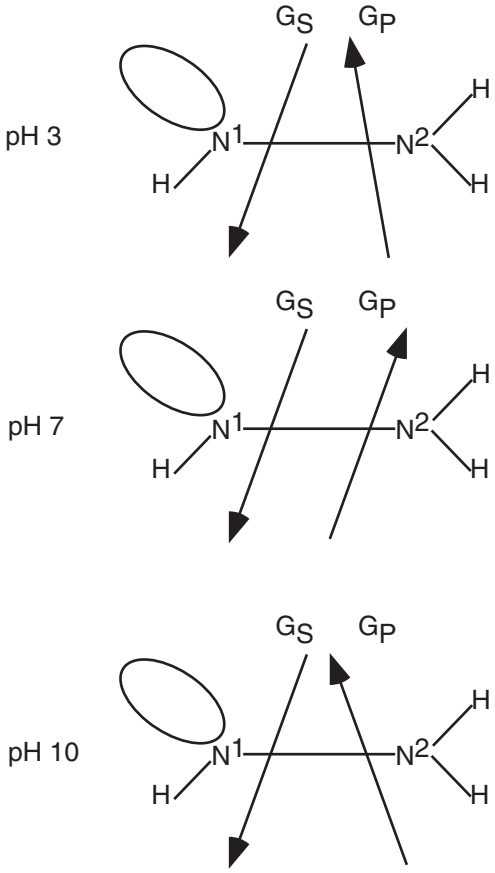

Fig. 5. *Sketches of the* ΛHT *atropisomer of* (S,R)*-pipenPt(5′-GMP)$_2$ at different pH values.* The tilt of G_S remains unchanged while the tilt of G_P changes dramatically with pH.

pen) hydrogen bonding. At higher pH (~7 to 8), the phosphate is deprotonated and the G^*_P tilt changes; the greater distance of G^*_P H(8) from the G^*_S base leads to a downfield shift. Finally, at higher pH, the G^*_P six-membered ring H1 deprotonates, probably again favoring G^*_P O(6)–NH(pipen) hydrogen bonding. The tilt change brings G^*_P H(8) close to G^*_S, leading to the upfield shift of the G^*_P H(8) signal. Similar shift behavior observed for the *ΔHT* rotamer can be explained similarly.

Further improvements in CCC ligand design led us to 2,2′-bipiperidine (Bip) (*Fig. 1*) [39][54]. The bulk of the Bip ligand is concentrated in the Pt coordination plane, a feature designed to slow dynamic processes of $BipPtG_2$ adducts. This design was validated in a study of $BipPt(5'\text{-GMP})_2$ complexes, for which we were able to examine the products of the coordination step for the second 5′-GMP before the products had time to redistribute [39]. The initial distribution was that expected from statistics, *i.e.*, ~50% *HH* adduct and 25% of each *HT* adduct. With time, equilibration occurred to give product distributions favoring an *HT* form, as is typical for fluxional systems; the *HH* rotamer became a minor species. The ^{1}H-NMR spectra of $BipPtG_2$ and $Me_2DABPtG_2$ adducts were very similar at equilibrium.

The volumes of H(8) NOE cross-peaks to NH, C^6H_{ax}, and C^6H_{eq} (*Fig. 1*) signals, assigned using 2D-NMR spectroscopy, were used to determine the absolute conformations of the rotamers; for example, if the relative ratio (volume of H(8)-NH cross-peak : volume of H(8)-C^6H_{ax} cross-peak) is > 1 or < 1, the 5′-GMP is oriented with the H(8) on the same or the opposite side, respectively, of the platinum coordination plane as the *cis* NH. The absolute conformations of the major *HT* rotamers at low pH of (*R,S,S,R*)-$BipPt(5'\text{-GMP})_2$ and (*S,R,R,S*)-$BipPt(5'\text{-GMP})_2$, assigned as *ΔHT* and *ΛHT*, respectively, depend on BipPt chirality [55]. For the *HH* atropisomers, cross-peaks between the H(8) signals confirmed the conformation since no exchange phenomena were observed in the 2D-NOESY spectra, in contrast to the $Me_2DABPt(5'\text{-GMP})_2$ spectra [50].

G*pG* and d(G*pG*) Models

The reaction of cisplatin with GpG or d(GpG) was found to yield one product with two H(8) ^{1}H-NMR signals [56–58]. These signals were shifted downfield of free GpG/d(GpG) H(8) signals as a result of Pt binding. The d(G*pG*) crosslink was determined to have an *anti*,*anti*-*HH*1 conformation with a 5′-N sugar and a 3′-S sugar. The 5′-N sugar appears to be a universal feature shown by NMR studies (as well as X-ray studies). The 3′ sugar is usually S, although the conformation in d(G*pG*) adducts is unclear [56][57]. This *HH*1 conformer has two recognized variants with different

directions of base canting in Pt-single-stranded (ss) oligomer complexes. The 5′-G* H(8) signal is downfield of the 3′-G* H(8) signal if the sugar is a ribose but upfield if the sugar is a deoxyribose [56]. *HH*1 crosslinks usually have one base canted toward the other, and the H(8) signal of the more canted base is upfield, an effect attributed to the ring-current effects of the less canted base [38][48]. Depending on which base is canted, opposite shift relationships are found for the 3′-G* H(8) and 5′-G* H(8) signals. The base tilting that leads to the 5′-G* H(8) downfield / 3′-G* H(8) upfield shift relationship is called an L1 conformation and has a localized left-handed helical sense (*Fig. 6*) [48]. The other tilt relationship, a right-handed conformation of the G* bases, is called an R2 conformation. Thus, the ribose to deoxyribose change was thought to change which base is canted [48]. As we will see later, these same two different tilts were proposed for Pt-ssDNA and Pt-dsDNA adducts. Both types of canting are observed in the crystal structure of the single-stranded species, *cis*-$Pt(NH_3)_2$(d(pGpG)), which has four independent molecules, all with the *HH*1 conformation [59][60]. Virtually all reports indicate that the *HH*1 conformer predominates, but many reports contain speculation that *HH*1 equilibrates with other forms that interconvert too rapidly for separate characterization by NMR spectroscopy [12][57] [61–64].

Other PtA_2(d(GpG)) and PtA_2(GpG) adducts have been studied. The A_2 ligands have chiral centers (*e.g.*, 1,2-cyclohexanediamine, dach) [65] or non-C_2-symmetrical ligands (designated here as AA′). For Pt(dach)(d(GpG)) or Pt(dach)(GpG) complexes, the same order of H(8) chemical shifts was observed as for the cisplatin analog. Thus, the chiral centers are too far removed to influence base canting [65]. Again, like the simple *cis*-PtA_2 compounds, Pt(dach) was found to cause a change in the 5′-G* sugar

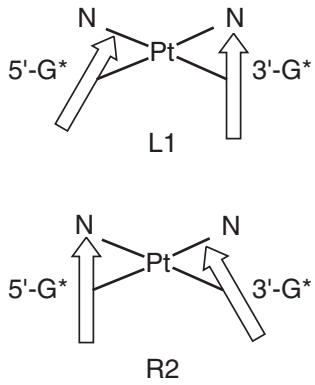

Fig. 6. *Schematic representation of the L1 and R2 conformations of a d(G*pG*) adduct*

pucker from S to N, as did unsymmetric PtAA′ complexes. However, the PtAA′ compounds did influence the H(8) chemical-shift order, depending on which base was next to the bulkier A group [66].

Less Dynamic Models

During the two decades of intense interest in the 1,2-intrastrand d(G*pG*) crosslink, the only *anti,anti* conformation proposed was *HH*1, and only this conformation was expected, before we studied (*R,S,S,R*)-BipPt(d(GpG)). Remarkably, we found two N(7)–Pt–N(7) crosslink products: one was an *HH*1 conformer, but the other was a new *HH* conformer (*HH*2) (*Fig. 7*). Each product had a pair of H(8) signals with a dispersion (*Fig. 8*) and a medium H(8)-H(8) NOESY cross-peak, features consistent with *HH* conformers [38][63][67][68]. The very similar H(8)-H(8) distance, estimated for both in the medium-distance (2.5–3.5 Å) range, is consistent with *HH* bases. For comparison, H(8)-H(8) distances in *HT* models are 4.5–5.5 Å. No H(8)-H(1′) cross-peaks were observed in the 300 ms mixing-time NOESY spectrum, indicating that all of the G*'s are *anti* since an intense H(8)-H(1′) cross-peak would be observed for a *syn*-G*.

For (*R,S,S,R*)-BipPt(d(GpG)), the downfield 5′-G* H(8) / upfield 3′-G* H(8) relationship for the *HH*1 adduct is opposite to that found for the *HH*2 adduct and for *cis*-$Pt(NH_3)_2$(d(GpG)) [56][57]. Only upfield 5′-G* H(8) / downfield 3′-G* H(8) shifts have been detected for deoxy single-stranded species except in two cases [69][70]. Of considerable interest, the *HH*1 form of (*R,S,S,R*)-BipPt(d(GpG)) is a third exception; it is unique in having fea-

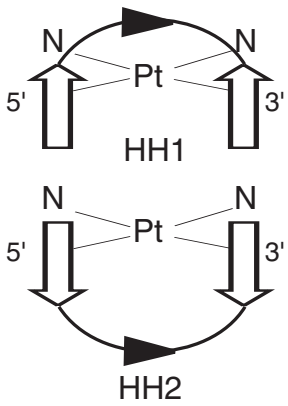

Fig. 7. *Representation of the* HH*1 and* HH*2 forms of (*R,S,S,R*)-BipPt(d(GpG))*

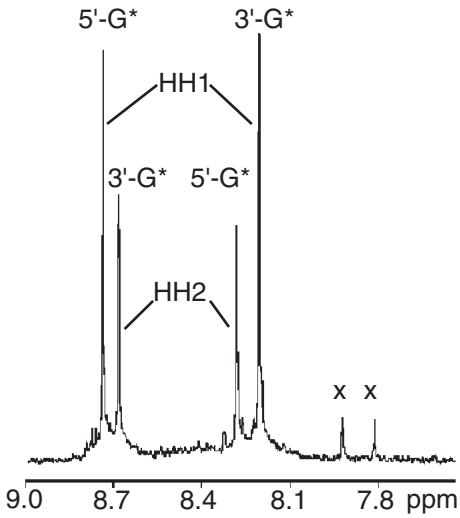

Fig. 8. *H(8)* ^{1}*H-NMR signals of* (R,S,S,R)*-BipPt(d(GpG)) at pH 3.5, 20 °C* (×: signals of a third (*R*,*S*,*S*,*R*)-BipPt(d(GpG)) species, probably an *HT* rotamer)

tures similar to the better defined *HH*1-duplex adducts for which downfield 5′-G* H(8) / upfield 3′-G* H(8) shift relationships [62][71–73] and ^{31}P shifts of *ca*. –3.2 ppm [62][72-76] are almost universal. Also, the 5′-G* H(8) shift of the *HH*1 adduct of (*R,S,S,R*)-BipPt(d(GpG)) is very close to this shift for most duplexes, ~8.7 ppm [62][71–73][77]. The upfield 3′-G* H(8) in duplexes indicates that the 3′-G* base is canted. The opposite shift relationship for the *HH*1 and *HH*2 conformers of (*R,S,S,R*)-BipPt(d(GpG)) is consistent with a difference involving which base is most canted.

The reaction of [(*S,R,R,S*)-BipPt(H_2O)$_2$]$^{2+}$ with d(GpG) also unexpectedly yielded two products of comparable abundance. One adduct was characterized to be the normal *HH*1 form. The G* H(8) shift pattern of this *HH* form, namely 5′-G* H(8) upfield and 3′-G* H(8) downfield, is the same as that found for *cis*-Pt(NH_3)$_2$(d(GpG)), suggesting that these two adducts have similar hydrogen bonding and base canting. The ^{31}P-NMR signal at –2.8 ppm is also a common feature of *HH* adducts. The second adduct, determined to be an *HT* conformer, has several unique spectral features. Of particular note, the relatively upfield shifts of *both* G* H(8) signals (7.91 and 7.77 ppm) and an upfield-shifted ^{31}P-NMR signal (–4.6 ppm) of the *HT* conformer are unprecedented for a major conformer of an adjacent G*-G* intrastrand crosslinked species.

The H(8) resonances of the *HT* product are unusually upfield and have a small signal separation. A small (≤ 0.2 ppm) H(8) separation has been observed in a few instances [67][69][70], but in all of these cases, the H(8)

signals are downfield of those from free d(GpG), and ^{1}H-NMR data indicate an *HH* base orientation. A strong 3′-G* H(8)-H(1′) NOE cross-peak and the absence of an H(8)-H(8) NOE cross-peak for the *HT* form indicate that it is an *HT* conformer with a *syn*-3′-G*. Upfield H(8) signals with small separation have been observed for interstrand G*-G* Pt crosslinks, determined to be *HT* adducts [21][22]. The H(8)-H(8) distance in the calculated *ΔHT* models was 4.6 Å. This distance is significantly larger than the H(8)-H(8) distance in our *HH*1 model (2.8 Å; 2.88 Å experimentally) but smaller than the H(8)-H(8) distance in *cis*-PtA_2G_2 adducts (5–5.5 Å), in which the G* bases are not tethered by a phosphodiester linkage.

The 5′-G* H(3′) signal at 3.9 ppm and the 3′-G* H(2′)/(2″) signal at 3.3 ppm of the *HT* conformer of (*S,R,R,S*)-BipPt(d(GpG)) have unusual shifts. Similar shifts have been reported in a hairpin-like structure, characterized as a '*head-to-side*' adduct (*Fig. 9*), with a *syn*-3′-G* residue [10][78]. These ^{1}H-NMR shifts may be diagnostic for identifying 3′-G* *syn*-residues in G*-G* adducts. Downfield H(2′) signals have been observed for Pt_2-d(G*pG*) adducts [79-81] and other modified DNA sequences [82][83] having *syn*-3′-G* residues. However, the differences in ^{31}P shifts of the d(G*pG*) moiety for the *ΔHT* form and the hairpin (–4.6 *vs*. –2.8 ppm) indicate that the backbone conformations are different.

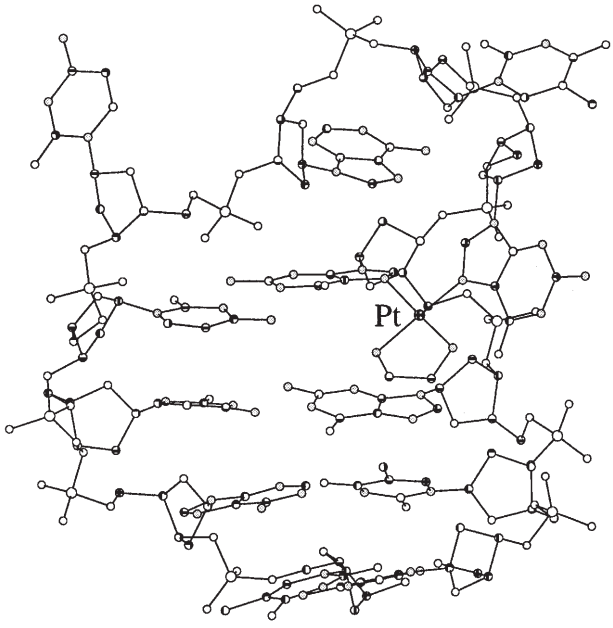

Fig. 9. *Ball-and-stick representation of the hairpin-like platinated LM4 adduct*

In summary, the BipPt(d(GpG)) adducts have several unusual features compared to typical d(G*pG*) complexes; two conformers are formed in roughly equal amounts by the two different $[BipPt(H_2O)_2]^{2+}$ stereoisomers. One conformer in each system has the normal *HH*1 backbone, but the *HH*1 atropisomer of (*R,S,S,R*)-BipPt(d(GpG)) is atypical because of the 5′-G* H(8) downfield / 3′-G* H(8) upfield chemical shift relationship. The second adduct in each system is unusual; in (*R,S,S,R*)-BipPt(d(GpG)), the second conformer is an *HH*2 atropisomer that has the opposite direction of propagation of the phosphodiester linkage compared to *HH*1, while for (*S,R,R,S*)-BipPt(d(GpG)) the second adduct is an *HT* atropisomer with a *syn* 3′-G* residue and upfield-shifted ^{31}P signal.

The observation of several d(G*pG*) conformers suggests that *cis*-$Pt(NH_3)_2$(d(GpG)) may be a mixture of rapidly interconverting atropisomers. Mixtures of several conformers also explain the many failures to obtain a crystal structure of *cis*-$Pt(NH_3)_2$(d(GpG)). The first crystallographic success (with *cis*-$Pt(NH_3)_2$(d(pGpG))) [59][60] revealed that it had the accepted *HH*1 conformation; this finding apparently confirmed the NMR interpretation. However, the crystal structure also revealed a stabilizing hydrogen bond between the 5′-phosphate group and the *cis*-NH_3 [59][60]. Our modeling studies with *cis*-$Pt(NH_3)_2$(d(pGpG)) also give *HH*1 conformers with this hydrogen bond [54]. The *HH*1 conformer is slightly more stable than the new *HH*2 conformer, which lacks this hydrogen bond. As we discuss below, the wide range of H(8)-NMR shifts for Pt-ssDNA adducts also supports the presence of multiple conformers.

Binuclear Models

Both Pt's of a new class of binuclear anticancer compounds, [{*trans*-$PtCl(NH_3)_2$}$_2${μ-$H_2N(CH_2)_nNH_2$}]$^{2+}$ and [{*cis*-$PtCl(NH_3)_2$}$_2${μ-$H_2N(CH_2)_nNH_2$}]$^{2+}$, have recently been shown by NMR methods to react with GpG, d(GpG), and d(TGGT) [79–81]. For the reaction of d(GpG) with [{*trans*-$PtCl(NH_3)_2$}$_2${μ-$H_2N(CH_2)_nNH_2$}]$^{2+}$, n = 3 or 6, the normal 3′-G* H(8) downfield / 5′-G* H(8) downfield chemical shift relationship was observed [79]. In both of these adducts, the 3′-G* residue was determined to be *syn* and to have a sugar with a high percentage of N character (72% and 70% for n = 3 and 6, respectively); the sugar of the 5′-G* residue remained mostly S-sugar. Neither of these findings is typical for normal d(G*pG*) adducts. Another unusual feature for the n = 3 adduct was the ^{31}P signal at –4.6 ppm, upfield of the usual –4.2 ppm ^{31}P DNA signal, whereas normal d(G*pG*)'s have a downfield-shifted ^{31}P signal. Reaction of the n = 6 compound with d(TGGT) yielded an adduct similar to the d(G*pG*) adduct, with the ex-

ception that the 3′-G* H(8) was upfield and the 5′-G* H(8) downfield [80]. These complexes were characterized as stepped *head-to-head* adducts. The reaction of $[\{cis\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}H_2N(CH_2)_nNH_2\}]^{2+}$ with $n = 4$ with d(GpG) gave an adduct similar to the *trans*-compound with $n = 6$. However, in this *cis*-adduct, the 3′-G* H(8) signal was broad, interpreted as indicating exchange between different conformations and restricted rotation about the 3′-G* N(7)–Pt bond [81]; the *trans*-complexes did not show this restricted rotation.

Other Dinucleotide Crosslinks

The second most abundant crosslink formed by cisplatin with DNA is d(A*pG*); the d(GpA) crosslink is not formed. The reaction of cisplatin with ApG gave primarily one adduct with two *anti N(7)*-platinated residues [84]. Like G*pG*, most of the characteristic NMR signal changes are for the 5′ residue; the A* H(8) signal is downfield of the G* H(8) signal, and the A* sugar has an N pucker. Reaction of cisplatin with d(ApG) yielded a mixture of A* *N(1)*- and A* *N(7)*-platinated products in a 1 : 4 N(1) : N(7) ratio at pH 6 [85]. For the N(7),N(7) crosslink, a 4 : 1 *anti*,*anti*/*anti*,*syn* d(A*pG*) ratio was observed. d(pA*pG*) formed only the N(7),N(7) *anti*,-*anti* adduct [85]. For *anti*,*anti* d(A*pG*), A* H(8) is downfield of G* H(8), unlike d(G*pG*), but the A* sugar is still N. The A* H(8) signal experiences a characteristically large downfield shift upon platination [58] and was calculated to experience a more inductive effect *vs*. G H(8) [86].

In several platinated NpN adducts with *syn*-residues and N = DNA base, the 3′ residue was found to be *syn* [87–89]. In these cases, N was a purine. This observation of a *syn* orientation for only the 3′ residue could be related to the 5′ residue having an N-sugar in these adducts; the N pucker favors an *anti*-orientation [90]. Also, in our *ΔHT* model of (*S,R,R,S*)-BipPt-(d(GpG)), there is a 3′-G* NH_2-phosphate hydrogen bond. This interaction could help stabilize the *syn*-orientation for the 3′-G* residue. In the one instance of a platinated NpN complex with a *syn* 5′ residue, the 5′ sugar pucker was N, but the 5′ base was a pyrimidine, cytosine [48].

Longer Single-Stranded Species

A number of Pt-ssDNA adducts platinated at d(G*pG*) sites have been studied utilizing ^{1}H, ^{31}P, ^{15}N, and ^{195}Pt-NMR spectroscopy [31][32] [38][56–58][61–63][67–70][87][91–97]. Both C_2- and non-C_2-symmetrical A_2 ligands have been used, but most of the results were similar, and both

classes will be included in this section. Self-complementary Pt-ssDNA molecules (≤ 6 bases in length) do not form a duplex structure; these sequences will also be discussed in this section [98–100]. Several general observations common to Pt-ssDNA molecules containing d(G*pG*) crosslinks have been noted. These trends, observed in simple d(G*pG*)/(G*pG*) adducts, are discussed next.

For Pt-ssDNA adducts, the H(8) chemical shift order is 5′-G* H(8) upfield / 3′-G* H(8) downfield, the same order observed for the simple d(G*pG*) adduct. Thus, the presence of a base to the 5′ or 3′ side of the platination site does not change the preferred canting in Pt-ssDNA adducts. In order to relieve some of the strain introduced by platination, the 5′-G* sugar pucker becomes N in Pt-ssDNA, while the 3′-G* sugar pucker remains mostly S, as deduced from ^{1}H-NMR coupling constant and NOE data [38][57][67][68][91–93]. This observation is again the same as found for d(G*pG*).

In contrast to the well-defined, relatively narrow shift ranges for duplexes (see below), single-stranded species have quite variable H(8) shifts, with the 5′-G* H(8) signal found from ~8.0 to ~9.0 ppm and the 3′-G* H(8) shift from ~8.5 to ~9.5 ppm [38][56][57][63][67][68][96]. This broad range of H(8) shifts is just one piece of evidence for dynamic exchange between multiple differently canted single-stranded conformers. Our BipPt(d(GpG)) results raise the possibility that dynamic exchange could involve *HH*2 and *HT* conformers as well. This possibility gains support from the broadness of the range of ^{31}P-NMR shifts reported for the single-stranded species, including values close to that of the *HH*2 form of (*R,S,S,R*)-BipPt(d(GpG)) [38][62][74]. Additional support for the possibility can be found in the very large ~7.8 to 8.8 ppm ranges of H(8) shifts for both 3′- and 5′-G*'s defined by the three observed conformers of BipPt(d(GpG)).

After the reaction of *cis*-PtA_2 compounds with DNA, a new ^{31}P signal at –3.2 ppm shifted downfield from the normal –4.2 ppm value (relative to TMP) [101] was observed [76]. In all *cis*-PtA_2-ssDNA adducts, a similar downfield ^{31}P signal has been observed [38][57][67][94]. This ^{31}P signal, assigned to d(G*pG*), has shifts from –2.6 to –3.6 ppm, depending on both the oligomer sequence and the A_2 ligand. The range of shifts has been attributed to differences in hydrogen bonding of an NH of A_2 with the phosphate group 5′ to the d(G*pG*) [38][67][94]. A platinated duplex was also thought to have phosphate-NH hydrogen bonds on the basis of an NH of A_2 coupling constants and sugar ring puckers [72]. Other factors affecting ^{31}P-NMR chemical shifts include the α (O(3′)–P–O(5′)–C(5′)) and ζ (C(3′)–O(3′)–P–O(5′)) torsion angles [102][103]; a *trans* (180°) value for either α or ζ leads to a downfield shift [104]. Narrowing of the O–P–O diester angle can lead to a downfield shift of the ^{31}P signal, while widening can lead to an upfield shift [103].

Although ^{13}C-NMR spectroscopy has been infrequently applied to Pt-ssDNA and Pt-dsDNA, it can provide useful structural information. Heteronuclear ^{13}C-^{1}H 2D-NMR data on Pt(en)(d(TGGT)) confirmed solid-state findings that an upfield shift of the sugar C(3′) ^{13}C signal was indicative of an N sugar pucker conformation [63]. This same technique has also been used in the identification of N-sugars in a Pt-duplex adduct (see below). The shift of G C(8) ^{13}C signals may also prove useful for determining *anti vs. syn* orientations; a downfield-shifted C(8) ^{13}C signal has been reported for *syn* residue [10].

Pt-ssDNA molecules (> 8 bases in length) have been shown to form stable duplexes with their complementary sequences in solution [12][51][61][62][71–74][105–108]. Some of these studies [12][61][108], emphasize ^{15}N/^{1}H-NMR data and are discussed by *Sadler* and coworkers in this volume.

^{195}Pt-NMR data have been reported for simple *cis*-Pt$(NH_3)_2G_2$ complexes [31][109] as well as for longer platinated ssDNA [62]. For *cis*-Pt$(NH_3)_2$(5′-GMP)$_2$, a ^{195}Pt-NMR signal was observed at –2455 ppm (referenced to K_2PtCl_6) [31]. For the Pt-ssDNA adduct, an almost identical shift of –2450 ppm was reported [62]. In ^{195}Pt-NMR studies of *cis*-Pt$(NH_3)_2$-dsDNA adducts, the major bifunctional adduct had a ^{195}Pt signal at –2445 ppm [97]. These results suggest that no unusual distortion has occurred at the Pt moiety, since ^{195}Pt chemical shifts are sensitive to changes in ligands or N–Pt–N bond angles [31][110].

Adducts Derived from Duplexed Oligomers

Platinated, self-complementary oligomers > 6 bases in length have also been studied [9][10][64][111][112]. In this section, we discuss adducts that form non-duplex structures or have more than one Pt-crosslink/duplex.

The self-complementary sequence d(GACCATATG*G*TC) forms a duplex [64], which has two d(G*pG*) crosslinks (one crosslink/strand) and is kinked (~40° from molecular modeling calculations) at both modified sites. No 5′-G* H(1) imino signal was observed, possibly because there is no hydrogen bonding between 5′-G* and its complementary C [64]. Weak or absent NOEs around the platination site provided further evidence for the disruption of this base pair. For (d([c^{7}G]CC[c^{7}G]CG*G*C))$_2$ ([c^{7}G] has C(7) instead of N(7)), the 5′-G* residue was *syn* and had an H(8) signal upfield of 3′-G* H(8). Two sets of ^{1}H-NMR signals that merged at high temperature suggested the presence of multiple conformers.

As mentioned, *cis*-PtA_2-DNA adducts have a new ^{31}P signal shifted downfield to –3.2 ppm [76]. A series of self-complementary d(GpG)-con-

taining oligomers from 8 to 14 bases in length treated with *cis*-PtA_2 compounds had at least one downfield-shifted ^{31}P-NMR signal [111]; no downfield-shifted ^{31}P signal was observed for *trans*-PtA_2 compounds or for sequences without GG sites but with A, AG, and GNG sites. The samples were kept under conditions favoring duplex formation, and imino signals were observed for the platinated species. For each of these oligonucleotides, the d(G*pG*) site was in a different location (5′-end (terminal) or internal). Thus, the downfield ^{31}P signal in Pt-DNA adducts is not a function of single or double strands, and the location of the d(G*pG*) adduct in the oligomer is not important. Pt compounds with *cis* leaving groups induced a downfield ^{31}P signal when reacted with poly(I)·poly(C) and completely disrupted duplex structure at a high Pt/DNA ratio [113]; compounds without *cis* leaving groups (transplatin, Pt(dien)Cl (dien = diethylenetriamine)) did not induce such spectral changes.

In spite of multiple potential platination sites on the parent duplex, d(ATGG*G*TACCCAT) (LM4) was formed for Pt(en) and *cis*-$Pt(NH_3)_2$, as studied by ^{1}H-, ^{13}C-, and ^{31}P-NMR spectroscopy [10][78]. Unlike the previously mentioned self-complementary sequences, the LM4 adduct has a hairpin-like structure (*Fig. 9*), as evidenced from ^{1}H-NMR, CD, and UV spectroscopy and electrophoresis [9][10][78][112]. Early ^{1}H-NMR studies showed that one G* residue had a *syn* orientation; the other G* H(8) signal could not be found [112]. Eventually, in a concentrated sample, it was noted that, although the hairpin-like structure was dominant, a small amount of a duplex form was identified [10]. The missing G* H(8) signal, identified through an exchange cross-peak from the duplex to the hairpin-like form and assigned as the 5′-G* H(8) signal, was found in the region of the spectrum where H(1′) resonances usually occur. In LM4, the 5′-G* base was base paired, while the 3′-G* base was not.

Interstrand G*-G* crosslinks formed by cisplatin and oligomers have also been studied by NMR spectroscopy (*Tables 1* and *2*) [21][22]. The interstrand G*-G* crosslink, like the intrastrand lesion, causes a kink in the DNA helix. However, unlike the intrastrand crosslink, the kink is toward the minor groove. A localized change from B-DNA structure to left-handed Z-DNA-like structure around the platination site was also observed. The G* H(1) signal was either not observed (PtLe) or was shifted upfield by >3 ppm (PtH) (*Table 2*), indicating that the platinated G*'s were not base paired with their complementary C's. In addition, these C's were extrahelical. The G* bases have an *HT* orientation, in contrast to the 1,2-intrastrand *HH*1 orientation. The G* H(8) signals of PtLe and PtH were shifted upfield and close in chemical shift (PtLe only; PtH is a self-complementary sequence) (*Table 2*).

Duplex Adducts: NMR Studies

The G H(1) and T H(3) signals can be observed only when these bases are paired and when H_2O signal suppression methods that do not saturate the solvent signal are used. In early studies of two different sequences, the observation of imino ^{1}H-NMR signals established the formation of a duplex by a single strand containing a d(G*pG*) crosslink on addition of the complementary strand [51][105]. These two platinated duplexes had comparable stability, reflected in the similar ~30 °C melting temperature [51][105]. From the chemical shift differences in R and PtR (*Table 1*), it was concluded that the helix must experience some structural distortion, most likely a kink toward the major groove since the N(7)-binding site is in the major groove (*Fig. 2*) [71].

As will be evident in the later parts of this section, the key unresolved issues about the platinum duplexes center on the 5′-G*·C base pair. In some studies, the 5′-G* H(1) signal was not observed, whereas in others it is very weak. The absence of this signal could indicate that the 5′-G* H(1) is

Table 1. *List of Interstrand and Intrastrand Duplex Adducts Studied by NMR Spectroscopy and Their Abbreviations and Sequences*

	Sequence	Reference
PtH	d(CATAG*CTATG)·d(CATAG*CTATG)	[21]
PtLe	d(CCTCG*CTCTC)·d(GAGAG*CGAGG)	[22]
PtK	d(CTCA*G*CCTC)·d(GAGGCTGAG)	[114]
PtR	d(TCTCG*G*TCTC)·d(GAGACCGAGA)	[62][71][74][105]
PtM	d(CTCCG*G*CCT)·d(AGGCCGGAG)	[107]
PtC	d(GCCG*G*ATCGC)·d(GCGATCCGGC)	[72][106]
PtW	d(CCTG*G*TCC)·d(GGACCAGG)	[73]
PtL	d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG)	[77]

Table 2. *List of Characteristic Chemical Shifts* [ppm] *around the Platination Site for Interstrand and Intrastrand Duplex and Duplex-like Adducts Studied by NMR Spectroscopy*

	5′-G*(A*) H(8)	3′-G* H(8)	5′-(XG*) X H(2′)	C/T(5′-G*/A*) H(2′)	C(3′-G*) H(2′)	C/T(5′-G*/A*) H(6)	C(3′-G*) H(6)	5′-CG*-3′ C–N(H_b)	5′-CG*-3′ C–N(H_e)	C(5′-G*) N(H_b)	C(5′-G*) N(H_e)
PtH	7.55	7.55	2.1	2.1	2.1	7.7	7.7	n/a	n/a		
PtLe	7.91	7.99	2.26	2.37	2.40	8.01	7.99	8.54	7.34		
PtK	9.01	8.81	0.87	1.84	1.90	7.29	7.38				
PtR	8.73	8.02	1.46	1.87	1.99	7.40	7.37	8.83	7.37	8.05	6.77
PtM	8.66	8.39	1.33	1.86	2.04	7.45	7.50	8.85	7.27	8.25	6.84
PtC	8.70	8.36	1.58	1.96	2.09	7.50	7.68	8.80	7.15	8.06	6.80
PtW	8.76	8.19	1.33	1.90	1.99	7.47	7.42	n/a	n/a	8.13	6.93
PtL	8.74	8.16	1.40	1.89	1.96	7.44	7.42	n/a	n/a	8.03	6.82

not needed in base pairing; on the other hand, there may be other reasons this signal was not observed in some cases. It is therefore of some interest that the PtR 5′-G* H(1) signal intensity is close to that of other imino signals (*Fig. 10*) [71][105]. For its time, the pioneering PtR study was very advanced, but the assignments based on 2D-NMR spectra obtained in D_2O for the non-exchangeable signals were far from complete [71][105]. We decided to reexamine PtR with more extensive methodology since the key 5′-G* H(1) resonance was so easily observed for PtR, and only 1D-NMR spectra were reported for H_2O.

In extending the PtR assignments using standard DNA sequential assignment methods (*Fig. 11*), we found several structural changes and distinct chemical shifts around the platination site. We report these features in *Table 3* and compare them to those of other duplexes with d(G*pG*) crosslinks studied by NMR methods [51][71-74][77][105][107]. These duplexes are generically referred to here as PtD (see *Table 1* for specific sequences and abbreviations). In addition, a duplex with a d(A*pG*) crosslink has been studied by NMR spectroscopy, and these data are also included in the

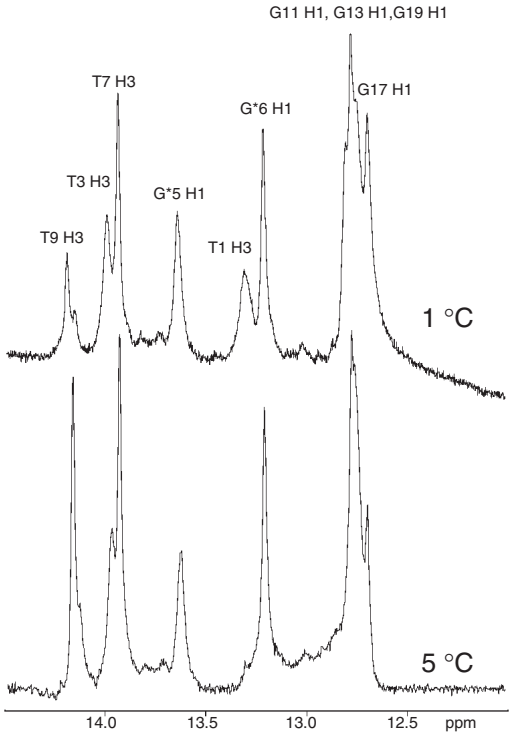

Fig. 10. *Imino ^{1}H-NMR region of PtR at 1 °C* (top) *and 5 °C* (bottom), *showing loss of the T1 H(3) signal between these temperatures*

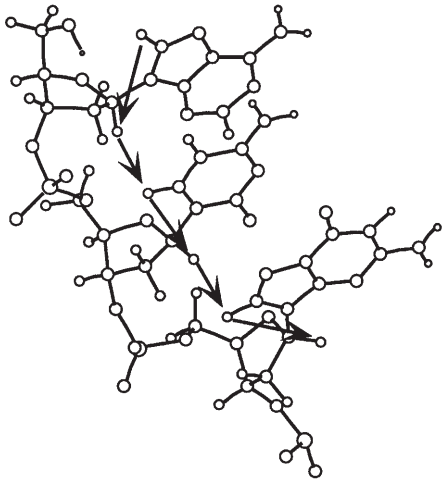

Fig. 11. *Sequential* $(H(8)/H(6))_n$-$H(1')_{n+1}$ *pathway followed for typical DNA NOESY assignment*

Table 3. *Non-exchangeable* (7 °C) *and Exchangeable* (5 °C) ^{1}*H-NMR Chemical Shifts of PtR*

	H(8)/H(6)	H(2)/H(5)/CH_3	H(1′)	H(2′)	H(2″)	H(3′)	H(4′)	H(1)/H(3)	NH_b	NH_e
T1	7.59	1.73	6.12	2.25	2.55	4.75	4.14	13.25	–	–
C2	7.76	5.78	6.08	2.26	2.56	4.83	4.22	–	8.42	7.08
T3	7.49	1.68	5.98	2.21	2.50	4.86	4.41	13.97	–	–
C4	7.44	5.71	5.89	1.46	2.46	4.70	4.07	–	8.83	7.37
G*5	8.73	–	6.11	2.59	2.68	5.10	4.28	13.65		
G*6	8.03	–	5.56	2.26	2.52	4.58	4.19	13.19		
T7	7.55	1.25	6.15	2.30	2.58	4.89	4.28	13.92	–	–
C8	7.61	5.62	6.03	2.16	2.57	4.78	4.17	–	8.47	7.15
T9	7.51	1.72	6.10	2.16	2.54	4.87	4.16	14.14	–	–
C10	7.66	5.83	6.28	2.55	2.26	4.58	4.02	–	8.30	7.25
G11	7.90	–	5.56	2.53	2.72	4.84	4.17	12.75		
A12	8.21	7.81	6.00	2.77	2.90	5.07	4.42	–		
G13	7.75	–	5.62	2.52	2.73	5.03	4.42	12.75		
A14	8.12	7.94	6.27	2.64	2.89	5.00	4.44	–		
C15	7.37	5.40	5.84	1.99	2.34	4.73	4.29	–	8.38	6.94
C16	7.40	5.42	5.57	1.87	2.23	4.77	4.02	–	8.05	6.77
G17	7.89	–	5.52	2.68	2.68		4.32	12.67		
A18	8.09	7.72	5.87	2.60	2.74	5.01	4.36	–		
G19	7.68	–	5.53	2.57	2.62	4.95	4.58	12.75		
A20	8.10		6.30	2.56	2.38	4.65	4.23	–		

comparison [114]. As we compare structural changes and chemical-shift data of the PtD adducts, we shall see numerous similar chemical-shift trends for all six PtD adducts. However, despite these similar spectral features, which presumably indicate that all adducts have the same main structural

features, many different models have been proposed in the literature. We shall describe some of these models and present results of our modeling study of the PtR duplex.

In comparing shifts of PtD adducts, we note that the G* H(8) signals are unusually downfield relative to the G H(8) signals for G residues within the same duplexes. The 5′-G* H(8) signal at 8.73 ppm of PtR was downfield of the 3′-G* H(8) signal at 8.03 ppm (*Table 3*). This same chemical shift relationship has been observed in all other intrastrand cross-linked duplexes [62][71-74][77][107][114]. The generally accepted explanation for this shift pattern is that the two platinated bases are in a right-handed (R2) *HH*1 arrangement (*Fig. 6*) [48]. In duplexes with d(G*pG*) crosslinks, the 5′-G* H(8) signal is always ~8.7 ppm (*Fig. 12*), suggesting a similar orientation of this base regardless of the flanking sequence. The chemical shift of the 3′-G* H(8) signal (*Fig. 12*), however, is clearly affected by the 3′ flanking base and potentially the base 5′ to the 5′-G*. The 3′-G* H(8) signal is found to be less downfield when the sequence is 5′-(G*G*T)-3′ vs. 5′-(G*G*C)-3′ [62][71–73][77][107]. It has been calculated that the ring current of C is greater than that of T [115][116], and this ring-current difference may be the source of the more upfield 3′-G* H(8) chemical shift.

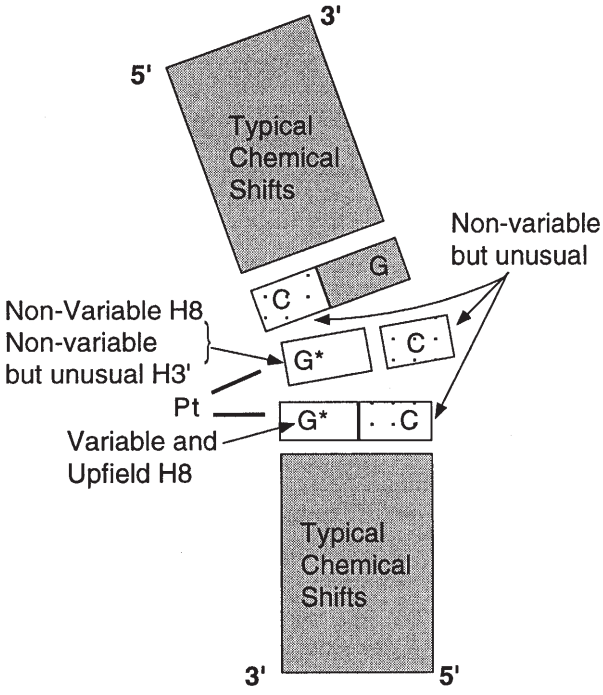

Fig. 12. *Diagram showing chemical shifts around Pt site*

Of the three PtD complexes with 5′-(G*G*T)-3′ sequences, two (PtW and PtL) have a T residue 5′ to 5′-G* and one (PtR) has a C. The 3′-G* H(8) signal of PtR is upfield of the analogous PtW and PtL signals by ~0.2 ppm, which might indicate that the base 5′ to 5′-G* influences the duplex structure in such a way as to affect the 3′-G* H(8) shift. However, the data are too limited to draw any definite conclusions.

Two lines of NMR evidence point to an N-sugar for the 5′-G*, a feature noted in earlier studies of PtR and in all studies of PtD adducts. First, a strong 5′-G*/A* H(8)-H(3′) NOE cross-peak indicates an N-sugar. This cross-peak, first noted for PtR, has also been reported for the other PtD adducts; it is the most often cited evidence of the N-sugar pucker [62][71][72][77][107][114]. Second, DQF-COSY coupling patterns in the H(1′)-H(2′)/(2″) region support the N-sugar pucker of 5′-G* for PtR (*Fig. 13*); H(1′)-H(2′)/(2″) coupling patterns (in either DQF-COSY data or 1D spectra) have been cited as additional evidence of the 5′-G* N-sugar for only three of the five other Pt-dsDNA complexes [72][107][114]. The duplex

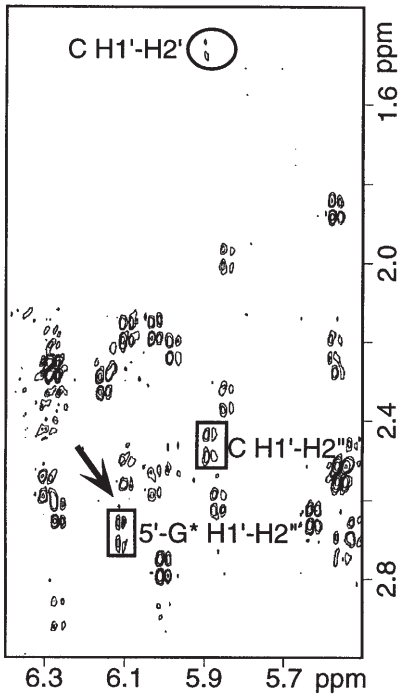

Fig. 13. *H(1′)-H(2′)/H(2″) region of the DQF-COSY spectrum of PtR*. The missing 5′-G* H(1′)-H(2′) cross-peak is indicated with an arrow and the weak C–H(1′)-H(2′) cross-peak is circled. The H(1′)-H(2″) cross-peaks for these same residues are boxed for comparison

PtM has these features of the N-sugar pucker. The N-sugar assignment of the 5′-G* of PtM has been confirmed by ^{13}C-NMR chemical shift data, the only Pt-dsDNA species so studied [107].

The NOESY and DQF-COSY data for PtR also indicate a high percentage of N character in the C sugar in the 5′-CG*-3′ sequence (*Fig. 13*); this assessment was not made in previous studies of this duplex [62][71][74][105]. On the basis of NOESY and H(1′)-H(2′)/H(2″) coupling constant (from DQF-COSY or 1D-NMR methods) data, the sugar of the base 5′ to 5′-G* has a high degree of N character in PtM and PtC [72][107] and is in an N/S equilibrium for PtK [114]. Although the pucker of this sugar was not explicitly stated, PtW models calculated using NOE restraints generated structures in which the T residue in the 5′-TG*-3′ sequence had an N-sugar [73]. Thus, the experimental data for PtW probably indicate that this T residue has a sugar with a large percentage of N character. This repuckering occurs in both 5′-CG*-3′ (PtR, PtM, PtC), 5′-TG*-3′ (PtW), and 5′-CA*-3′ (PtK) steps. No mention of this sugar pucker was made for PtL, and analysis of the PtL NOESY spectrum does not clearly indicate an N-sugar for the T residue in 5′-TG*-3′ [77]; however, NOESY data are only one line of evidence, and a clearer picture may be obtained through the use of COSY and ^{13}C data. Therefore, the presence of the N-sugar pucker for 5′-([C/T]G*)-3′ or 5′-CA*-3′ sequences seems to be a general feature in 1,2-intrastrand crosslinked adducts that is independent of the flanking sequences and influenced only slightly by the nature of the crosslink.

For the 5′-(CC)-3′ sequence complementary to 5′-(G*G*)-3′ for PtM and PtC, the NOESY data were consistent with a predominantly N-like and predominant S-sugar pucker for C(3′-G*) and C(5′-G*) (C's complementary to the 3′-G* and 5′-G*) [72][107]. NMR data indicated S-sugars for PtR. The C(3′-G*) and C(5′-G*) sugar puckers were both S for PtL and S and N, respectively, for PtW.

The C–H(2′) signal in the 5′-CG*-3′ sequence was upfield-shifted to 1.46 ppm in PtR (*Table 3*), as had been previously noted [62][71]; the normal shift range for H(2′)/H(2″) signals is 1.8–3 ppm [117]. A similar upfield shift (to 1.33–1.58 ppm) has been reported for the H(2′) signal of the base 5′ to 5′-G* in the other Pt-dsDNA adducts (*Table 2*) [72][73][77][107]. This shift was observed regardless of identity of the flanking base [72][73][77][107] and was attributed to shielding of this proton by the 5′-G* base [72][107]. For the PtK duplex, with the 1,2-intrastrand d(A*pG*) crosslink, an even higher upfield-shifted H(2′) signal (at 0.87 ppm) was attributed to shielding by 5′-A* in modeling studies [114]. Therefore, the fact that replacement of 5′-G* with the more anisotropic 5′-A* shifts the H(2′) signal for the 5′ flanking residue more strongly indicates that the cause of the upfield shift is shielding by the 5′-G*/A* base. This upfield-shifted H(2′)

signal has also been correlated to the N-sugar for the residue 5′ to 5′-G*/A* as described above. It was concluded from modeling studies that this H(2′) will point toward 5′-G* if the 5′-flanking residue has a N-sugar [72]. All six PtD sequences have this upfield H(2′) signal. In model structures of five of the six PtD sequences, this sugar has an N pucker, although some studies have also generated structures with an S-sugar to explain the experimental S/N equilibrium of this sugar [72][73][107][114]. It is likely that this sugar pucker change is found in all PtD adducts, at least as part of a major contributing conformation, since all PtD sequences have an upfield-shifted H(2′) signal for this residue.

The C(5′-G*) and C(3′-G*) in PtR had the most upfield-shifted base signals (*Table 3*). This result was generally true for PtM, PtW, PtL, and PtK, although these sequences had a signal overlapping the more downfield signal from the complementary C/T base (*Table 2*). For PtC, these CH(6) signals were not the most upfield shifted; eight other C/T H(6) signals were upfield of the more downfield signal of the complementary C [72]. The chemical shift difference between C(3′-G*) H(6) and C(5′-G*) H(6) was small for PtR (0.03 ppm), a finding also true for PtM, PtW, PtL, and PtK [73][77][107][114]. The separation of these CH(6) signals for PtC was ~0.18 ppm [72], much larger than for the other PtD sequences. The shift and separation of these signals probably reflect the composition of the sequences flanking the platination site. In PtR, PtM, PtW, PtL, and PtK, the CC sequence complementary to d(G*pG*) is preceded by a purine, whereas in PtC the sequence is preceded by T. Upfield-shifted H(6) and H(5) signals have been observed when C is preceded by A relative to when C is preceded by T [118]. The AT / GC base-pair ratio is roughly the same for the 5′-flanking sequence *vs*. the 3′-flanking sequence within a PtD sequence except for PtC where this ratio is 0 : 3 for the 5′-flanking sequence *vs*. 2 : 3 for the 3′-flanking sequence. Thus, the slight difference for PtC may also reflect altered duplex stability on one side of the platination site. Another potential explanation related to base content of PtC is that each strand has a 50 : 50 purine / pyrimidine ratio, whereas the other PtD sequences have only two purines in the platinated strand and only two pyrimidines in the unplatinated strand (*Table 1*).

The C–N(H_b) and N(H_e) signals in the 5′-CG*-3′ sequence of PtR were the most downfield-shifted C-amino signals (*Table 3*); this result was true for PtM, PtC, and PtK, which also have 5′-(C[G*/A*]G*)-3′ sequences [72][107][114]. The downfield shift of the C N(H_b)/N(H_e) signals may reflect a structural feature common to all PtD adducts, even though it is not possible to determine the shift for PtW and PtL on the basis of the same data since these sequences have a T residue in this position. Conversely, the C(5′-G*) N(H_b) and C(5′-G*) N(H_e) signals of PtR were the most upfield

C-amino signals. The analogous C N(H_b) signals of PtM, PtC, PtW, and PtL are also the most upfield observed for a non-terminal C base; PtK has a T(5′-A*) (T complementary to A*). No clear trend was observed for the C(5′-G*) N(H_e) signals in the PtD adducts. Interpretation of the CNH_2 chemical shifts is difficult since, in addition to shielding/deshielding effects of the surrounding sequence, hydrogen bonding also influences the chemical shifts.

Important evidence for the similarity of the PtD adducts is found in the downfield-shifted d(G*pG*) ^{31}P-NMR signal, observed for PtR, PtM, and PtC (^{31}P-NMR data not reported for PtW, PtL, and PtK). The ^{31}P assignment of PtR has been previously reported and was not repeated [62][74]. As discussed earlier, a downfield ^{31}P signal was observed for Pt-ssDNA and Pt-dsDNA adducts. For PtC and PtR, the downfield shift of the d(G*pG*) ^{31}P signal was interpreted as evidence of a α^t, γ^t conformation at the 3′-G* residue [62][72][74]. However, current models of PtR (see below) do not have *trans*-α-angles. Furthermore, a *trans*-α- or -ζ-angle is not typically observed in the X-ray structures [60]. Consequently, although the downfield shift of the d(G*pG*) ^{31}P-signal is a common observation for cisplatin-DNA adducts and this shift is observed in DNA, no convincing structural explanation for the shift has been offered so far.

Additional evidence for the similarity of the PtD adducts is the sharpness of the 3′-G* H(1) signal, indicating good base pairing, and the evidence of weaker base pairing by the 5′-G*. The evidence for this weakness includes the broadness of the 5′-G* H(1) signal for PtR, PtC, and PtM [72][106][107] and the absence of the 5′-G* H(1) signal for PtW or PtL [73][77]. The 5′-G* H(1) signal of PtR was the sharpest of the 5′-G* H(1) signals detected. For PtR (*Fig. 10*), PtC, and PtM, the 5′-G* H(1) signal is relatively sharp at low temperature but broad at 25 °C. The broadening or absence of this signal is due to exchange with water. PtK has an upfield-shifted T(5′-A*) H(3) signal at 12.5 ppm *vs.* a normal T H(3) signal between 13–15 ppm [119]. The 5′-A*·T base pair is not the weakest in PtK since in PtK melting studies, the G H(1) signal disappeared shortly before the T H(3) signal in 5′-(C·G)(A*·T)-3′ [114].

As discussed below, NMR-based models can easily accommodate the 3′-G*·C base pair, but the 5′-G*·C base pair is difficult to model. The NMR shifts are consistent with normal, if somewhat weak, base pairing. However, the rapid exchange with water and the difficulty in modeling might suggest an unusual tautomeric form for 5′-G*. Observation of the T(5′-A*) H(3) and 5′-G* H(1) signals suggests that the 5′-G*/A* base is not in an unusual tautomeric form. In summary, the 5′-G* H(1) and T(5′-A*) H(3) signals showed evidence of enhanced exchange consistent with weak base pairing for all sequences.

The broadness of the 5′-G* H(1) signal was coupled to weak NOE cross-peaks from 5′-G* H(1) for PtR, PtM, and PtC [72][107]. The PtW, PtC, PtL, and PtK samples were prepared in the presence of 3–50 mM phosphate, while desalted samples were used for PtR and PtM. Phosphate anion is known to be a good catalyst of NH-proton exchange [120]. Therefore, the sharper 5′-G* H(1) signals in PtR and PtM could be due to the absence of salt effects rather than to sequence-related structural effects.

A striking feature, not easily explained, of the 5′-G* H(1) signal (when observed) is its downfield chemical shift (*Table 3* for PtR). In fact, as mentioned above, the T(5′-A*) H(3) signal of PtK is ~12.5 ppm, upfield from the usual T H(3) shift region, was attributed to interconversion between two conformers, one of which lacked the 5′-A* N(1)-T(5′-A*) H(3) hydrogen bond [114]. Thus, if the 5′-G* H(1)·C N(3) *Watson-Crick* (*W-C*) hydrogen bond was weak or absent, the 5′-G* H(1) signal should be upfield. It is possible that 5′-G* H(1) is hydrogen bonded to a base on the unplatinated strand in a non-*W-C* fashion, as has been observed in other modeling studies [72][121]. It is difficult to rationalize the similar shifts observed for the other signals near the Pt binding site of all PtD species if some have the 5′-N* base paired while others do not.

In addition to shift patterns, it is possible to examine NOE cross-peak patterns for all six duplexes. The NOE intensity patterns suggested more distortion from the normal B-DNA geometry on the 5′-side than on the 3′-side of the platination site, 5′-([G*/A*]G*)-3′. On the platinated 5′-([C/T][G*/A*]G*)-3′ strand, there is no apparent dependence of the NOE intensity patterns on the sequence. Most importantly, sequential NOE cross-peaks from [C/T] H(1′)/H(2′) to 5′-G*/A* H(8) and from 5′-G*/A* H(1′)/H(2′) to 3′-G* H(8) were absent or relatively weak, although overlap in PtL obscures accurate NOE cross-peak intensity interpretation for the 5′-G* H(2′)-3′-G* H(8) NOE cross-peak; the 5′-G*/A* H(8) – 3′-G* H(8) NOE cross-peak was strong regardless of the sequence. On the non-platinated 5′-([C/T][G/A])-3′ strand (complementary to 5′-([C/T][G*/A*])-3′), the [C/T] H(2′)-[A/G] H(8) NOE cross-peak is relatively weak for all sequences. Overall, the NOE cross-peak intensities around the binding sites are very similar.

All aspects of NMR studies (similar NOE cross-peak patterns, unusual ^{1}H- and ^{31}P-NMR chemical shifts, imino-proton exchange patterns for all sequences (including a d(A*pG*) cross-link) suggest strongly that the main structural features should be the same for all six duplexes. Modeling studies, however, provide differing interpretations of these NMR observations.

Duplex Adducts: NMR-Based Molecular Modeling

The molecular models in the literature differ in the mode of base pairing, base stacking, backbone conformation, pucker of some sugars, and the out-of-plane distortions of the Pt–N(7) bond. There is also a conflict in these modeling studies about whether the adducts adopt one or more than one conformation. The reader can conclude that it is difficult to account for the results using normal structural features in the models. In this discussion, we often refer to one model. However, it must be recalled that in all cases, the duplex is dynamic. Any one model represents a compromise designed to reflect the average 'equilibrium' position of a species undergoing limited movements. In such cases, small populations of different conformers, such as species with disrupted base pairs involved in DNA 'breathing' are thought to exist but to have only a minimal effect on the experimental observations. In a few cases, investigators have concluded that a combination of models is necessary. These each undergo the normal limited dynamic motion. However, in these cases, the conformers exist in a high percentage, have very distinct structures, and interchange rapidly. In this section, after first describing some literature results, we focus mostly on our recent modeling studies on the PtR duplex, employing a combination of NOE-restrained energy-minimization (EM) and molecular-dynamics (MD) calculations. We also compare the modeling results for PtR to results from other modeling studies.

Early PtR models were derived from unrestrained energy-minimization calculations. These belong to two main families (*Fig. 14*), which have unkinked and kinked helices [121–123]. Since experiments established kinking, structures in the unkinked model family were not considered. Later, an additional kinked family of models was generated in studies modeling PtC [72]. The main structural difference between the kinked families was the stacking or non-stacking of the C bases complementary to the G* bases [72]. These two classes are referred to here as parallel (LL) or non-parallel (NP) (*Fig. 14*) and are used to characterize the PtR models. It should be noted that some modeling studies have concluded that equilibria exist between conformers, represented by LL and NP models.

An unrestrained molecular-mechanics approach was used for PtC and PtK, and the resulting models were evaluated on the basis of energy, chemical shifts, coupling constants, and a small number of NOE cross-peaks [72][114]. An important conclusion of the PtC modeling study was that only a combination of conformers from both the LL and NP families explained all the experimental NMR data, while the PtK study concluded that the data was best fit by two models in the LL family (see below). An approach based solely on NOESY data was used for PtW and PtL [73][77], resulting in one

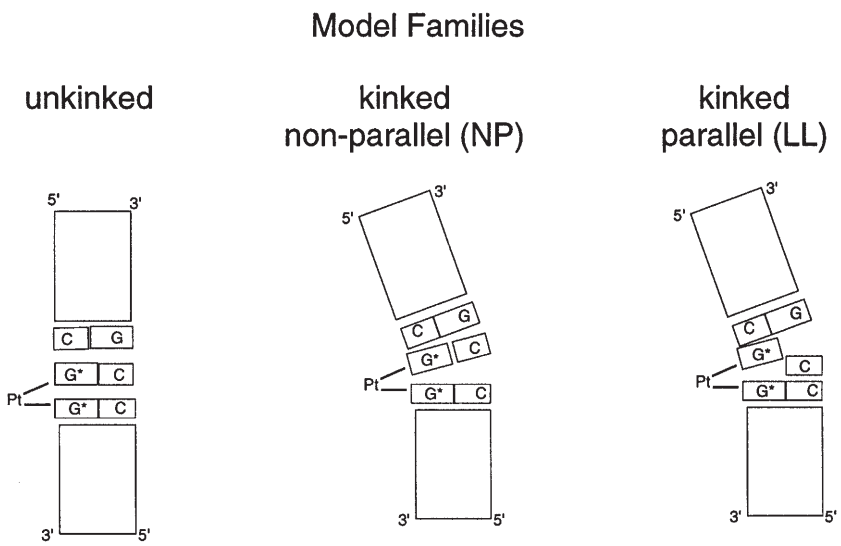

Fig. 14. *Representation of base stacking in the different classes of calculated model families*

model or model family for each that best fit the NOESY data as evaluated by *R*-factors and simulated NOESY spectra. The proposed PtW family of models and PtL model are in the NP class. The PtW model gave a low NOE *R*-factor but provided no explanation for the upfield shift of the T H(2′) signal in the 5′-(TG*G*)-3′ sequence or for the absence of the 5′-G* H(1) signal since the 5′-G*·C base pair had all three *W-C* hydrogen bonds in the model. The PtL model also did not explain the upfield shift of the T H(2′) signal in the 5′-(TG*G*)-3′ sequence but had no hydrogen bond to the 5′-G* H(1) [77]. In both the PtW and the PtL modeling studies, the minor groove was found to be shallower and wider than for normal B-DNA. For PtW, this minor groove opening accompanied the deepening and narrowing of the major groove.

In the one modeling study of the d(A*pG*) crosslink, the T(5′-A*) residue remained stacked on the neighboring 5′-C residue in the two preferred models that the authors felt explained the H(2′) shielding, corresponding to an LL model [114]. The T(5′-A*) H(3) signal was upfield but observable, indicating hydrogen bonding of this proton and no unusual tautomer for the 5′-A* base. In one model, T(5′-A*) H(3) was hydrogen bonded to 5′-A* N(1), but the T(5′-A*) O(4) was hydrogen bonded to CNH_2 in 5′-(CA*)-3′ [114]; in the other model, T(5′-A*) H(3) was not hydrogen-bonded, but the T(5′-A*) O(4)-5′-A* NH_2 hydrogen bond was maintained. An equilibrium between a direct NH_3-phosphate and a water-mediated NH_3-phosphate hydrogen bond was suggested on the basis of the experimentally determined

S/N equilibrium for the C sugar in 5′-(CA*)-3′. In previous modeling studies, a change from direct to water-mediated NH_3-phosphate hydrogen bonding was found to change the sugar pucker of the residue 5′ to 5′-G* [72].

We turn now to consider recent work on PtR and discuss here only the four most representative PtR models, 1-4, along with a model, Model 4bi, derived from Model 4 with different hydrogen bonding of the base pairs (*Table 4*). All of the PtR models have helical kinks as determined experimentally (*Fig. 15* for the representative Model 4bi). These models fall either within the LL family (1) or between the NP and LL families with mainly LL (2) or NP (3, 4, and 4bi, *Fig. 16*) features. Since the key structural chang-

Table 4. *Shift, Slide, and Ring-Current Effect Values for Models 1–4bi of PtR*

Model	Shift [Å]	Slide [Å]	5′-G* Ring-current effect [ppm]	Minor-groove width [Å]
1	–0.21	–1.29	+0.02	10–12
2	–0.02	–0.58	+0.4	10–10.5
3	0.12	0.31	+0.45	11–13
4	0.22	1.72	+1.15	11–12
4bi	0.16	1.63	+0.94	11–12
B-DNA	0	–0.76		

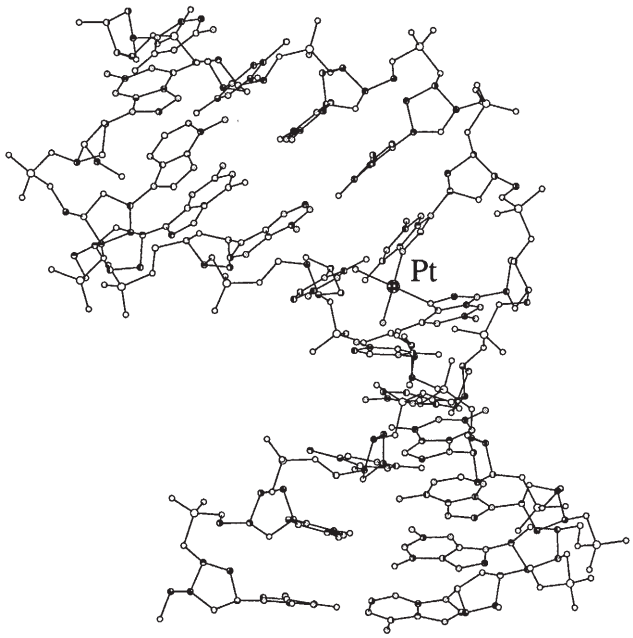

Fig. 15. *Model 4bi of the PtR duplex*

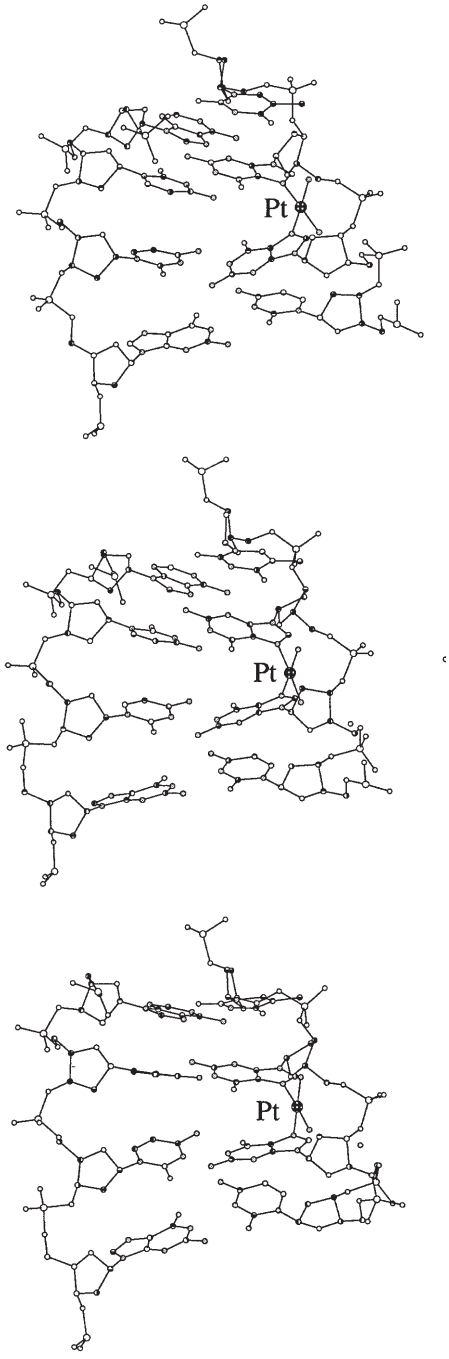

Fig. 16. *Partial Model 3* (top), *Model 4* (middle), *and Model 4bi* (bottom) *of the PtR duplex showing the 5′-(CG*G*T)-3′ region*

es to the duplex involve the 5′-G*·C base pair, several different stacking arrangements between this 5′-G*·C base pair and the C·G base pair 5′ to the 5′-G* were considered. We hoped to find an explanation for, among other observations, the upfield shift of the C H(2′) signal and the unusual C-5′-G* and C(5′-G*)-G interresidue NOE cross-peak intensity patterns. For this reason, other restraints in addition to NOE and *W-C* hydrogen-bond restraints were used. The 5′-(C·G/5′-G*·C)-3′ base-pair stacking was modified by adding (Model 4 and 4bi) or not adding (Models 1, 2, and 3) restraints that kept C H(2′) in the shielding cone of the five-membered ring of the 5′-G* base. The 5′-(C·G/5′-G*·C)-3′ base-pair stacking was also altered by forming direct hydrogen bonds between the 5′-NH_3 and 5′-(CpG*G*)-3′ phosphate group (Models 2 and 3), or by restraining a water molecule between these two groups (Models 1, 4, and 4bi); the latter approach also allowed us to assess water-mediated hydrogen bonding. Models 1-4 have all three 5′-G*·C *W-C* hydrogen bonds, while in Model 4bi, two of the three normal *W-C* hydrogen bonds (5′-G* H(1)-C(5′-G*) N(3) and 5′-G* N(H_b)-C(5′-G*) O(2)) are present as well as a bifurcated hydrogen bond, C(5′-G*) N(H_b) to 5′-G* O(6) and 3′-G* O(6).

The 5′-G* H(8) signal was downfield of the 3′-G* H(8) signal in PtR as well as in the other d(G*pG*) duplexes, suggesting that the 3′-G* H(8) is shielded by the 5′-G* base. In this arrangement, the twist or rotation of the 3′-G* base relative to the 5′-G* base has the same direction as in a right-handed helix. This relative positioning of the 5′-G* and 3′-G* bases is accurately reflected in Models 1–4bi of PtR as well as the PtC, PtM, PtW, PtL, and PtK models (*Table 5*) [72][73][77][107][114]. The strong 5′-G* H(8)-3′-G* H(8) NOE cross-peak indicated an *HH* arrangement of the bases in PtR. The backbone should have the propagation direction in *HH*1 since, in the *HH*2 form originally identified with (*R,S,S,R*)-BipPt(d(GpG)) [54], the G* bases would be extruded and G* H(1) solvent exposed. However, NOE data in H_2O indicate interstrand NOEs between the exchangeable G* H(1) and the complementary C's, suggesting that the *HH*1 form is the major *HH*1 form present in PtD with intrastrand crosslinks. The NOE-estimated 5′-G* H(8)-3′-G* H(8) distance is in the range of 3.0–3.4 Å. Although the distance (3.6 Å) is slightly long in Model 1 of PtR, Models 2, 3, 4, and 4bi have H(8)-H(8) distances in the correct range. This distance is suitably short in the other favored models of PtD duplexes (*Table 5*) [72][73][77][107][114].

On the basis of ring-current effects calculated using standard methods [116], we find that C H(2′) is well shielded by the 5′-G* base in the 5′-(CG*)-3′ sequence in Models 4 and 4bi and partially shielded by the five-membered ring of the 5′-G* base in Models 2 and 3 (*Table 4*); Model 1 has minimal shielding of this H(2′). The main parameters describing relative base-pair positioning are slide, shift, and twist (*Fig. 17*). There is a reduced

(relative to B-DNA) helical twist in all models and a positive increase in slide value going from Model 1 (–1.29 Å) through to Model 4 (1.72 Å) (*Table 4*). Thus, with a more positive slide value, C H(2′) is shielded. The calculated ring-current effects suggest that Model 4 shields C–H(2′) too much, while Models 2 and 3 provide shielding more in keeping with experimental observations. However, slight decreases in the slide and shift lessen the

Table 5. *Comparison of Explanations of Experimental Data by the PtD Models for 5′-(X·Y)(G*·C)-3′* [a])

Model	X H(2′) shielded by 5′-G*	X N-sugar[b])	X S-sugar[b])	Weak X H(1′)/H(5′) G* H(8) NOE[c])	Weak X H(2′)/H(5′) G* H(8) NOE[c])	5′-G* H(1) signal shift observable	Weak C H(2′)-Y H(8)NOE
PtR[d])							
2	+[e])	++	–	++	–	++	–
3	+	++	–	++	–	++	–
4	++	++	–	–	+	++	+
4bi	++	++	–	–	+	++	+
PtC[f])							
NP_{0N}	+	++	–	++[g])	–	++	–
NP_{0S}	–	–	++	++	+	++	–
NP_{I}	+	++	–	++	–	++	–
LL_{0N}	+	++	–	++	–	–	–
LL_{0S}	–	–	++	++	+	–	–
LL_{1A}	++	++	–	++	+	–	–
LL_{1B}	+	++	–	++	–	–	–
PtW	+	++[h])	–	++	–	++	–
PtL	–	–[h])	++	++	?	–	–

[a]) All models considered have appropriate kink, correct G* H(8) chemical-shift order, short G* H(8)-G* H(8) distance, 5′-G* N-sugars. This table focuses on experimental/structural features that differ between studies.
[b]) These observations are related and no model explains both observations simultaneously.
[c]) These observations are related and no model explains both observations simultaneously.
[d]) PtR models give very similar results to PtM models, which are not included in this table.
[e]) ++ Indicates model explains well, + indicates model partially explains, and – indicates model does not explain.
[f]) Number indicates number of NH_3-phosphate hydrogen bonds, and N or S indicates sugar pucker of residue 5′ to 5′-G*. 1A and 1B differ in location of sugar of residue 5′ to 5′-G* relative to 5′-G* base.
[g]) This cross-peak was not observed for PtC and the distance between protons is probably long in models.
[h]) The internucleotide NOEs T H(2′)/H(2″) to 5′-G* H(8) suggest an S-sugar for PtW and an N-sugar for PtL; however, models proposed have N-sugar for PtW and S-sugar for PtL. The intranucleotide NOE T H(6)-H(3′) suggests an S-sugar for PtL; this NOE is in overlap for PtW.

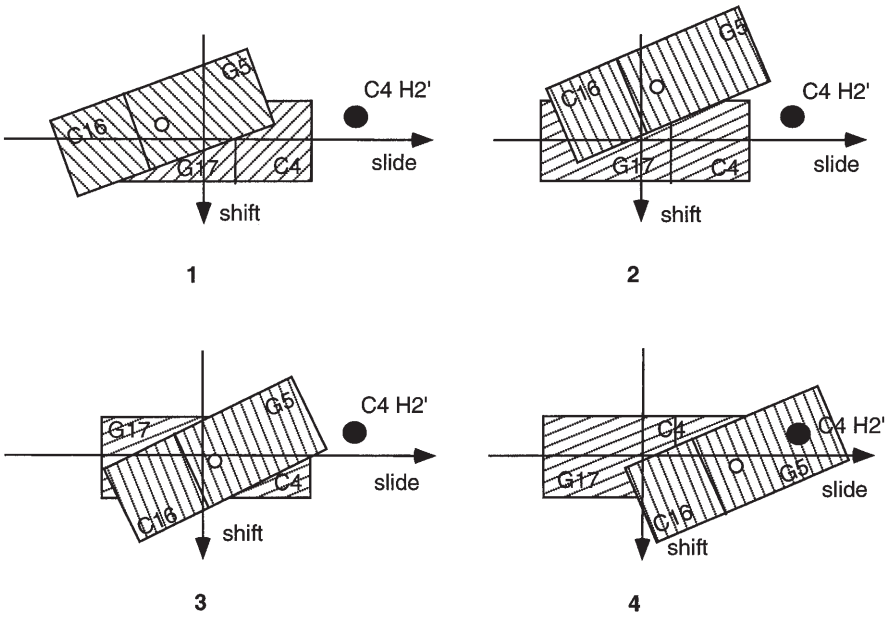

Fig. 17. *Schematic representation of PtR Models 1–4 showing the C·G/5′-G*·C base pairs as a function of slide and shift and how well CH(2′) is shielded by 5′-G* in each model.* The 5′-G*·C base pair is on top. The arrows indicate the direction of positive shift and slide changes.

shielding in Model 4bi relative to Model 4. Although the ring current most likely causes the CH(2′) shielding, the shielding may have another, less obvious cause. There appears to be no one clear cause for the positive slide, although positive slide may help minimize steric interactions between the 5′-NH_3 and the C residue. As mentioned above, Pt adducts exhibit many established unusual features. Also, many unusual features are not understood, even in simple models. Therefore, it is quite likely that modeling directed at explaining the features of six PtD species using already known structural forces in normal (unplatinated) DNA may fail to explain fully the Pt-dsDNA features. As yet unrecognized interactions, which are unprecedented, may be influencing the structure.

The upfield-shifted H(2′) signal was a general observation for all sequences. Therefore, our modeling suggests that, regardless of the bases in the intrastrand crosslink and the surrounding sequence, conformers with increased positive slide and reduced helical twist of the 5′-G*·C base pair should be the major conformers for all PtD adducts in solution. The ring-current shielding of this CH(2′) in PtC models was reported as 0 to 0.95 ppm [72]. This information suggests a positive slide in the structures with the

higher shielding, but coordinates allowing us to calculate the shielding were not reported. The two favored PtK models both shield this C H(2′) proton, but no calculated ring-current effects were reported [114]. Interestingly, the PtW model, which belongs to the NP family, has a negative slide (–0.58) and shift (–0.97 Å) for the corresponding 5′-(T·A/5′-G*·C)-3′ stack. Furthermore, the 5′-(T·A/5′-G*·C)-3′ stack in the PtL X-ray structure, which also belongs to the NP family, has large negative values for both slide and shift (–1.59 and –1.51 Å, respectively). We calculate ring-current effects [116] of 0.35 and 0.12 ppm for PtW and PtL, respectively; these values are less than those experimentally observed. Thus, these models do not suggest an explanation for the upfield shift of the H(2′) signal (*Table 5*).

The 5′-G* H(1) signal was observed for PtR (*Fig. 10*), PtC, and PtM [72][107], suggesting 5′-G* H(1) is involved in hydrogen bonding. In Models 1–4, 5′-G* H(1) is hydrogen-bonded to C(5′-G*) N(3) in a normal *W-C* base pair. Well formed 5′-G*·C *W-C* base pairs were present in NP PtC models with zero or one NH_3-phosphate hydrogen bond (*Table 5*) [72]. In LL family PtC models with zero or one NH_3-phosphate hydrogen bond, none of the usual 5′-G*·C *W-C* hydrogen bonds are present. Instead, two bifurcated hydrogen bonds connecting C(5′-G*) O(2) with 5′-G* H(1) and 5′-G* NH_2, and connecting C(3′-G*) N(H_b) with 5′-G* O(6) and 3′-G* O(6) exist [72]. Thus, the proposed LL/NP equilibrium of conformers for PtC would lead to a potentially weaker 5′-G* H(1)-C(5′-G*) N(3) hydrogen bond. Although no 5′-G* H(1) signal was observed, the 5′-G* H(1)-C(5′-G*) N(3) hydrogen bond is present in the PtW model [73]. The PtL solution model does not have 5′-G* H(1) involved in any hydrogen bonding [77]. However, the NMR data and trends for the PtD duplexes, including an d(A*pG*) adduct, suggest similar structures for all adducts. It may be that the 5′-G* H(1)-C(5′-G*) N(3) hydrogen bond is intact while the two other C groups, instead of forming the 5′-G*·C *W-C* hydrogen bonds, form hydrogen bonds bifurcated between the 5′-G* and flanking bases as in Model 4bi.

CN(H_b)/CN(H_e) Signals in the 5′-(CG*)-3′ sequence are the most downfield of the amino signals. Either strong hydrogen bonding or deshielding by nearby residues could account for this downfield shift. Deshielding sources are not so easily evaluated as shielding sources. Deshielding could result from this group being in a base plane. However, other than the G complementary to this C, there are no other bases appropriately positioned in the PtR models. The C(5′-G*) N(H_b) signal is slightly upfield shifted. Again, evaluating the source of this apparent upfield shift is difficult. This atom is not clearly in the shielding cone of a nearby base, although it is close to the C base in the 5′-(CG*)-3′ sequence in Model 4. It could be that the C(5′-G*) NH_b-5′-G* O(6) hydrogen bond is weaker than in the other G·C base

pairs or that C(5′-G*) N(H_b) is bifurcated as in Model 4bi. Alternatively, this CNH_2 group may not be involved in hydrogen bonding at all, which would shift the signals upfield, and what is actually being observed is a downfield shift of the non-hydrogen-bonded C(5′-G*) 'NH_b' signal. Since neither the PtR models nor any published models clearly indicate the source of the C amino shifts, these shifts cannot be used to evaluate the models.

As described above, a distinctive NOE cross-peak pattern was observed around the platination site for PtR and the other PtD duplexes. Thus, this pattern is characteristic of the crosslink and suggests that the conformations of the PtD sequences are all closely related. These NOE intensities were used to evaluate the models. The sequential CH(1′)-5′-G* H(8) NOE cross-peak was very weak for PtR. The corresponding distance is long (4.9–5.2 Å), but in the NOE-detectable range in Models 1, 2, and 3; this distance is too long (6.3 and 6.1 Å) in Models 4 and 4bi, respectively. This NOE cross-peak was not observed for PtC, and the models contain a long C H(1′)-5′-G* H(8) distance [72]. This distance is 5.2 Å in the PtW model, in good agreement with the experimental data [73]. Although the coordinates for the PtL model have not been released at this time, this distance appears to be suitably long in this model as well [77].

In PtR, the CH(2′)-5′-G* H(8) NOE cross-peak is weaker than other sequential H(2′)-H(8)/H(6) NOE cross-peaks; the equilibrium distance is probably toward the upper end of the experimentally estimated 3.2–4.0 Å range. This CH(2′)-5′-G* H(8) distance is of appropriate length only in Models 4 and 4bi (~3.6 Å) (*Table 5*). The strength of this NOE cross-peak corresponds well with the CH(2′) shielding by the 5′-G* base; only when 5′-G* is positioned to shield this H(2′) is the distance appropriately large. This CH(2)′-5′-G* H(8) distance is shorter when this C residue has an N-sugar. The PtR experimental data indicate that this sugar has a high percentage of N character but is not completely N. Thus, there is possibly at least one conformer of PtR present with a more S-like sugar, which would increase this average distance. This CH(2′)-5′-G* H(8) distance ranges from 2.47 to 3.84 Å in PtC models. The PtC models with a longer distance have an S-sugar for this C residue and minimal shielding of this CH(2′), in poor agreement with the observed upfield shift [72]; however, these S-sugar models were not the PtC models preferred by the authors. The T H(2′)-5′-G* H(8) distance in the PtW model is 2.8 Å, while the T H(2″)-5′-G* H(8) distance is 3.7 Å, even though this latter NOE cross-peak is more intense than the former. However, this NOE intensity discrepancy, which is also a problem for the PtR models, may reflect the need to explain experimental data with multiple conformers having different sugar puckers for this residue, as discussed above. The T H(2′)-5′-G* H(8) distance is probably suitably long

in PtL because this T residue has an S-sugar in the model. However, this S-sugar does not properly position T H(2)′ for shielding by 5′-G*.

On the non-platinated strand, the 5′-3′ sequential C(5′-G*) H(2′)-G H(8) NOE cross-peak was very weak for PtR. This distance is quite short in all PtR models, 2.4–3.8 Å. The weakness of this C(5′-G*) H(2′)-G H(8) NOE cross-peak could be due to a duplex structure very different from any model discussed here. Both the NP and LL PtC models have distances from 2.42 to 3.06 Å, which would probably result in NOE cross-peaks more intense than those observed experimentally. In the PtW model, the corresponding C(5′-G*) H(2′)-A H(8) distance is 3.0 Å, too short for the observed weak NOE cross-peak [73]. This distance also appears to be short in the PtL models [77]. Thus, although experimental data indicate a large separation of the C(5′-G*) H(2′) and G H(8) atoms, the force fields used in modeling calculations brings these two moieties close together.

On the non-platinated strand, no C(5′-G*) H(6)/H(5)-G H(8) NOE cross-peaks were observed experimentally. The corresponding C(5′-G*) H(6)/H(5)-G H(8) distances for PtR models are < 5 Å in Model 1 and > 5 Å in Models 2, 3, 4, and 4bi. The distances were not given for the PtC and PtL models. In the PtW model, the C(5′-G*) H(6)/H(5′)-G* H(8) distance is 4.5 Å, while the C(5′-G*) H(5)/H(5′)-G* H(8) distance is 5.9 Å.

As described earlier, a downfield-shifted ^{31}P signal has been attributed to a *trans* (180°) value for the d(G*pG*) α or ζ torsion angles [104] or narrowing of the O–P–O diester angle [103]. In Models 1-4bi of PtR, the d(G*pG*) α and ζ torsion angles all fall within the normal *gauche*$^-$ (*ca.* –60°) range. The d(G*pG*) O–P–O diester angles are all wider (~104°) in the PtR models than the normal B-DNA value (101.5°). Thus, the source of the downfield ^{31}P signal is not readily determined. Although the literature contains no ^{31}P data for PtW or PtL, we believe both duplexes would give the normal downfield ^{31}P d(G*pG*) signal. The PtW model has *gauche*$^-$ α and ζ angles and an O–P–O diester angle of 103.4° [73]; these parameters would not lead to a downfield shift of the ^{31}P signal. The PtL solution structure has a *trans* ζ angle for d(G*pG*), which would explain the downfield ^{31}P d(G*pG*) signal. However, this structure also has *trans* α and ζ angles for phosphate groups in parts of the duplex where no unusual ^{31}P signals are typically observed [77]. Thus, no published model accounts for reported or expected ^{31}P data for PtD duplexes.

In the PtK adduct, 5′-A* H(2)-G NH_2 (G in 5′-T(5′-A*)G-3′ on the non-platinated strand) and 5′-A* NH_2-C(3′-G*) NH_2 NOE cross-peaks indicated the close proximity of these moieties. In the PtM sequence, NOEs in H_2O were observed between the CNH_2 groups of C(5′-G*), C(3′-G*), and C in 5′-CG*-3′ [107]. The CNH_2 groups that have NOEs in PtM are within NOE-

detectable distance in the PtR models, as are the moieties analogous to the PtK sequence that give NOEs in H_2O, although there are no H(2) and observable NH_2 signals for 5′-G* in PtR. This result points again to the similarity of the conformation of the PtD sequences.

For PtR, the large out-of-plane distortion of the 5′-G* N(7)–Pt bond is the most noteworthy shortcoming of Model 4bi, compared to the other models (Models 3 and 4) that account well for the ^{1}H-NMR data. The PtL X-ray structure also had substantial out-of-plane displacement (~1 Å) of the Pt from the 5′-G* base plane [124][125]. However, in Models 3 and 4 of PtR, there is much less out-of-plane distortion of the Pt (0.6–0.7 Å). As mentioned earlier, ^{195}Pt-NMR data indicate no substantial distortions around Pt when coordinated to a duplex DNA [97]. Thus, we believe the out-of-plane distortion of the Pt–N(7) bond is probably much smaller in solution than that found in the PtL X-ray study. The distortion in the models may arise from the limitations of the force field.

Conclusion

As the reader can judge from *Table 5*, no reported model satisfactorily accounts for all the results. To rationalize this limited success we advance the following hypotheses:

Hypothesis I: Unprecedented structural feature(s) present in PtD duplexes in solution remain to be discovered.

Points in support of *Hypothesis I*: *1*) Numerous unprecedented structural features are already well accepted for PtD duplexes. *2*) Many unexplained spectral features have been found for other Pt adducts. *3*) Extremely unusual spectral features found for a hairpin with an intrastrand crosslink can be explained only in part by unprecedented structural components. *4*) Unique, in some cases unpredicted, structures have been found even recently in some small d(G*pG*) complexes containing CCC diamines. (5) The 5′-G* H(1) signal is intense in PtR and PtM and has a downfield shift; however, 5′-G* H(1) undergoes rapid water exchange. *6*) The ^{1}H- and ^{31}P-NMR signals do not shift significantly with temperature below the melting point, suggesting that one form is present. *7*) The similar spectral features in the PtD duplexes are most easily rationalized if only one major conformer is present.

Hypothesis II: Substantial populations of two interconverting PtD conformers exist.

Points in support of *Hypothesis II*: *1*) No one model with ‘standard’ d(G*pG*) features can account for the experimental results. *2*) A mixture of conformers with the 5′-G* H(1) hydrogen-bonded to N(3) of the comple-

mentary C in both conformers could explain the 5′-G* H(1) spectral features. *3*) A mixture of forms has been identified in an adduct of a PtD duplex with an HMG protein [12][61]. *4*) Evidence is growing that d(G*pG*) intrastrand crosslinked adducts are highly dynamic, and it is reasonable that such a dynamic nature might facilitate exchange between mixtures of significantly different conformers. *5*) The absence of shifting of NMR signals could be rationalized since conformers with nearly equal population would have nearly the same energy, and the equilibrium would probably not be temperature dependent.

Hypothesis III: The known force fields, designed to reproduce typical DNA structures, fail to meet the challenge presented by accommodating NMR restraints from a distorted duplex with an intrastrand crosslink.

Points in support of *Hypothesis III*: *1*) Most models are unable to account for the weak C(5′-G*) H(2′)–G H(8) NOE cross-peak unless special restraints are included (*Table 5*). *2*) The force field does not account explicitly for water, and a charged Pt moiety should alter the water structure. *3*) The modeling assumes one correlation time, and the d(G*pG*) moiety may lead to a greater divergence than normal in the correlation times of various protons in the duplex.

In our opinion, the most satisfactory conclusion from the NMR-based modeling work is that there is essentially one conformer regardless of sequence. We believe this conformer has features which have not been found in other DNA's and which cannot be easily modeled. Therefore, numerous related models have been proposed to fit the data obtained for different PtD duplexes under various experimental conditions. Thus, additional efforts to define the relationship between NMR-spectral features and adduct conformation are needed.

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^{195}Pt- and ^{15}N-NMR Spectroscopic Studies of Cisplatin Reactions with Biomolecules

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The direct detection of ^{195}Pt- and ^{15}N-NMR signals was widely used in early studies of reactions of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) with various biomolecules. The ^{195}Pt chemical shift is sensitive to the nature of the bound donor atoms as well as to geometrical isomerism and chiral centres. Both ^{15}N chemical shifts and $^{1}J(^{195}\text{Pt-}^{15}\text{N})$ coupling constants can be used to identify the *trans*-ligands in Pt ammine and amine complexes. The direct detection of ^{195}Pt- and ^{15}N-NMR is limited by their low sensitivity. The use of inverse detection (^{1}H-detected ^{15}N) can greatly improve the sensitivity of ^{15}N, and at the same time simplify the spectrum. The combined detection of ^{1}H and ^{15}N in an inverse 2D heteronuclear single (or multiple) quantum coherence (HSQC or HMQC)-NMR experiment is especially useful for the detection of low concentrations of intermediates formed during reactions of platinum complexes with biomolecules under physiological conditions. NMR studies of the activation of cisplatin and related complexes, DNA platination, reactions with amino acids, peptides and proteins are discussed in this chapter.

Introduction

The high efficacy of cisplatin in the treatment of several types of cancers has made it the most widely used anticancer drug. The use of ^{195}Pt- and ^{15}N-NMR spectroscopy has made a major contribution, along with other methodology, in the understanding of its molecular mechanism of action, including the detection of intermediates in reactions with DNA, amino acids (proteins) and metabolites in body fluids [1]. In particular the use of inverse detection combined with pulsed-field gradients has allowed some cisplatin chemistry and that of related platinum anticancer complexes to be elucidated under physiologically relevant conditions [2][3]. In this chapter, we describe the use of ^{195}Pt- and ^{15}N-NMR methods, with emphasis on the application of 2D [^{1}H,^{15}N]-NMR, for investigations of cisplatin activation, metabolism, DNA and protein binding.

Cisplatin. Edited by Bernhard Lippert

^{195}Pt- and ^{15}N-NMR Spectroscopy

^{195}Pt-NMR

^{195}Pt is a reasonably sensitive nucleus for NMR detection, with natural abundance of 33.8%, nuclear spin quantum number $I = {}^1/_2$, and a receptivity relative to ^{1}H of 3.4×10^{-3}. The limit of detection (*ca.* 10 mM) precludes detection of natural abundance ^{195}Pt signals in physiological fluids. The receptivity can be improved by a factor of three by isotopic enrichment of ^{195}Pt (> 95%). The spin-lattice relaxation time (T_1) for ^{195}Pt is usually in the range of 0.3 to 1.3 s.

The ^{195}Pt chemical-shift range is very large, about 15,000 ppm (usually in the range from –600 to 9000 ppm relative to $[PtCl_6]^{2-}$), and often allows easy differentiation between Pt^{II} and Pt^{IV}, which tend to have chemical shifts at the high-field and low-field ends of the range, respectively. The ^{195}Pt chemical shift in monomeric complexes is sensitive primarily to the set of bound donor atoms, see *Table 1*. Some caution is required in searching for peaks, because the shifts of Pt^{IV} halides alone span 12000 ppm. Also, usually there are ^{195}Pt chemical-shift differences between geometrical isomers and between diastereomers (chiral ligands). For ^{15}N-enriched ligands, the splitting pattern in the ^{195}Pt spectrum indicates the number of coordinated non-equivalent ^{15}N atoms. These characteristics of the ^{195}Pt chemical shift can be utilized in the detection of different intermediates formed during the reactions of platinum complexes with biomolecules. Sometimes even isotopomers are distinguishable: the ^{195}Pt isotope shift difference for ^{195}Pt-$^{35/37}$Cl is 0.17 ppm and for ^{195}Pt-$^{79/81}$Br is 0.03 ppm [4]. Therefore in principle it is possible to count the number of Cl and Br ligands bonded to Pt *via* the isotope-splitting pattern. In practice it is difficult to resolve because of line broadening, which is usually due to either relaxation

Table 1. *^{195}Pt Chemical Shifts of* cis*-Pt Adducts with Different Donor Atoms*

cis-Pt Complexes	$\delta(^{195}Pt)$ range [ppm] [a]	References
cis-$[PtCl_2(NH_3)_2]$	–2149	[22]
cis-$[Pt(NH_3)_2(O)_2]$	–1460 to –1598	[52][53]
cis-$[PtCl(NH_3)_2(O)]$	–1806 to –1841	[52]
cis-$[Pt(NH_3)_2(N)(O)]$	–2067 to –2147	[53]
cis-$[PtCl(NH_3)_2(N)]$	–2297 to –2369	[22][54][55]
cis-$[Pt(NH_3)_2(N)_2]$	–2434 to –2660	[22][52][53]
cis-$[Pt(NH_3)_2(S)(O)]$	–2618 to –2800	[31][56]
cis-$[Pt(NH_3)_2(S)(N)]$	–2800 to –3218	[31][56]
cis-$[Pt(NH_3)_2(S)_2]$	–3200 to –3685	[31][56]

[a]) Relative to Na_2PtCl_6.

mechanisms or poor temperature control of the sample. The latter is a problem because of strong temperature dependence of ^{195}Pt-NMR resonances (0.5 to 1.1 ppm K^{-1}).

The quadrupolar effects of natural abundance ^{14}N (*i.e.*, 99.6% ^{14}N, I = 1) from ammines which coordinate to Pt can broaden the ^{195}Pt resonances. Such quadrupolar effects of ^{14}N have the beneficial effects of shortening the ^{195}Pt relaxation times and allowing rapid pulsing without saturation effects. ^{195}Pt-^{14}N couplings in ^{195}Pt-NMR spectra are usually better resolved at higher temperature because of the decreased quadrupolar relaxation rate of ^{14}N, due to the decrease in correlation time. Even in the absence of ^{14}N ligands, ^{195}Pt resonances can still be very broad owning to chemical shift anisotropy (CSA) relaxation, which can be the dominant relaxation mechanism for platinum complexes at high magnetic-field strength. Similarly, ^{195}Pt satellites in ^{15}N and ^{1}H spectra of Pt^{II} complexes are often broadened beyond detection owing to CSA relaxation of ^{195}Pt [5]. The linewidths of ^{195}Pt satellites of ^{1}H-NMR resonances are dependent on the spin-lattice relaxation time of ^{195}Pt:

$$\Delta\nu_{1/2}(\mathrm{H}) = [\pi T_2^{*}(\mathrm{H})]^{-1} + [2\pi T_1(\mathrm{Pt})]^{-1}$$

where $[\pi T_2^{*}(\mathrm{H})]^{-1}$ is the natural linewidth plus the contribution from magnetic inhomogeneity broadening (measurable from the linewidth of the centre peak). The contribution to ^{195}Pt T_1 relaxation from CSA is given by:

$$[T_1(\mathrm{Pt})]^{-1}(\mathrm{CSA}) = (6/7) \times [T_2(\mathrm{Pt})]^{-1}(\mathrm{CSA}) = (2/15) \times \gamma_{\mathrm{Pt}}^2 \times B_0^2 \times \Delta\sigma^2 \times \tau_c$$

In general, ^{195}Pt satellites (and ^{195}Pt resonances) are sharper in Pt^{IV} complexes which are six coordinate and hence more symmetrical (smaller anisotropy $\Delta\sigma$), and are broader at higher fields of measurement (B_0) and in larger molecules (longer correlation time τ_c).

^{15}N-NMR

^{14}N-NMR spectroscopy can be useful for ammine and amine complexes, but ^{14}N is a quadrupolar nucleus, and quadrupolar relaxation is dominant when the environment of ^{14}N has a low symmetry. This can lead to very broad lines and a consequent reduction in sensitivity. On the other hand, short relaxation times also have the advantage of allowing rapid pulsing so that a large number of transients can be acquired. Thus it is possible to follow reactions of cisplatin in blood plasma and cell-culture media at millimolar drug concentrations and to detect ammine release [6].

By using ^{15}N-substituted ammine complexes, the broadening of ^{195}Pt signals caused by the quadrupolar effects of ^{14}N can be avoided. Both ^{15}N-

NMR chemical shifts and $^{1}J(^{195}Pt\text{-}^{15}N)$ coupling constants are sensitive to the nature of the *trans*-ligand in Pt ammine and amine complexes, which can provide useful information for identifying the ligands in the coordination spheres of both Pt^{II} and Pt^{IV} complexes. Typical ^{15}N and ^{1}H shift ranges for Pt^{II}-NH, Pt^{II}-NH_2 and Pt^{II}-NH_3 and $^{1}J(^{195}Pt\text{-}^{15}N)$ values are shown in *Fig. 1* and *Fig. 2*, respectively. In general, ligands with high *trans*-influences give rise to smaller $^{195}Pt\text{-}^{15}N$ coupling constants (S < I < Br < Cl < H_2O) and cause a low-field shift of the ^{15}N resonance. The dominant contribution to one-bond coupling constants between ^{195}Pt and ^{15}N is usually interpreted in terms of the Fermi contact interaction involving Pt 6s and N 2s orbitals [7]. The usefulness of $^{1}J(^{195}Pt\text{-}^{15}N)$ values is limited by the difficulty in determining them for larger molecules especially at high observation frequencies on account of the dominance of relaxation *via* chemical-shift anisotropy [2]. The $^{1}J(^{195}Pt\text{-}^{15}N)$ values for Pt^{IV} are smaller by a factor of about 1.5 (in theory based on the change in hybridization from dsp^2 to d^2sp^3) to 1.2 (in practice) and are $1.4\times$(*i.e.*, $\gamma^{15}N/\gamma^{14}N$) larger than $^{1}J(^{195}Pt\text{-}^{14}N)$ values.

The low receptivity of ^{15}N (3.85×10^{-6} relative to ^{1}H) limits to some extent its usefulness for directly-detected ^{15}N-NMR studies of Pt ammine and amine complexes. However, the sensitivity of detection can be improved

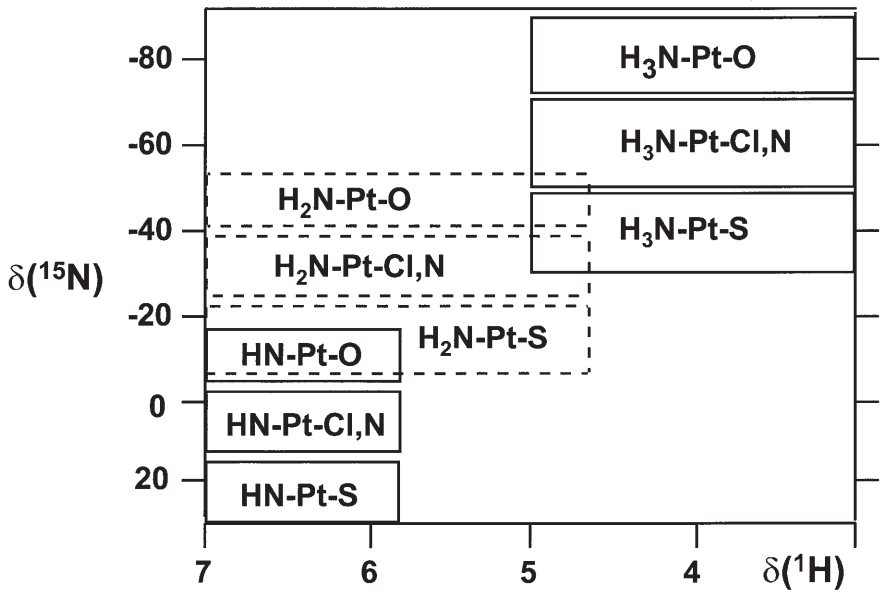


Fig. 1. *Variation of ^{1}H- and ^{15}N-NMR chemical shifts with the* trans-*ligand in Pt^{II}-NH, Pt^{II}-NH_2 and Pt^{II}-NH_3 complexes*

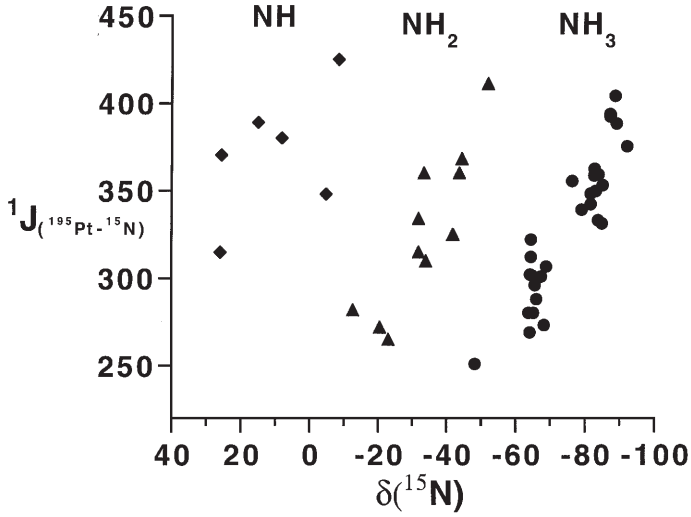


Fig. 2. *Plot of* $^{1}J(^{195}Pt\text{-}^{15}N)$ vs. $\delta(^{15}N)$ *for Pt-NH*$_3$*, Pt-NH*$_2$ *and Pt-NH, showing a similar dependence on the* trans*-ligand.* Data are taken from [12][59–62].

by ^{15}N isotopic enrichment combined with enhancement by polarization transfer from ^{1}H (*e.g.*, ^{15}N-{^{1}H} DEPT and INEPT pulse sequences). The maximum enhancement in ^{15}N signal intensity achievable *via* polarization transfer is only 9.8× (*i. e.*, $\gamma^{1}H/\gamma^{15}N$), which means that inverse (^{1}H-detected) ^{15}N methods are usually preferred due to the superior enhancement for ^{15}N-detection (*vide infra*). The repetition time of the pulse sequence is governed by the ^{1}H rather than the longer ^{15}N spin-lattice relaxation time (T_1), which is an additional advantage because it allows more rapid pulsing. For example, ^{15}N-{^{1}H} DEPT sequences enable detection of rapidly-changing intermediates in the reaction of ^{15}N-cisplatin with glutathione [8], and also ammine release following reaction of ^{15}N-cisplatin with intracellular components in intact red blood cells at concentrations as low as 0.1 mM [8]. Direct ^{15}N-{^{1}H} DEPT/INEPT methods can be of value in situations where ^{1}H-NMR resonances are very broad.

Inverse Detection Methods

The sensitivity of ^{15}N can be greatly improved by the use of inverse detection methods (^{1}H-detected ^{15}N), by a theoretical maximum of 306 $\{(|\gamma H|/|\gamma N|)^{5/2}\}$ with respect to directly detected ^{15}N (*Fig. 3*), such that signals can be detected in aqueous solutions at concentrations of physiological relevance (5 μM). ^{1}H-Detected inverse methods are applicable to any

system which contains a ^{15}N atom with a measurable spin-spin coupling to ^{1}H (*i.e.*, $^{1}J(^{15}N, ^{1}H)$ in ammine, primary and secondary amines, but not tertiary amines although a longer range coupling can sometimes be utilized). In practice the best applications are for those systems with large one-bond couplings (*e.g.*, *ca.* 73 Hz for $^{15}NH_3$). Besides the high sensitivity, inverse detection also brings a simplification of complicated spectra because it detects only those protons directly attached to the labelled ^{15}N atoms in the sample. This is very important for investigations of ^{1}H-NMR spectra of body fluids or cell culture media which consist of thousands of overlapping resonances.

Although ^{1}H-NMR resonances can be detected from NH protons with ^{14}N present in natural abundance (99.6%), they are often broad because of the quadrupolar relaxation of ^{14}N ($I = 1$). It is also necessary to work in H_2O (as opposed to D_2O), since NH protons in platinum ammine and amine complexes usually exchange with deuterium within minutes. The exchange of NH protons with solvent is much faster for Pt^{IV} than for Pt^{II} complexes at

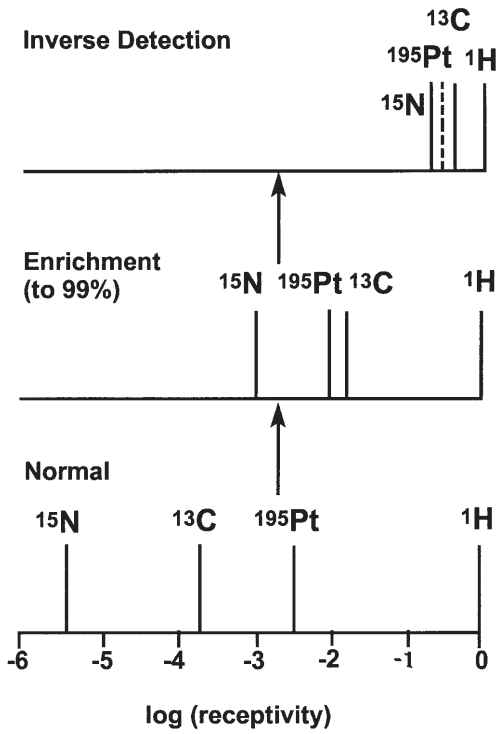


Fig. 3. *The theoretical increase in receptivity (*abundance × sensitivity*) obtainable by isotope enrichment and inverse ^{1}H detection of ^{13}C, ^{15}N and ^{195}Pt.* In practice, inverse ^{1}H-{^{195}Pt} detection is limited by the broad linewidths of the ^{195}Pt satellites.

neutral pH, since, for example, NH_3 ligands on Pt^{IV} have lower pK_a values. Introduction of ^{15}N by synthetic labelling usually gives rise to a sharp ^1H-NMR doublet for a Pt-^{15}NH group in H_2O together with (CSA-broadened) ^{195}Pt satellites. The resonances move progressively to lower field on changing from Pt-NH_3, to Pt-NH_2 to Pt-NH, and Pt^{IV}-^{15}NH and ^1H-NMR resonances for Pt^{IV}-NH are to lower field of those for Pt^{II} (see *Fig. 1*). Pt^{IV} anticancer complexes can be studied by the ^1H-{^{15}N}-NMR if the NH exchange is slowed down by lowering the pH or by other means.

[^1H,^{15}N]-NMR Spectroscopy

The Pt-^{15}NH protons can be detected selectively by the use of heteronuclear single (or multiple) quantum coherence (HSQC and HMQC) pulse sequences. A 1D ^1H spectrum containing only resonances from Pt-^{15}NH species is obtained by acquiring only the first increment in a two-dimensional experiment; resonances for CH and OH (including water) are eliminated. This is particularly useful in studies of body fluids or cell culture media, where only the signals from platinum complexes are detected and thousands of other overlapping ^1H resonances are filtered out. If ^{15}N decoupling is employed during acquisition (*e.g.,* the GARP method), then each type of Pt-NH resonance appears as a singlet, sometimes together with broadened ^{195}Pt satellites. In practice the water resonance is so intense that it is usually necessary to use additional solvent suppression techniques (*e.g.* presaturation). The addition of an H_2O T_2 relaxation agent (*e.g.*, 0.5M $(NH_4)_2SO_4$) can also be helpful to detect NH peaks very close to the H_2O peak. A large improvement in water suppression is achieved by the use of pulsed field gradients for coherence selection, for example, by use of the HSQC sequences of *Stonehouse et al.* [9]. We have been able to detect NH peaks within a few Hz of the water resonance at concentrations as low as about 10 μM without the need for additional solvent-suppression techniques.

The combined detection of ^1H and ^{15}N in a 2D inverse NMR experiment is especially powerful, since both the ^{15}N-NMR chemical shift (*Table 2* and *Fig. 1*) and the one-bond coupling constant $^1J(^1H\text{-}^{15}N)$ (*Fig. 2*) are diagnostic of the *trans*-ligand. As shown in *Fig. 4*, the ^{195}Pt satellites (when not broadened beyond detection by the effects of CSA relaxation) in a 2D [^1H,^{15}N] spectrum appear as diagonal peaks which correspond to the $^2J(^{195}Pt\text{-}^1H)$ coupling constant in the $F_2(^1H)$ dimension and to the $^1J(^{195}Pt\text{-}^{15}N)$ coupling in the $F_1(^{15}N)$ dimension. Pt^{II} and Pt^{IV} ammine and amine complexes can be distinguished by the combination of ^1H and ^{15}N shifts.

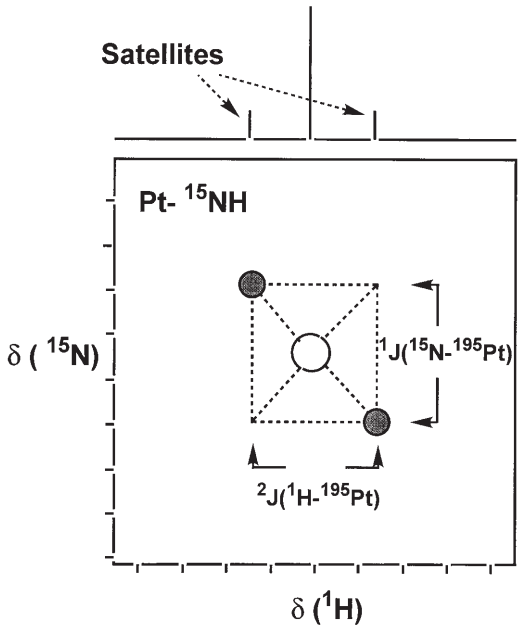


Fig. 4. *General appearance of a 2D [^{1}H, ^{15}N] HMQC or HSQC spectrum.* The ^{195}Pt satellites are usually more intense for symmetrical Pt species (Pt^{IV} rather than Pt^{II}).

Table 2. *Variation of ^{15}N-NMR Chemical Shifts with the* trans*-Ligand in Pt-NH_3, NH_2 and NH Complexes*

trans-Ligand	δ(^{15}N) Range [ppm] [a])	References
NH_3-Pt-O	–75 to –90	[41][44]
NH_3-Pt-N or Cl	–55 to –70	[41][44]
NH_3-Pt-S	–40 to –50	[41][44]
NH_2-Pt-O	–40 to –50	[20][32][42][57]
NH_2-Pt-N or Cl	–25 to –35	[20][32][42][50]
NH_2-Pt-S	–5 to –15	[20][32][42][50]
NH-Pt-O	–10	[32]
NH-Pt-N or Cl	10	[32]
NH-Pt-S	30	[32]

[a]) The ^{15}N chemical shifts of NH_2-Pt and NH-Pt are mainly based on ring-closed $\{Pt(en)\}^{2+}$ and $\{Pt(dien)\}^{2+}$ complexes.

Activation of Cisplatin

The mechanism of action of cisplatin is believed to involve activation *via* hydrolysis inside cells where the Cl^- concentration is much lower (*ca.* 4 mM) than outside cells (*ca.* 100 mM) [10]. Pt^{II}-OH_2 bonds are more reac-

Cisplatin Transplatin

tive towards DNA (*e.g.*, guanine N(7)) than either Pt-Cl or Pt-OH bonds [11]. Therefore, it is very important to determine the hydrolysis rates and pK_a values of the hydrolysis products. By observing the variation of δ_N *trans* to water/hydroxide with pH, the pK_a values of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ and *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ have been determined by ^{15}N-NMR spectra [12]. Direct observation of ^{15}N peaks requires the use of high concentrations of Pt (*ca.* 100 mM). For $[Pt(H_2O)_2(NH_3)_2]^{2+}$ oligomerization is a problem, and the detection had to be carried out at 278 K. By the use of [^{1}H,^{15}N] HMQC spectroscopy, rapid measurement at low concentrations (mM) allowed accurate determination of their pK_a values (*Fig. 5*) [13]. By observation of ^{1}H- and ^{15}N-NMR chemical shifts as a function of pH, the pK_a val-

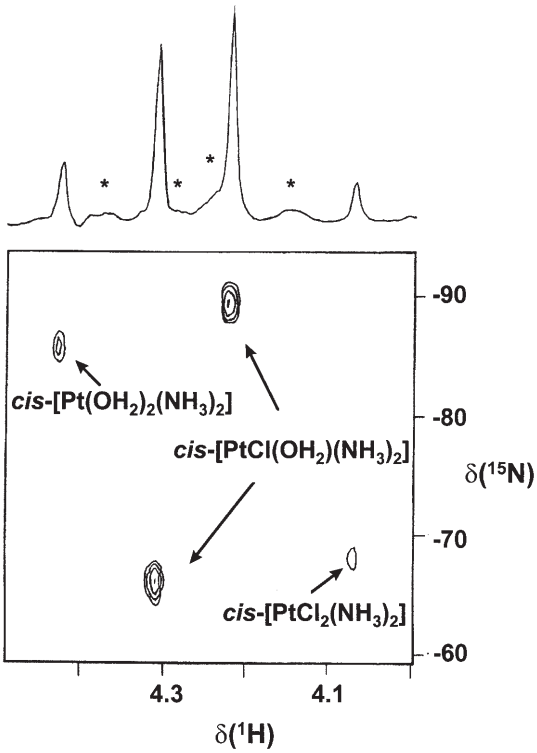
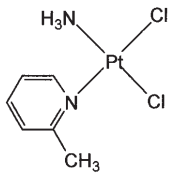


Fig. 5. *[^{1}H, ^{15}N] HMQC-NMR Spectrum of a 5 mM solution containing cisplatin and its hydrolysis products in 95% H_2O/5% D_2O, pH 4.72, 300 K.* ^{195}Pt Satellites are marked with an asterisk, $^2J(^1H\text{-}^{195}Pt)$ *ca.* 64 Hz. (Adapted from [13]).

ues of the monoaqua and diaqua adducts were determined to be 6.41, and 5.37 and 7.21, respectively. In principle, the pK_a values of aqua ligands on any Pt^{II} ammine or (primary or secondary) amine complex can be determined by the same method [14][15], and any hydroxo-bridged species which forms during the course of the reaction can be detected. Such information is valuable in understanding structure-activity relationship because of the reactivity of bound aqua ligands but inertness of hydroxo ligands.

The time-dependence of the [^{1}H,^{15}N] 2D-NMR spectra can also be used to determine the hydrolysis rates for each individual chloride ligand in the dichloride complex and in the monoaqua monochloro complex, by fitting the curves of the concentration changes of each species with time [14]. The NMR spectrum of ^{15}N-cisplatin in water at 310 K for 40 h (at equilibrium) contains [^{1}H,^{15}N] resonances assignable to unreacted cisplatin, the monoaqua and diaqua adducts in a ratio of 0.64:0.35:0.01, respectively, from which an equilibrium constant of 2.72 for the first stage of cisplatin hydrolysis was calculated [13].

The sterically-hindered anticancer complex *cis*-$PtCl_2(NH_3)$(2-methylpyridine) (AMD473) is now on clinical trial [16]. By ^{15}N-labelling the NH_3 ligand, the two monoaqua and one diaqua adducts can be clearly distin-



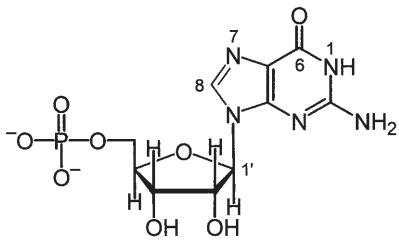
AMD473

guished in 2D [^{1}H,^{15}N] HSQC-NMR experiments [14]. The hydrolysis rates for each chloride ligand and the pK_a values of the monoaqua and diaqua adducts have been determined. Compared with cisplatin, both the slow hydrolysis and the dominance of inert hydroxo species under intracellular conditions may contribute to the greatly reduced reactivity of this complex.

Reactions with Nucleotides and DNA

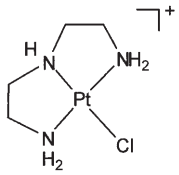
Using ^{15}N-edited one-dimensional ^{1}H-NMR spectroscopy and 2D [^{1}H,^{15}N] HMQC-NMR spectroscopy, reactions between ^{15}N-labelled *cis*-$PtCl_2(NH_3)_2$ and guanosine 5′-monophosphate (5′-GMP) have been studied in aqueous solutions [17]. The short-lived aqua-chloro intermediate is detectable during the early stages, followed by the formation of the mono- and

bis-GMP adducts. The large low-field shift of the NH_3 ^{1}H-NMR resonance for *cis*-$[Pt(GMP)_2(NH_3)_2]^{2+}$ was notable.



5′-GMP

From the combined pH and temperature dependence of Pt-NH ^{1}H-NMR shifts, hydrogen-bonding interactions were suggested between Pt-NH_3 and the deprotonated 5′-phosphate of GMP. Similar behaviour was also observed with $\{Pt(en)\}^{2+}$ adducts of GMP and AMP [18]. The stereospecific H-bonding is the strongest for NH protons held rigidly in a chelate ring (*e.g.*, ethylenediamine (en)), when the phosphate is fully deprotonated, and the rotation about Pt-N(7)(*head-to-tail* isomerism) is slow, as in the case of $[Pt(en)(5'\text{-AMP-}N7)_2]^{2+}$. ^{195}Pt-NMR signals for two slowly interconverting '*head-to-tail*' rotamers for $[Pt(en)(5'\text{-AMP-}N(7))_2]^{2+}$ were observed by *Reily et al.* [19]. Pt-NH-5′-phosphate H-bonding is detectable for pGpG adducts but not for GpG adducts [20]. In the model monofunctional DNA adduct $[Pt([^{15}N_3]dien)(5'\text{-GMP-}N(7))]^{2+}$, all the NH protons of dien in the 2D



$[Pt(dien)Cl]^+$

$[^1H,^{15}N]$ HSQC-NMR spectrum are magnetically non-equivalent (*Fig. 6*), which has been attributed to H-bonding interactions involving Pt-NH and the 5′-phosphate and C(6)O carbonyl groups of GMP [21]. These interactions may be important in the stabilization of adducts of *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with guanine bases in GG oligonucleotides.

With ^{195}Pt-NMR spectroscopy, the kinetics and mechanism of binding of cisplatin and its inactive *trans*-isomer to fragments of chicken erythrocyte DNA (*ca*. 40 base pairs) have been investigated by *Bancroft et al.* (*Fig. 7*) [22]. Both cisplatin and transplatin bind to DNA by two successive pseu-

do-first-order processes, forming monofunctional adducts (^{195}Pt chemical shift near –2300 ppm) that subsequently become bifunctional lesions (chemical shift near –2450 ppm). The rate constants for the first DNA binding steps are similar to the rate of hydrolysis of the first chloride ion of *cis*-DDP and *trans*-DDP in the solution. The monofunctional adducts are predominantly at N(7) of guanosine and retain a chloride ligand. The rate constants for macrochelate ring closure of the monofunctional adducts are similar to those for the second hydrolysis step of cisplatin, which appeared to indicate that the loss of chloride is the rate-limiting step in bifunctional chelate for-

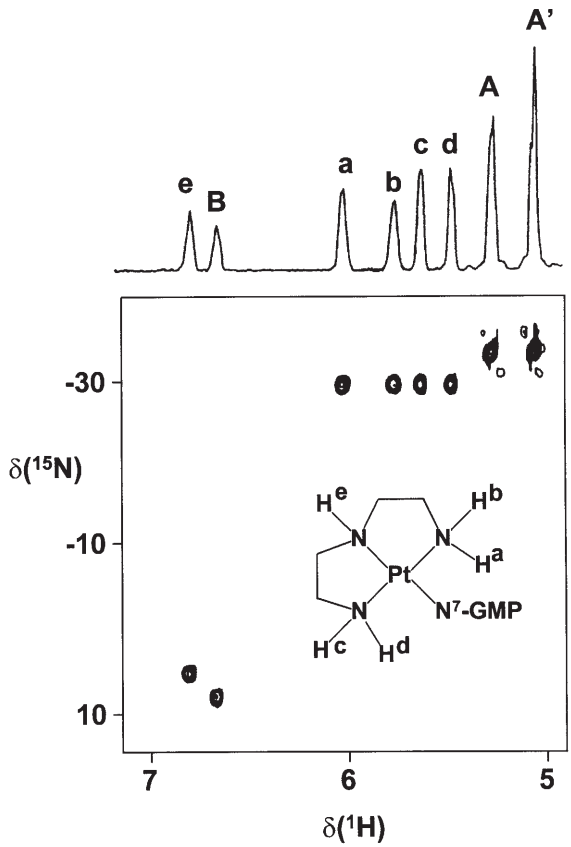


Fig. 6. *Two-dimensional [^{1}H,^{15}N] HSQC-NMR spectrum from the reaction of [Pt([$^{15}N_3$]dien)Cl]$^+$ with 5′-GMP recorded 1.2 h after mixing.* NH_2 and NH peaks for [Pt([$^{15}N_3$]dien)Cl]$^+$ are labelled as A, A′ and B, respectively, and for the GMP adduct labelled *a–e* (all five NH protons are non-equivalent). The large downfield shift of peak *a* is notable. ^{195}Pt Satellites in both the ^{1}H and ^{15}N dimensions are evident for [Pt([$^{15}N_3$]dien)Cl]$^+$ but not for the GMP adduct (satellites broaden with increase in molecular size and chemical-shift anisotropy). (Adapted from [21]).

mation. The linewidths of the ^{195}Pt-NMR resonances increase from *cis*-DDP to the mono and then bifunctional adducts. This is because of an increase in nuclear quadrupolar relaxation resulting from ^{14}N coordination, with the increased rotational correlation time due to the attachment of platinum to large DNA fragments, and the presence of a variety of local magnetic environments [22].

Even with enriched ^{195}Pt (to 97.28%) and high concentrations of DNA oligomers (*ca*. 14–32 mM), aquated cisplatin could not be detected by ^{195}Pt-NMR during the course of DNA reactions [22]. The detailed kinetics for the reaction of ^{15}N-cisplatin with the decamer oligonucleotide d(ACATGGTACA) and with the duplex containing the complementary strand have been investigated [23]. The major species in the pathways of platination of both single- and double-stranded GG oligonucleotides by ^{15}N-cisplatin can all be detected simultaneously by [^{1}H,^{15}N]-NMR spectroscopy (*Fig. 8*). This has allowed a direct determination of the lifetime of the aqua-chloro intermediate (8 min at 310 K) which was present at only micromolar concentrations. Kinetic data (*Fig. 9*) obtained by [^{1}H,^{15}N]-NMR spectroscopy are in close agreement with those determined by ^{195}Pt-NMR spectroscopy [22].

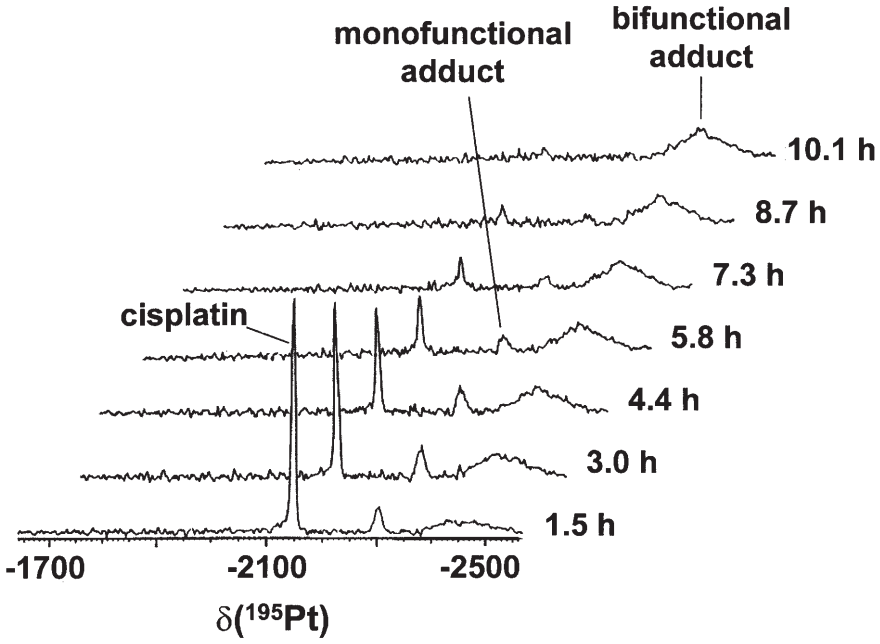


Fig. 7. *Time-dependent ^{195}Pt-NMR spectra of the reaction between* cis*-DDP and chicken erythrocyte DNA at 37 °C in 3 mM NaCl, 1 mM NaH_2PO_4, at a drug-to-nucleotide-ratio (D/N) of 0.07.* (Adapted from [22]).

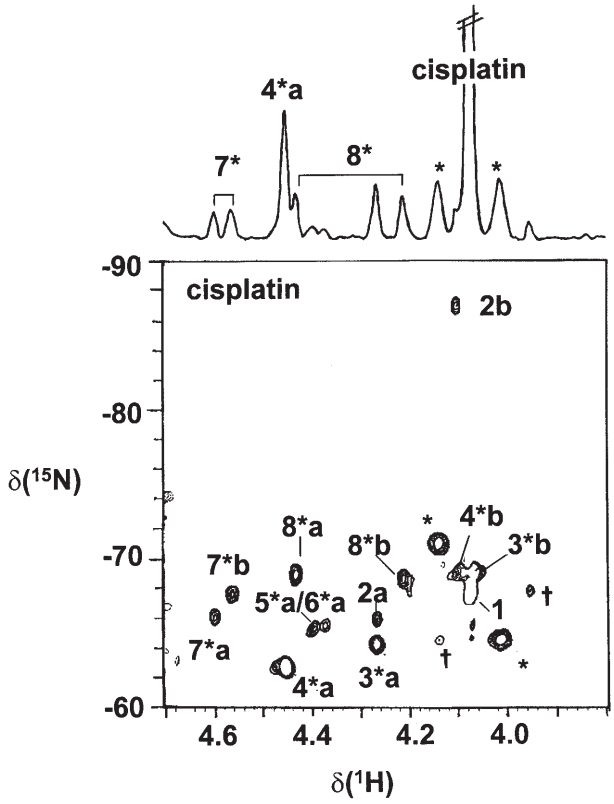


Fig. 8. *2D [^{1}H,^{15}N] HSQC-NMR spectrum (500 MHz) at 298 K of 14-mer duplex d(ATACATGGTACATA)·d(TATGTACCATGTAT) after reaction with [^{15}N]cisplatin for 8 h.* Labels: *: ^{195}Pt satellites, †: artefact. Peaks are assigned: *cis*-[$PtCl_2(NH_3)_2$] (1); *cis*-[$PtCl(H_2O)(NH_3)_2]^+$ (2); *cis*-[$PtCl(N(7)G(7))(NH_3)_2$] (3); *cis*-[$PtCl(N(7)G(8))(NH_3)_2$] (4); *cis*-[$PtCl(N(7)G(18/25))(NH_3)_2$] (5/6); *cis*-[$Pt(N(7)G(7)N(7)G(8))(NH_3)_2$] (7, 8: distorted and kinked forms). (Adapted from [24]).

In both single strands containing GG sequences and the corresponding DNA duplexes, NMR studies show that one of the two G's is platinated faster than the other (by a factor of ca. 4) [23][24], in line with the findings of *Chottard*, *Kozelka* and co-workers using HPLC methods [25]. Remarkably, ring closure on the duplex to form the GG chelate occurs about an order of magnitude faster for one monofunctional adduct than for the other. The long-lived monofunctional adduct, which was later characterized as a 5′-G monofunctional species [26], has distinctive ^{1}H- and ^{15}N-NMR chemical shifts, and the Pt-Cl bond may be protected from hydrolysis by the duplex. In contrast, the two monofunctional adducts on the GG single strand undergo ring closure at about the same rate.

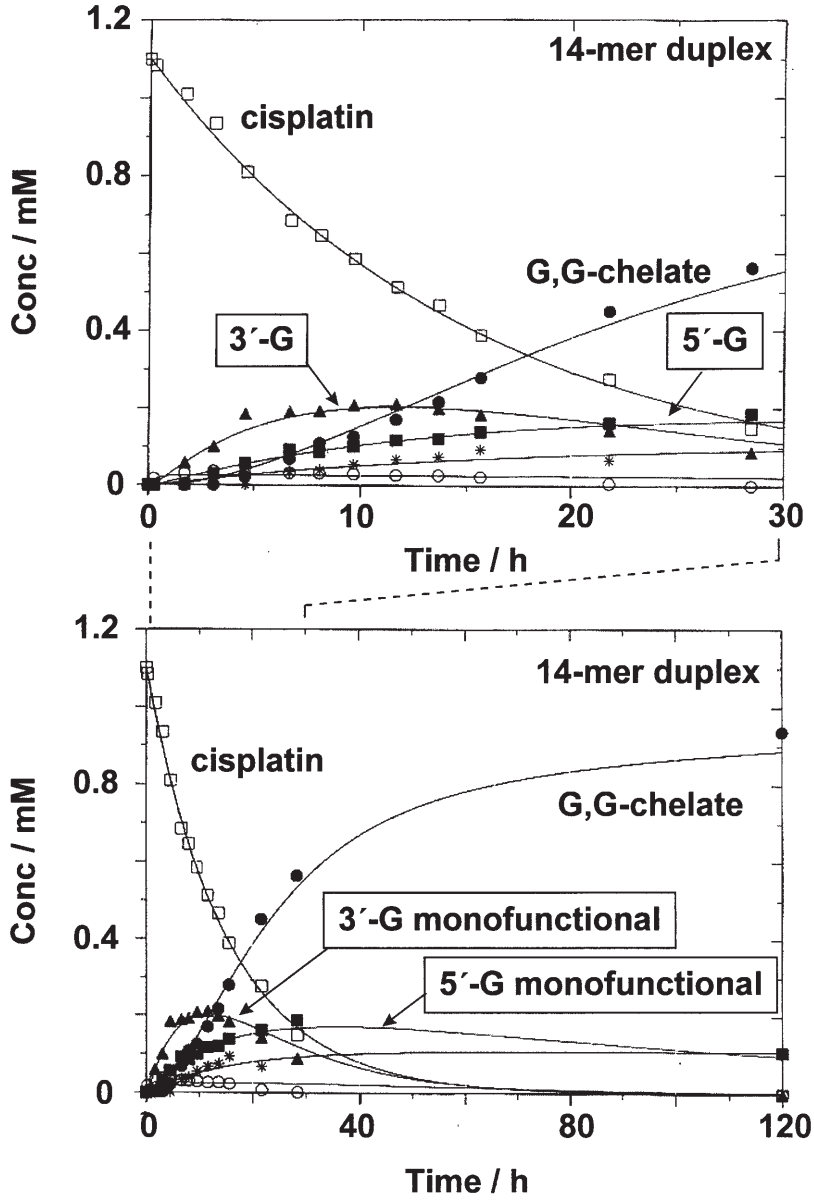


Fig. 9. *Plots of relative concentrations of species observed during reactions at 298 K of 14-mer duplex d(ATACATGGTACATA)·d(TATGTACCATGTAT) with [^{15}N]cisplatin.* Labels according to *Fig. 8*. For the purpose of the fit, the sum of the concentrations of the two forms of the GG chelate 7 + 8 was used. (Adapted from [24]).

The [^{1}H,^{15}N]-NMR shifts of a duplex platinated with cisplatin to give a bifunctional GG adduct are sensitive to duplex melting [23]. A large low-field shift of one of the Pt-NH_3 resonances is observed just after the duplex melts, which suggests that a platinated single strand still possesses some secondary structure.

The DNA-binding properties of *cis*-[$PtCl_2(NH_3)(C_6H_{11}NH_2)$], a metabolite of an orally active Pt^{IV} anticancer drug [27], have been studied by NMR. The geometry at platinum for the two orientational isomers of d(GpG) adduct was deduced by a double-labelling experiment, in which both the ammine and the N(7) position of the 3′-guanine base were ^{15}N labelled. ^{15}N-^{15}N coupling (6 Hz) was observed in ^{15}N{^{1}H}-NMR spectra for the isomer with labelled ammine ligand *trans* to the labelled 3′-base, but not for the isomer with cyclohexylamine ligand *trans* to the 3′- base (*Fig. 10*).

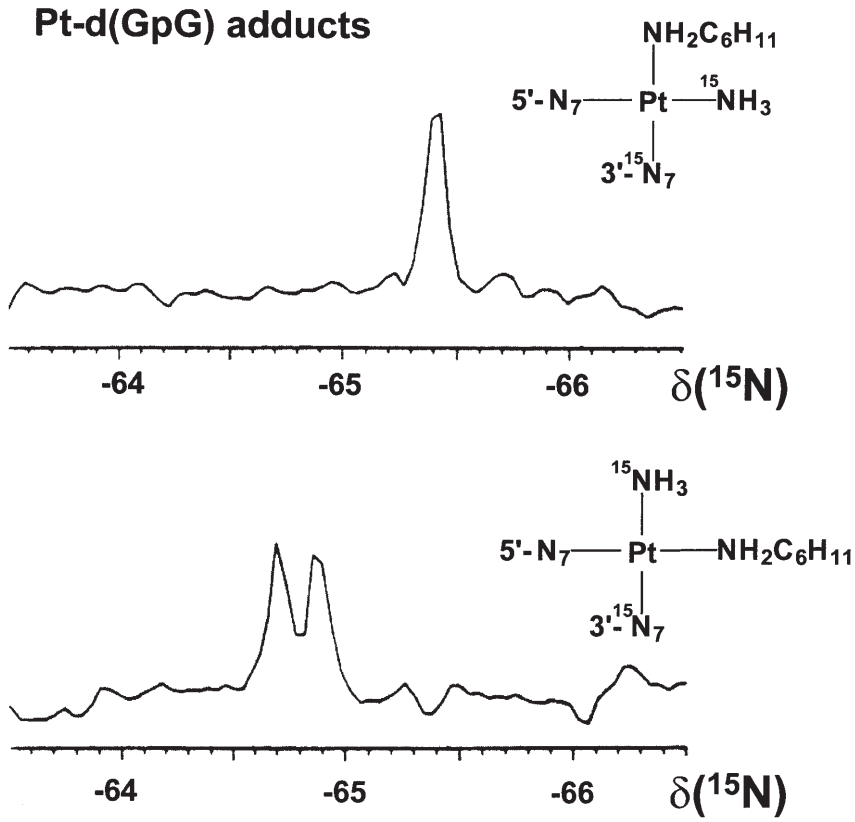


Fig. 10. *^{15}N{^{1}H}-NMR spectra of the two linkage isomers of the adducts of* cis-*[$Pt(NH_3)(C_6H_{11}NH_2)Cl_2$] with d(GpG).* Chemical shifts are reported in ppm downfield of 5M $^{15}NH_4^+$ as $^{15}NH_4{}^{15}NO_3$ in 2M $D^{15}NO_3/D_2O$. (Adapted from [27]).

Reactions with Methionine, GSH, and Other Amino Acids

Pt^{II} being a 'soft' metal ion is known to have a very high affinity for 'soft' ligand atoms such as sulfur. Sulfur-containing ligands, *e.g.* glutathione, *N*-acetyl-L-cysteine, have been investigated as rescue agents for the removal of Pt from the body. The amino acid and thioether L-methionine (L-HMet) is thought to play an important role in the metabolism of cisplatin, and one of the few characterised metabolites of cisplatin is $Pt(L\text{-}Met)_2$ which has been isolated from urine [28]. This complex was originally assigned a *trans*-configuration, but ^{195}Pt- and ^{15}N-NMR data have suggested that $Pt(Met)_2$ consists of a mixture of *cis*- and *trans*-isomers in aqueous solution, with the *cis*- isomer predominating (10:1) [29]. Three possible diastereomers ((*R,R*); (*R,S*)/(*S,R*); (*S,S*)) are resolved in ^{195}Pt-NMR spectra (*Fig. 11*). The assignment of ^{195}Pt-resonances was greatly aided by the use of

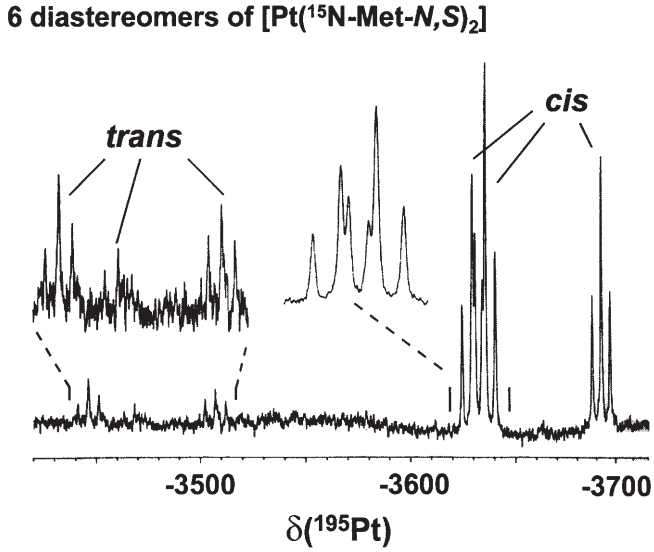
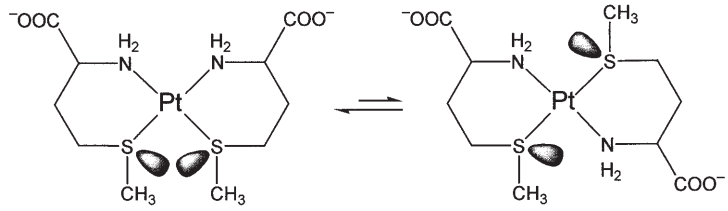


Fig. 11. *^{195}Pt-NMR Spectrum of [Pt(L-^{15}N-Met)$_2$], a metabolite of the cisplatin, showing three sets of triplets for each of the* cis- *and* trans-*isomers.* The three diastereomers for each geometrical isomer arise from slow inversion (on the NMR time scale) of chiral coordinated sulfur. (Adapted from [29]).

^{15}N-enriched methionine. The *cis*-and *trans*-isomers of this complex have been separated by HPLC and detailed studies of the facile *cis-trans*-isomerization in aqueous solution have been made by 2D [^{1}H,^{15}N] HMQC-NMR spectroscopy [30]. NMR data allow characterization of the three diastereomers which arise from the presence of two chiral coordinated S atoms for

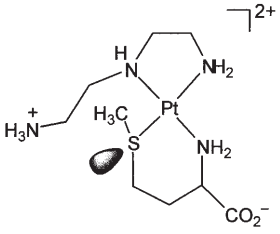
each geometrical isomer, and suggest that chelate ring conformation is dependent on the chirality of the coordinated sulfur. At neutral pH, the isomers interconvert extremely slowly (half-lives of 22.4 h and 3.2 h for the *cis*- and *trans*-isomers, respectively, at 310 K) with the *cis*-isomer predominating at equilibrium ($K = 7.0$).



cis *trans*

The pH-dependent *S*,*O*- *vs*. *S*,*N*-chelation in the reaction of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with ^{15}N-labelled *S*-methyl-L-cysteine (MeCysH) and L-methionine has been studied by *Appleton et al.,* using ^{1}H-, ^{13}C-, ^{15}N- and ^{195}Pt-NMR [31]. The chelate products $[Pt(NH_3)_2$(MeCys-*S,N*)$]^+$ and $[Pt(NH_3)_2$(Met-*S,N*)$]^+$ were observed at pH values near 5, with ^{195}Pt chemical shifts in the region typical of PtN_3S. In strongly acidic conditions (pH $\leq$ 0.5), the initial product from the reaction with MeCysH is $[Pt(NH_3)_2$(MeCysH-*S,O*)] with a ^{195}Pt chemical shift in the region of PtN_2SO. This species slowly converts to the *S*,*N*-chelate. A similar reaction sequence occurs with methionine, but *cis*-$[Pt(NH_3)_2$(MetH-*S*)$_2]^{2+}$ is also formed in competition with the *S,O*- chelate. Slow release of ammonia was observed for all these complexes by ^{15}N-NMR spectroscopy.

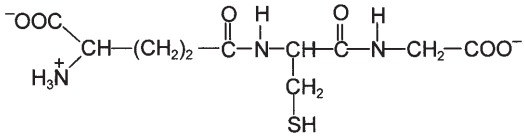
The interconversion between *S*- and *N*-bound L-methionine adducts of $\{Pt(dien)\}^{2+}$ *via* dien ring-opened intermediates has been observed by using [^{1}H,^{15}N] 2D-NMR spectroscopy and HPLC [32]. The complex [Pt(^{15}N-dien)(L-MetH-*S*)$]^{2+}$ was dominant at neutral pH and converted partially and reversibly into [Pt([^{15}N]dien)(L-MetH-*N*)$]^+$ at pH > 8. The dien ring-opened intermediate [Pt(dienH-*N,N'*)(L-MetH-*S,N*)$]^{2+}$ was observed when the pH was lowered from 8 to 3, and this converted slowly into the *S*-bound complex. The ring-opened intermediate was separated by HPLC, and was



[Pt(dienH-*N,N'*)(L-MetH-*S,N*)$]^{2+}$

surprisingly long-lived. It was characterized by [^{1}H,^{15}N] 2D-NMR spectroscopy as a mixture of four diastereomers (due to chiral centers at S and NH) present in a 2:2:1:1 molar ratio (*Fig. 12*). Intramolecular H-bonding was observed for both the complex [Pt([^{15}N]dien)(L-MetH-*S*)]$^{2+}$ and dien ring-opened intermediates.

The S-containing tripeptide glutathione (GSH) is present in cells at mM concentrations, and the formation of Pt:GSH complexes may play an important role in the biological activity of platinum complexes. By multinu-



Glutathione (GSH)

clear NMR (^{15}N,^{195}Pt,^{13}C,^{1}H), a product containing a dinuclear Pt_2S_2 four-membered ring has been observed from reactions of *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ with GSH [33]. The transient intermediates formed during the reaction of *cis*-[PtCl$_2$(^{15}NH$_3$)$_2$] with GSH have been monitored by ^{15}N-{^{1}H} DEPT-NMR [34]. The mono-substituted complex *cis*-[PtCl(SG)(^{15}NH$_3$)$_2$] was

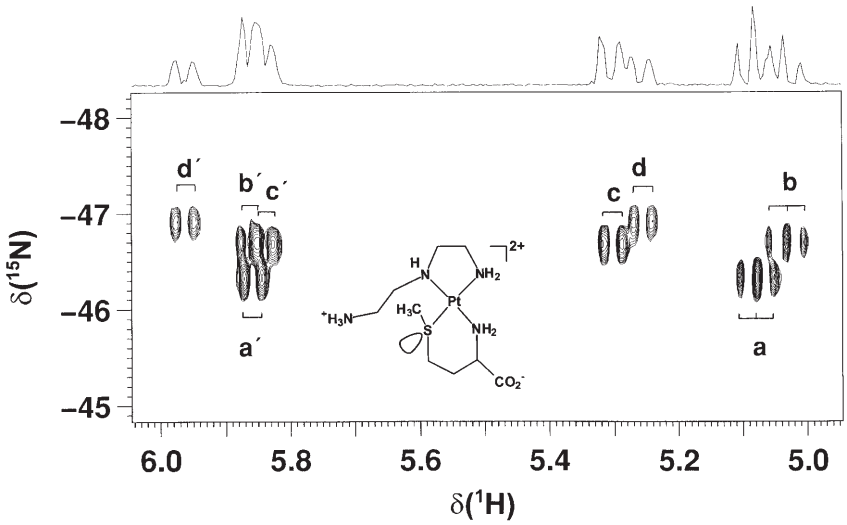


Fig. 12. *The 2D [^{1}H,^{15}N] HSQC-NMR spectrum of the HPLC-isolated dien ring-opened complex at pH 4.0.* Only the NH$_2$ group of L-MetH was ^{15}N-labelled, and the four sets of cross-peaks (peaks *a*, *a´* to *d*, *d´*) can be assigned to the non-equivalent Pt-NH$_2$ groups in the four diastereomers of [Pt(dienH-*N,N´*)(^{15}N-L-Met-*S,N*)]$^{2+}$. All peaks have ^{2}J(NH$_a$, NH$_b$) of *ca.* 12 Hz, while only peaks *a* and *b* have an additional ^{3}J(α-CH,NH) of *ca.* 13 Hz. (Adapted from [32]).

formed first, but several other species containing a $^{15}NH_3$-Pt-S linkage were present within a few minutes. Release of $^{15}NH_3$ ligands was observed within 10 min of reaction and final product contained no coordinated $^{15}NH_3$. The ^1H, ^{13}C, and ^{195}Pt-NMR spectra of the final product were consistent with a high-molecular mass polymer with a 1:2 Pt/GSH stoichiometry.

The effect of glutathione (GSH) upon macrochelate ring closure of *cis*- and *trans*-DDP monofunctional adducts on DNA has been investigated using ^{195}Pt-NMR spectroscopy [22]. From the ^{195}Pt chemical shift, the new products from cisplatin have a PtN_3S coordination sphere (–2800 to –3200 ppm), and the solution slowly developed a yellow color, consistent with formation of a reported polymeric species having two GSH ligands bound per platinum atom. Compared with cisplatin, the reaction between GSH and *trans*-DDP monofunctional adducts leads more rapidly to the formation of sulfur-bound, glutathione-trapped monofunctional adducts. Glutathione does not appear to react with *cis*- or *trans*-DDP bifunctional adducts. These results are consistent with proposals that the biological inactivity of *trans*-DDP may arise from selective trapping of monofunctional adducts before they ring-close to form bifunctional lesions.

^1H- and ^{195}Pt-NMR investigations have shown that therapeutic nucleophilic agents for cisplatin, such as Na(ddtc) (sodium diethyldithiocarbamate) and thiourea, can help to remove Pt from certain proteins [35]. The mechanism may be based on the relatively easy reversal of Pt binding to methionine side chains. In contrast, nephrotoxicity, thought to be caused by formation of Pt-cysteine adducts (Pt^{II} thiolate bonds), cannot be reversed by Na(ddtc) and thiouera.

Reactions of cisplatin with the amino acids $^+NH_3(CH_2)_nCO_2^-$ (LH) ($n = 1$ (Glycine, glyH); $n = 2$ (β-alanine); $n = 3$ (γ-aminobutyric acid)) have also been studied by ^{15}N- and ^{195}Pt-NMR [52]. Initially, glycine forms oxygen-bound complexes with cisplatin, and then ring closes to form *N*,*O*-chelated complexes, which are thermodynamically more stable. Ring closure becomes difficult as the chain length (n) increases. Intramolecular migration of the model fragment $\{Pt(dien)\}^{2+}$ from sulfur to imidazole-N^1 in histidylmethionine (His-MetH) has been investigated by HPLC and NMR methods [36]. The adducts were characterized by multinuclear (^1H,^{195}Pt) NMR spectroscopy. Under acidic conditions, the dominant Pt complex is $[Pt(dien)(His\text{-}MetH\text{-}S)]^{2+}$, while the imidazole-$N^1$-bound complex, $[Pt(dien)(His\text{-}MetH\text{-}N^1)]^{2+}$ becomes the major species at pH values higher than 6.1. The N^1-bound species is characterized by the lack of a δ-CH_3 downfield shift and the equivalence of $^3J(^1H\text{-}^{195}Pt)$ values (19 Hz) for the imidazole-H(2) and -H(5) resonances. The dinuclear intermediate $[\{Pt(dien)\}_2(His\text{-}MetH\text{-}N',S)]^{4+}$ was observed during the slow isomerization. ^{195}Pt and ^1H, ^{13}C-NMR spectroscopy has been used to identify the complexes

formed between *cis*-DDP and *trans*-DDP with the tetrapeptide Boc-Cys(SMe)-Ser-Ala-Cys(SMe)-$CONH_2$ (CSAC), which is a model for metallothionein (MT) [37]. MT is a low-molecular-weight protein rich in cysteine (~30%) thought to be responsible for cisplatin detoxification. The reaction of CSAC with cisplatin gives a mixture of different diastereoisomers and polymeric species, with NH_3 liberation due to the strong *trans*-effect of sulfur. *trans*-DDP, on the other hand, forms a 2:1 complex coordinated to the -S-CH_3 groups, and no amine release was observed.

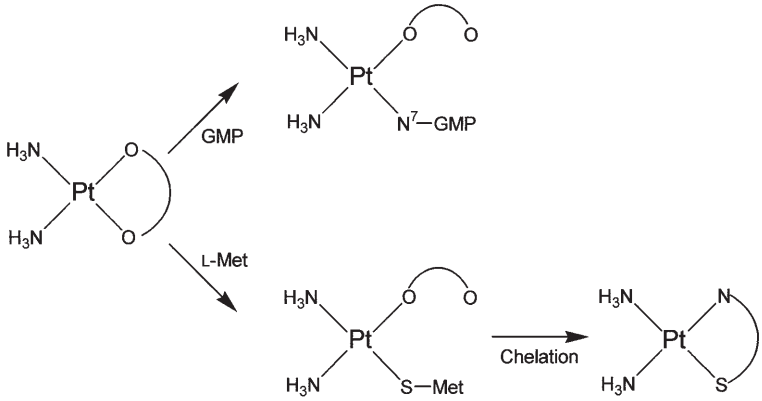
Displacement of Methionine Sulfur by Nucleotides

Although the kinetic reactivity of sulfur is high, the Pt-thioether bond is labile in the presence of other nucleophiles [38]. This could provide novel pathway for DNA platination. NMR studies of the kinetics and thermodynamics of the competitive binding of Met, His, 5′-GMP, 5′-AMP, 5′-TMP and 5′-CMP to $\{Pt(dien)Cl\}^+$ in aqueous solution show that 5′-GMP selectively displaces *S*-bound Met. Initially only L-Met coordinates to Pt with little GMP coordination, but in the later stages of the reaction coordinated L-Met is displaced by N(7)of GMP [38]. It is notable that thioethers such as L-Met react with Pt^{II} amines faster than thiols such as GSH, and reactions of thiols tend to be irreversible [39]. Intramolecular migration of $\{Pt(dien)\}^{2+}$ from S to guanosine-N(7) in *S*-guanosyl-L-homocysteine has been observed by *van Boom et al*. [40] (see also chapter by *J. Reedijk* and *J. M. Teuben* in this book). The reaction of cisplatin with 5′-GMP in the presence of L-Met has also been investigated [41]. Novel intermediates including *cis*-$[Pt(GMP\text{-}N(7))(Met\text{-}S)](^{15}NH_3)_2]^{2+}$ and *cis*-$[Pt(GMP\text{-}N(7))(Met\text{-}S,N)](^{15}NH_3)]^+$ (N(7) *trans* to S) were detected and characterized according to the ^{15}N chemical shifts of *trans*-ammine ligands. Ammine release was observed during the formation of intermediates. Monodentate *S*-bound L-HMet can coordinate to Pt reversibly, whereas *S*,*N*-chelated L-Met is much more inert. Interestingly the reaction of 5′-GMP with cisplatin is faster in the presence of L-Met than in the absence.

Highly stable monofunctional adducts are formed during reactions of monodentate *S*-bound *N*-acetyl-L-methionine complexes $[Pt(en)(MeCO\text{-}Met\text{-}S)Cl]^+$ and $[Pt(en)(MeCO\text{-}Met\text{-}S)_2]^{2+}$ (MeCO-Met = *N*-acetyl-L-methionine) with 5′-GMP and GpG [42]. Two intermediates were observed, which were assigned to 5′-G or 3′-G bound monofunctional adducts. From the wide range of ^1H chemical shifts, a hydrogen-bond network which could stabilize the monofunctional adducts was suggested. Such adducts formed by methionine and its derivatives could play a role in the trapping of monofunctional adducts of platinum anticancer drugs with DNA *in vivo*.

Ring Opening of Carboplatin

The lower side-effects and toxicity of carboplatin [$Pt(NH_3)_2$(CBDCA-*O,O′*)] (H_2CBDCA: cyclobutane-1,1-dicarboxylate) compared with cisplatin, can be attributed to its lower reactivity caused by the presence of the chelating CBDCA ligand. It could be a pro-drug for cisplatin but the rate of hydrolysis is very slow (half-life in water > 4.4 years). A ring-opened carboplatin adduct containing monodentate CBDCA can be detected using ^{1}H-, ^{15}N- and [^{1}H,^{15}N]-NMR during reactions of carboplatin with 5′-GMP [43]. It has unusual ^{1}H-NMR chemical shifts, with each proton on the four-membered cyclobutane being magnetically non-equivalent. Modelling showed that there is close hydrophobic contact between the cyclobutane ring of monodentate CBDCA and the purine ring of 5′-GMP bound by N(7). The fast reactions of carboplatin with 5′-GMP compared with nitrate, phosphate and Cl^- suggest that direct attack of nucleotides on carboplatin may be of importance in the mechanism of action of this drug [43].



Ring-opened adducts of carboplatin can form not only from reactions with nucleotides, but also by the attack of sulfur amino acids [44]. Reactions with thioether ligands are much more rapid compared with thiols. Surprisingly very stable ring-opened species such as [Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] are formed, which has a half-life for Met-S,N closure of 28 h at 310 K. Such an intermediate could also play a role in the biological activity of this drug.

Characterization of Metabolites in Urine

[^{1}H,^{15}N]-NMR spectroscopy can be used to detect a wide range of metabolites in animal urine samples after dosing with ^{15}N-labelled Pt complex-

es. In initial investigations of urine samples from mice treated with ^{15}N-cisplatin, about 20 different types of Pt-NH_3 species were detected, including at least four with sulfur as the *trans*-ligand (thioethers or thiols). A species with shifts very similar to the ring-opened carboplatin complex [Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] was detected as a major metabolite in the urine of animals treated with ^{15}N-labelled carboplatin (*Fig. 13*) [45]. Also notable is the presence of peaks for other metabolites, one of which (–45.5 /3.89 ppm) may be a glutathione conjugate.

Detection of Platination Sites on Proteins

Cisplatin is able to bind to a number of extra- and intracellular proteins. Most of the platinum (65–98%) in blood plasma is protein-bound one day after rapid intravenous infusion of cisplatin [46]. [^{1}H,^{15}N]-NMR spectroscopy can be used to study binding sites of Pt^{II} ammines and amines on these proteins, such as albumin and serum transferrin.

Reactions between cisplatin and serum albumin are thought to be the main route for platinum binding in human blood plasma. Several clinical and experimental observations have suggested that albumin-bound platinum may be anticancer active [47][48]. Additionally, albumin binding may reduce some of the side-effects of cisplatin treatment, especially its nephrotoxicity [49]. The reaction of cisplatin with intact and chemically modified recombinant human albumin (rHA), and with HSA (human serum al-

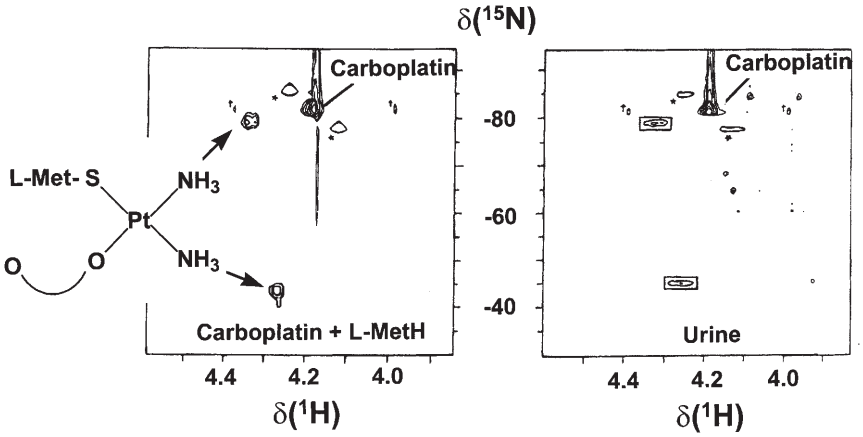


Fig. 13. *[^{1}H,^{15}N]-NMR Spectra of a solution containing carboplatin and* L*-methionine in a 1:1 mole ratio 3.5 h after mixing* (left)*, and of urine collected from mice treated with carboplatin* (right). (Adapted from [45]).

bumin) has been studied using 1D ^{1}H and 2D [^{1}H,^{15}N] HSQC-NMR spectroscopy to characterize the platinum sites [50]. Contrary to previous reports, it was found that the free thiolate group of Cys-34 of albumin is not the major locus for cisplatin binding. The [^{1}H,^{15}N]-NMR data, obtained *via* the use of *cis*-[$PtCl_2(^{15}NH_3)_2$], suggest that the major binding site involves a Met-*S,N*-macrochelate, together with minor monofunctional sites involving Met-*S* and Cys-34. Eventually platinum-bound NH_3 ligands were displaced due to the high *trans*-effect of Cys-*S* and Met-*S*.

Transferrin is a single-chain glycoprotein which has two similar binding sites for Fe^{III} ions situated in interdomain clefts in the N-terminal half (N-lobe) and C-terminal half of the molecule. Diferric transferrin is taken up by cells *via* receptor-mediated endocytosis. It is possible that transferrin delivers Pt to tumor cells which are known to overexpress such receptors. The combination of ^{1}H-, ^{15}N- and ^{13}C-NMR spectroscopy (^{15}N-cisplatin and ^{13}C-Met-transferrin) has shown that one of the major cisplatin binding sites is Met-256 in the N-lobe which is solvent-accessible [51].

Conclusions

^{195}Pt- and ^{15}N-NMR spectroscopy is a powerful combination for the study of reactions of cisplatin and related complexes with molecules of biological importance. The ^{195}Pt chemical shift is very sensitive to the oxidation state and coordination sphere of ^{195}Pt. However ^{195}Pt resonances in larger Pt complexes are broadened *via* chemical shift anisotropy relaxation, which can be severe at high observation frequencies. The use of inverse detection of ^{15}N, and 2D HSQC experiments with ^{15}N-labelled complexes, allows studies of Pt anticancer complexes at concentrations approaching physiological relevance and is providing detailed insight into the thermodynamics and kinetics of reactions with proteins and DNA. The ^{15}N chemical shifts in particular are diagnostic of the *trans*-ligand in Pt-NH systems. Even though it is necessary to work in H_2O rather than D_2O, effective water suppression can be achieved using pulsed field gradients. The 2D method provides a marked simplification of spectra allowing, for example, the detection of metabolites of platinum anticancer drugs in intact biological media such as body fluids.

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Structural Aspects of Pt-Purine Interactions: From Models to DNA

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This review surveys structural details of model systems for platinum-DNA adducts containing intrastrand d(GpG) and d(ApG) crosslinks. Included in this discussion are most of the salient features of various systems from complexes containing modified nucleobases, to those with di-, and trinucleotides, to the assemblies with larger DNA fragments. Chemical and structural behavior of purine bases, as the most important components of the cisplatin-DNA interactions, is reviewed. A large number of studies indicate the strong preference of platinum for binding to the N(7) site of guanine. However, several studies on platinum binding to adenine and its role in the cisplatin action as an anticancer drug are also examined.

Preamble

Cisplatin (*cis*-dichlorodiammineplatinum(II) or *cis*-DDP) has been widely recognized as one of the most potent anticancer drugs, especially effective against testicular, ovarian, and head tumors [1]. It was postulated quite early that cellular DNA is the primary target of cisplatin reactivity [2]. Consequently, over the years, many reviews have been written which cover the interactions of nucleic acid components (nucleobases, nucleosides and nucleotides) with platinum [3] and other metal ions [4], including both studies in the solid state (X-ray structure determinations) as well as in solution. In this article, we will summarize how our picture of Pt-binding to DNA has evolved during the past thirty years, from studies of simple model compounds to those of more elaborate fragments of nucleic acids. Most of this article will be on the interactions between cisplatin and purine bases, which have been the main focus of investigators in this area. Because of space limitations, we have not attempted to be thorough or exhaustive. For more com-

Cisplatin. Edited by Bernhard Lippert

prehensive reviews, the reader is referred to the many excellent articles that are available elsewhere [3].

Background

The discovery by *Rosenberg* and co-workers that *cis*-$Pt(NH_3)_2Cl_2$ and related compounds possess antitumor properties [5] has prompted an intensive search for their possible target sites. Suspicion that DNA could be the target of Pt-binding arose for a number of reasons. Among them was the original observation by the *Rosenberg* group that some platinum compounds, under certain conditions, caused the growth of *E. coli* cells into giant filaments [6]. This phenomenon of filamentous growth was also triggered by bifunctional alkylating agents, a known class of anti-tumor compounds. Since alkylating agents were suspected to act by cross-linking DNA, it naturally followed that DNA was suspected, at the very outset, to be the target of Pt-binding [7].

Once DNA was implicated as a likely site for Pt-complexation, the next question was where, along the DNA double helix, this binding may take place. There are numerous sites which contain potential donor atoms for metal binding: the phosphate and/or ribose oxygen atoms and the various N- and O-atoms of the purine and pyrimidine bases. Although initially it was not clear which of the nucleobases were involved, attention quickly shifted to guanine as the most probable site after it was shown that the amount of Pt-bound to a polynucleotide was related to its guanine (G), cytosine (C) content [8]. In addition, the fact that guanine is also believed to be the target of other electrophilic antitumor compounds, such as alkylating agents, [9] gave further credence to this point of view. Subsequently, however, it was discovered [10] that adenine (A) may also play an important role in the mechanism of crosslinking induced by cisplatin.

An examination of the purine bases (*Fig. 1*) shows a number of potential metal-binding sites: the five nitrogen atoms (each of which has a formal lone pair of electrons) and the carbonyl oxygen atom (in the case of guanine). However, the lone pair electrons on N(1), N(9), and the exocyclic NH_2 groups are part of the delocalized π-electron system of the heterocyclic molecule, and therefore unavailable for metal complexation. This leaves atoms N(3) and N(7) (and O(6) of guanine) as the only sites with 'directed', sp^2-hybridized lone pairs. Because N(3) is in a sterically crowded position, with the glycosidic N(9)–C(4′) bond nearby, N(7) was first proposed as the most likely binding site. Furthermore, the fact that N(7) is not involved in *Watson-Crick* base pairing, and is exposed in the major groove of the DNA double helix, makes it an even more attractive target for Pt-complexation.

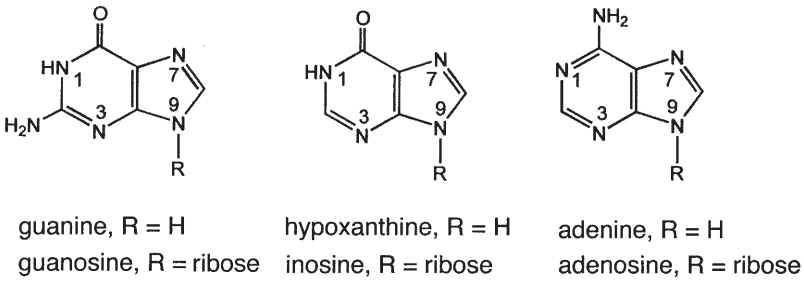

Fig. 1. *Structural formulas and numbering system for the purine nucleobases and nucleosides*

It should be noted, however, that alternative Pt-binding sites other than N(7) of purines were subsequently discovered, especially under conditions where the nucleobases were deprotonated (*vide infra*).

A large number of experimental data including stability constant measurements [11] [12] indicate that, at neutral pH, N(7) of guanine is a better metal-binding site than N(7) of adenine. This observation is also supported by modern quantum-chemical calculations with the inclusion of electron correlation effects which reveal that polarity of the bases and the corresponding basicity of the N(7) site decreases in the order: guanine > inosine > adenine > 2-aminoadenine [13].

Pt-Guanine and Related Complexes

The main task of several early investigations was to confirm the Pt–N(7) bonding model. One of the first reported structures was that of the 1:2 complex of a *cis*-$(NH_3)_2Pt^{II}$ fragment with inosine monophosphate (5′-IMP)

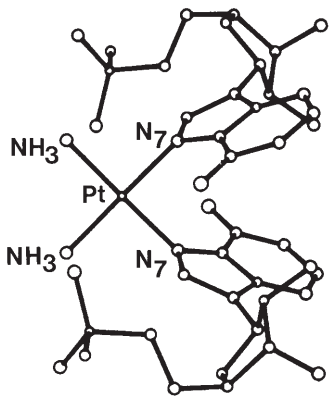

Fig. 2. *The structure of the* cis-*$[Pt(NH_3)_2(5'\text{-}IMP)_2]^{2-}$ anion [14], showing the common* head-to-tail *orientation of the purine rings*

(*Fig. 2*), a nucleotide closely related to 5′-GMP (inosine is an analog of guanosine without the 2-NH_2 group) [14]. This structure clearly shows the monodentate attachment of the purine to Pt through the N(7) atom. The Pt–N(7) distances are 2.02 Å and the N(7)–Pt–N(7) angle is 89°. In this paper the authors speculated on the possible involvement of an N(7)–O(6) chelate from guanine to platinum, even though such a chelate was not actually present in the structure of *cis*-$[Pt(NH_3)_2(5'\text{-IMP})_2]^{2-}$. The issue of the N(7)–O(6) chelate will be discussed later in this article.

Soon thereafter, proof of Pt-N(7) binding was extended to derivatives of guanine itself. Basically the same 1:2 *cis*-complexation pattern was reported in the structures of $[Pt(en)(guanosine)_2]^{2+}$ (en = $NH_2CH_2CH_2NH_2$) (*Fig. 3*) [15], *cis*-$[Pt(NH_3)_2(guanosine)_2]^{2+}$ [16], and *cis*-$[Pt(NH_3)_2(5'$-

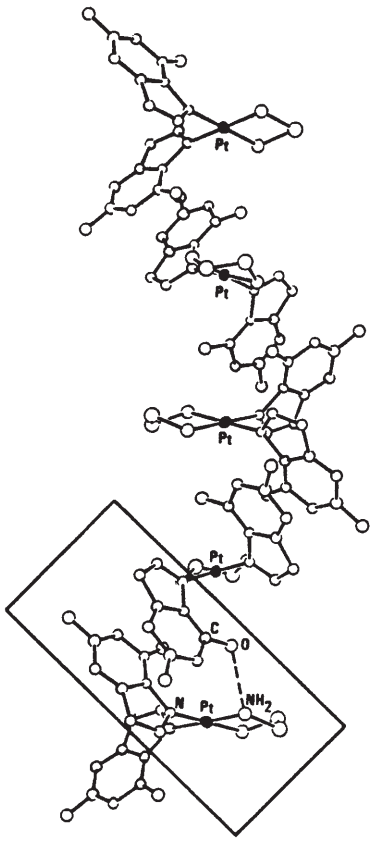

Fig. 3. *A portion of the intermolecular packing between* $[Pt(en)(guanosine)_2]^{2+}$ *cations* [15]*, showing the stacking of guanine bases from adjacent cations*. Note H-bonding (dotted lines) from the ammine groups of the ethylenediammine ligand to the O(6)-atom of a neighboring guanine ring (see text).

GMP)$_2$]$^{2-}$ [17]. In all of these cases, the guanine rings assume an antiparallel (*head-to-tail*) arrangement, and are almost perpendicular to each other (dihedral angles 71–74°).

A closer examination of the structure of [Pt(en)(guanosine)$_2$]$^{2+}$ revealed a hydrogen bond formed between the NH_2 group of the ethylenediamine ligand and the O(6) oxygen atom of a neighboring complex cation (*Fig. 3*). Hydrogen bonding involving the ammine groups would later be found in virtually all subsequent X-ray structures of Pt-nucleotide and related compounds. In each case, hydrogen bonds employed either the exocyclic oxygen atoms of the bases or the oxygen atoms of the phosphate groups. This finding was quite significant as it indicated that hydrogen bonding involving the ammine ligands of *cis*-Pt(ammine)$_2$X$_2$ complexes could be an important factor in the stabilization of Pt-DNA interactions. For example, differences in the ability to form hydrogen bonds could help explain the observation that the anti-tumor activity of *cis*-Pt(ammine)$_2$X$_2$ complexes drops off markedly in the sequence $NH_3 \approx NH_2R > NHR_2 \gg NR_3$ [18].

In the following years, a few more structures appeared in the literature on Pt-complexes of nucleosides and nucleotides [4a]. In general, they reinforced the conclusions from earlier studies indicating the predominant Pt–N(7) binding with a *head-to-tail* arrangement of guanine bases. However, the number of structural studies reported was rather small because of the difficulties in obtaining single crystals.

The motivation for studying Pt-nucleotide-complexes, as opposed to those of simpler ligands such as the nucleobases and nucleosides, is often instigated by the need to assess interactions with a complete basic unit of DNA. These studies can, for instance, offer an estimation of the complexation abilities of the phosphate oxygens, compared to those of other potential ligating sites, such as the ribose oxygens and the heteroatoms of the nucleobases. The results of such comparisons brought about several interesting conclusions. In general, direct platinum-phosphate covalent bonding has not been found, except in the dimeric pyrimidine complex [Pt(en)(5′-CMP)]$_2$ [19]. However, since this is not the normal complexation mode of cytidine monophosphate (which usually takes place through the N(3) atoms of the pyrimidine rings [20]), it is generally acknowledged that platinum binding to the phosphate groups is not especially significant. Furthermore, complexation to the ribose oxygens has never been found, so it is safe to assume, as has been done all along, that the nucleobases constitute the primary targets for platinum binding.

In marked contrast to the relatively sparse structural results on Pt-nucleoside and Pt-nucleotide complexes, platinum complexes of modified nucleobases (*i.e.*, without the sugar and phosphate groups) have been quite plentiful. They are surveyed in an extensive review by *Lippert*, *Randaccio*

et al. [3b]. A major criticism of some of the early work on these model compounds was that virtually all of the early structures showed the purine and pyrimidine bases in a *head-to-tail* disposition, leading to a molecule with C_2 symmetry. This orientation was considered to be a poor model for Pt-DNA interaction, since native DNA would not normally contain adjacent guanine rings in such a *head-to-tail* conformation. The situation changed in 1984 when *Lippert* and co-workers reported the structures of four complexes of the type *cis*-$[Pt(NH_3)_2(9\text{-EtGH-}N(7)_2]^{2+}$ (9-EtGH = neutral 9-ethylguanine) (*Fig. 4*) [21], containing the guanine rings in a *head-to-head* conformation, which attracted considerable attention as the first 'realistic' model of a Pt-DNA-complex. The dihedral angles between the guanine rings in these compounds are large (68 and 70°), indicating the lack of any substantial intramolecular base-base stacking interactions.

An example of an octahedral Pt^{IV}-complex with 9-methylguanine (9-MeGH) has also been investigated [22]. The analysis of [Pt(diaminocyclohexane)(9-MeGH-$N(7)_2Cl_2]^{2+}$ (*Fig. 5*) showed that the guanine rings are much less perpendicular to each other (dihedral angle 46°) than in square-planar Pt^{II}-complexes. The preparation of some other Pt^{IV}-complexes has been attempted, but it was found that in many cases they became inadvertently reduced to Pt^{II} compounds [23].

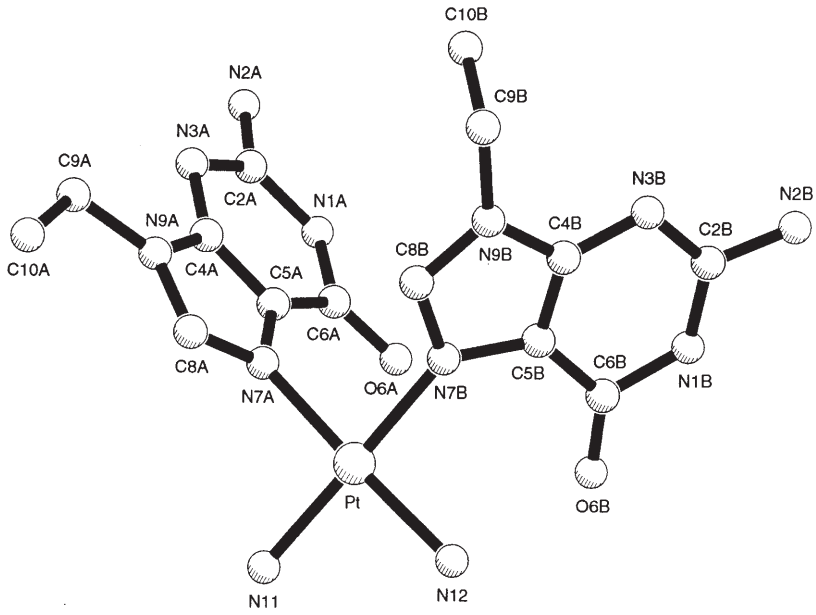

Fig. 4. *Structure of the* cis-*$[Pt(NH_3)_2(9\text{-ethylguanine})_2]^{2+}$ cation, the first* head-to-head *guanine complex characterized by X-ray crystallography*. Coordinates taken from [21a].

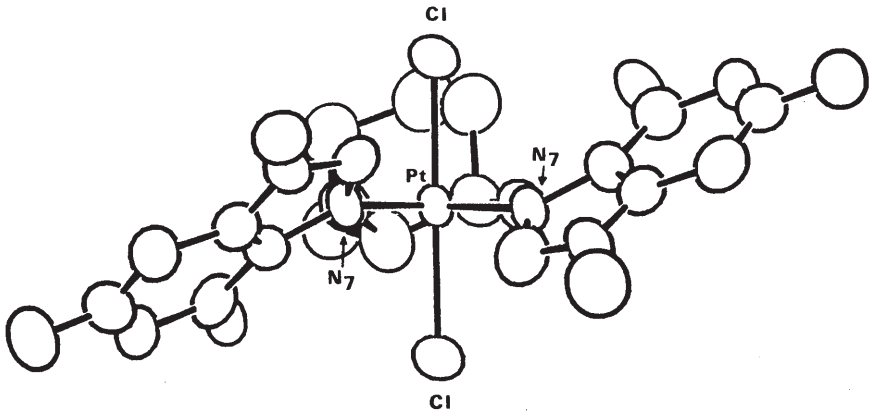

Fig. 5. *Structure of the six-coordinate Pt^{IV} complex, the [(diaminocyclohexane)Pt(9-methylguanine)$_2$Cl$_2$]$^{2+}$ cation* [22]. The diaminocyclohexane is partially obscured behind the Pt-atom. Note how the bulk of the axial chloride ligands forces the guanine rings to be in a less perpendicular orientation (dihedral angle 46°) than in other (square-planar) bis-guanine complexes.

Disputed Existence of N(7)–O(6) Chelation

Several models for the site of interaction of cisplatin with the DNA duplex have been postulated in early stages of this research [24]. One of these models considered a bidendate chelate complex between *cis*-diammineplatinum(II) and the guanine base through the formation of Pt–N(7) and Pt–O(6) covalent bonds. The hypothesis of N(7)–O(6) chelation gained several supporters, as well as an equal number of staunch opponents. Experimental evidence for such a chelate was derived mostly from IR and photoelectron spectroscopy. However, some of these early spectroscopic data have subsequently been reinterpreted or simply dismissed [29]. To date, there has been no unambiguous structural information supporting the existence of N(7)–O(6) chelates in Pt^{II} complexes, but chelation of this kind has previously been observed in a few related sulfur analogs, such as a Pd^{II}-complex with 6-thio-9-benzylpurine [25] and in a Cu^{II}-complex with 6-thio-9-methylpurine [26]. On the other hand, in a square-pyramidal Cu^{II}-complex with theophylline (1,3-dimethylxanthine) [27], considered a potential compound having chelate binding, an observed Cu–O(6) separation of 2.92 Å was too long to be indicative of a covalent Cu–O bond.

Following his analysis of the structure of a Cu^{II}-complex with 9-methylhypoxanthine, *Sletten* [28] concluded that the formation of N(7)–O(6) chelate may be impossible or at least very difficult, for several steric reasons. To accommodate a metal ion in the chelate system, *Sletten* argued, the di-

rection of the N(7) orbital would have to be distorted significantly away from its normal position. Furthermore, such a chelate system would have a very strained structure. For instance, the C(5)–N(7)–M angle would have to be approximately 90°, whereas the observed angles (for a normal non-chelating situation) are close to 135°. In the corresponding 6-thiopurine complexes, however, chelation exists because of the strength of the metal-sulfur bond, which is the dominant factor in the stabilization of the strained chelate ring.

Over the years, our understanding of the chelate controversy has significantly improved. Presently, it is believed that the N(7)–O(6) chelate involving neutral guanine will not form in the presence of water ligands [3d]. Instead, an indirect chelation with a water molecule serving as a bridge between O(6) and the N(7)-bound metal ion is expected to occur [29]. Finally, it should also be noted that in octahedral Pt^{IV}-complexes there exist well defined examples of N(7)–O(6) chelation: in a hexameric trimethylplatinum–theophylline system, the theophylline ligand binds to an octahedral Pt^{IV} through the N(7)- and O(6)-atoms, with the Pt–N(7) and Pt–O(6) distances being 2.17 and 2.34 Å, respectively [30].

Additional Pt-Binding Sites in Guanine

Pt-binding sites other than N(7) have been studied in a number of compounds. *Lippert* and co-workers [31] reported the preparation and characterization of a series of mono-, di-, and trinuclear Pt^{II}-complexes with anionic 9-methylguanine (9-MeG), containing a Pt bound to the deprotonated N(1) position or simultaneously to N(1) and N(7). The bridging properties of 9-methylguanine have been explored in the complexes $\{[(dien)Pt]_2(9\text{-MeG-}N(1),N(7))\}^{3+}$ and *cis*-$[(NH_3)_2Pt(1\text{-MeU-}N(3))(9\text{-MeG-}N(1),N(7))\text{-}Pt(dien)]^{2+}$ (dien = diethylenetriamine; 1-MeU = 1-methyluracilate). In both compounds, the base binds to two different Pt-atoms through N(1) and N(7). The Pt–N(7) distances are 2.02 Å, whereas the Pt–N(1)-bond lengths are slightly longer (2.06 and 2.04 Å).

The N(3)-atom of guanine is not usually considered a good Pt-binding site. However, the increased basicity of guanine, due to the proton replacement by Pt at N(1) and Pt-binding to N(7), may lead to the formation of a Pt–N(3) bond. An example of this simultaneous binding to N(1), N(3) and N(7) is provided by the structural analysis of $\{[(NH_3)_3Pt]_3(9\text{-EtG-}N(1),N(3),N(7))\}^{5+}$ (9-EtG = 9-ethylguaninate) [32]. The Pt–N(1), Pt–N(3), and Pt–N(7) distances are 2.03, 2.05 and 2.03 Å, respectively. Several significant changes in bond angles of deprotonated 9-ethylguanine were found when compared to the neutral guanine or its protonated forms. These dif-

ferences are related mostly to the N(9)-, C(4)-, and C(5)-atoms. For instance, the C(4)–N(9)–C(9′) angle is larger by 6°, whereas the adjacent angle C(9′)–N(9)–C(8) decreases by *ca*. 8°.

Under physiological conditions N(1) of guanine is involved in *Watson-Crick* base pairing and, therefore, not available for metal binding. However, Pt-binding to this site could happen in single-stranded DNA or in a duplex with guanine involved in the *Hoogsteen* base pairing. Several complexes containing the Pt–N(1) bond have been prepared [33]. One example of these complexes is (en)Pt(9-MeG-*N(1)*)$_2$, where two anionic 9-methylguanine moieties are arranged in a *head-to-tail* fashion. As compared to *N*(1), *N*(7)-diplatinated compounds, there are no significant differences in the geometries of the bases.

Pt-Adenine and Mixed Pt-Adenine, Guanine Systems

Interest in Pt-binding to adenine has been stimulated by the finding that the (A-*N(7)*)p(G-*N(7)*) intrastrand crosslink represents the second most abundant DNA adduct of cisplatin [10][24]. Structures of several Pt-complexes containing single adenine ligands, in their neutral or protonated forms [35][36], have been determined. The complex $[(NH_3)_3Pt(9\text{-MeA})]^{2+}$ (9-MeA = 9-methyladenine) [34] contains an adenine ligand bound to Pt through N(7) (the Pt–N(7) distance is 2.00 Å) and is almost perpendicular to the Pt-coordination plane. Bis(adenine) systems have also been studied. In the complex *cis*-$[(NH_3)_2Pt(3\text{-MeA})_2]^{2+}$ (3-MeA = 3-methyladenine) [37], the two adenine ligands are arranged in a *head-to-tail* fashion, with an interbase dihedral angle of 90.6°. A virtually identical geometry was found in the complex with 9-methyladenine, *cis*-$[(NH_3)_2Pt(9\text{-MeA-}N(7))_2]^{2+}$ [38] where the *head-to-tail*-disposed bases form an angle of 90.7°.

Recently, the group of *Arpalahti* [39] studied the *cis*-$[(NH_3)_2Pt(\text{Ado-}N(7))_2]^{2+}$ (Ado = adenosine) system. There are two independent complex cations in the unit cell, both exhibiting a *head-to-tail* orientation. The Pt–N(7) distances are within the range 2.00–2.05 Å, and the dihedral angles between the bases are 83.5° and 86.6°.

Very few structural studies have been concentrated on models of ApG adducts of cisplatin. Conformational analysis of two rotameric forms of the complex *cis*-$[Pt(NH_3)_2(9\text{-MeA-}N(7))(9\text{-EtGH-}N(7))]^{2+}$ has recently been described [40]. One of the forms, crystallized as a PF_6^- salt, can be characterized as a right-handed helicoidal model for the intrastrand ApG crosslink in double-stranded DNA. The bases in this compound assume a *head-to-head* orientation (*Fig. 6*) with the interbase dihedral angles of 81.8° and 87.5°. There are two independent complex cations in the unit cell. The left-

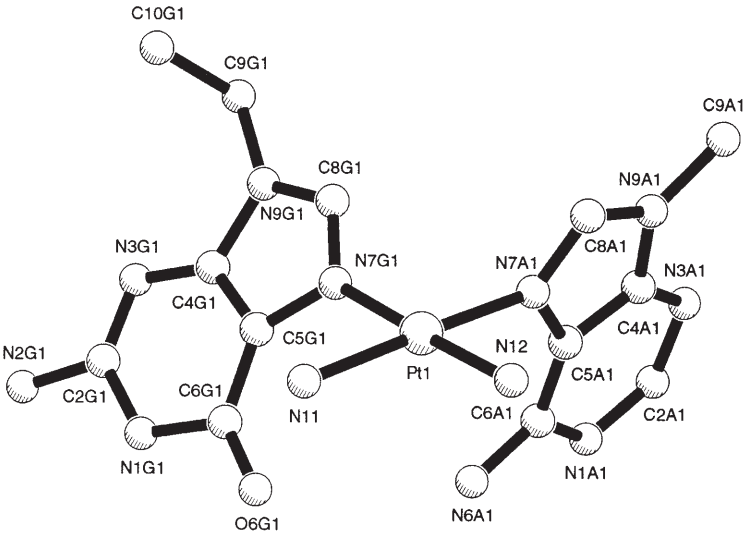

Fig. 6. *Structure of the* cis-*[Pt(NH$_3$)$_2$(9-ethylguanine)(9-methyladenine)]$^{2+}$, a model for the ApG crosslink*. Coordinates taken from [40b].

handed rotamer, crystallized as an NO_3^- salt, has similar metric parameters. The intramolecular distance O(6)(G)···N(6)(A) is 3.11 Å in this rotamer, indicating a weak hydrogen-bonding interaction.

Pt-Dinucleotide Complex

A complete model of an intrastrand Pt-DNA crosslink was provided by the study of a Pt-complex with the deoxydinucleotide d(pGpG), published by *Sherman*, *Lippard* and co-workers [41]. In order to avoid crystal degradation and partial occupancy problems, the authors decided not to soak pregrown crystals of d(pGpG) in platinum solutions, but to prepare the Pt-d(pGpG) complex first and to purify it before crystal-growth attempts. The resulting compound, *cis*-Pt(NH$_3$)$_2$[d(pGpG)], crystallizes in two different space groups having essentially the same structure, and in both cases there were four independent molecules in the unit cell. These independent molecules form an assembly with an approximate two-fold rotation axis. The major feature of this structure is the presence of bidentate chelation of a *cis*-(NH$_3$)$_2$Pt^{II} fragment *via* the N(7)-atoms of two separate guanine rings with the whole arrangement forming a 17-membered ring (*Fig. 7*). The two guanine rings arranged in a *head-to-head* fashion are severely distorted from their normal parallel base-stacked arrangement, making an average dihedral

angle of 81° with each other. Pt-binding to adjacent N(7)(G) atoms in the major groove decreases the N(7)···N(7) distance from 4.2 Å in duplex B-DNA to 2.8 Å. As a result, the guanine bases open up toward the minor groove. The structure is stabilized by an extensive hydrogen-bond network. The hydrogen bond between terminal 5′-phosphate oxygen atoms and ammine ligands seems to be of special significance. Such hydrogen bonding is believed to be an important factor in the stabilization of Pt-DNA adducts.

Although many of the features found in the crystal structure of *cis*-$Pt(NH_3)_2$[d(pGpG)] had been indicated by prior NMR work [42] the significance of the X-ray work cannot be overestimated. Among other things, it conclusively proved that it is indeed possible for two adjacent guanine rings on the same strand to bond covalently to a platinum atom in a *cis*-disposition. These guanine rings were found to maintain an approximate *head-to-head* configuration. Furthermore, the analysis provided details of the distortions in the main backbone parameters (changes in the torsion angles at the ribose and phosphate groups *etc*.) upon formation of an intrastrand Pt-DNA crosslink.

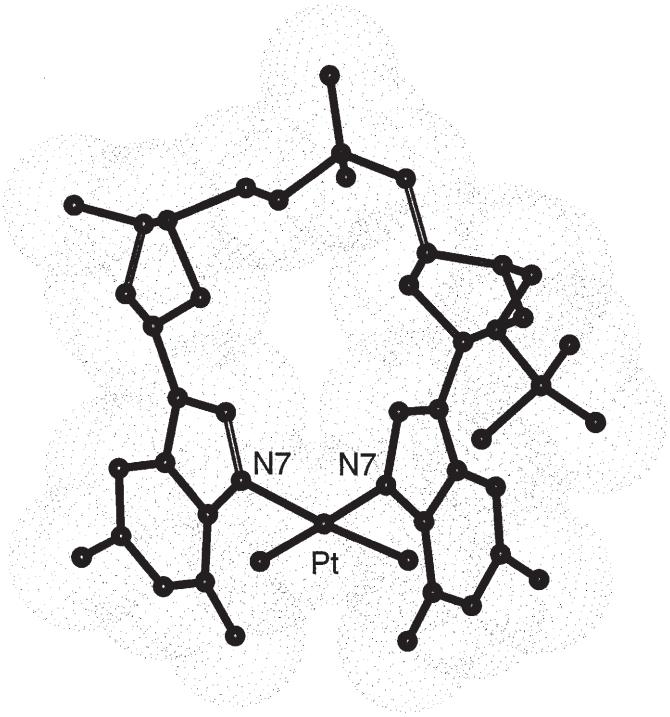

Fig. 7. *Molecular structure of the* cis-*[$Pt(NH_3)_2${d(pGpG)}] complex* [41] *showing the* head-to-head *arrangement of the guanine rings and their near-perpendicular orientation (*dihedral angle ≈ 80°*).* Coordinates taken from the Protein Data Bank [49].

Pt-Trinucleotide Structures

The structure analysis of a platinum with the trinucleotide d(CpGpG) posed several problems due to poor diffraction quality and extensive disorder of the crystals [43a]. Nevertheless, it was possible to establish the most important structural properties. Thus, the trinucleotide complex $Pt(NH_3)_2$[d(CpGpG)] showed essentially the same features as those found in the dinucleotide complex: two *head-to-head* guanine rings in an approximately perpendicular conformation (dihedral angle 80–84°). Significantly, the third nucleotide (cytosine) was found to be no longer involved in a parallel stacked orientation with respect to the central guanine base (*Fig. 8*). This shows that Pt-DNA binding disrupts not only the molecular conformations around the immediate binding site (guanine), but also those of its neighbors (in this case cytosine). As previously found in the dinucleotide complex, the structure is stabilized by hydrogen bonding involving NH_3 ligands and O(6)(G), as well as phosphate O-atoms.

The X-ray structure of another trinucleotide complex of platinum, Pt(dien)[d(ApGpA)] (dien = diethylenetriamine), was also reported by *Reedijk* and co-workers [43b]. In this complex Pt binds only to N(7) of the central guanine. Unlike the situation in the CpGpG complex, Pt-binding leaves the base stacking undisturbed.

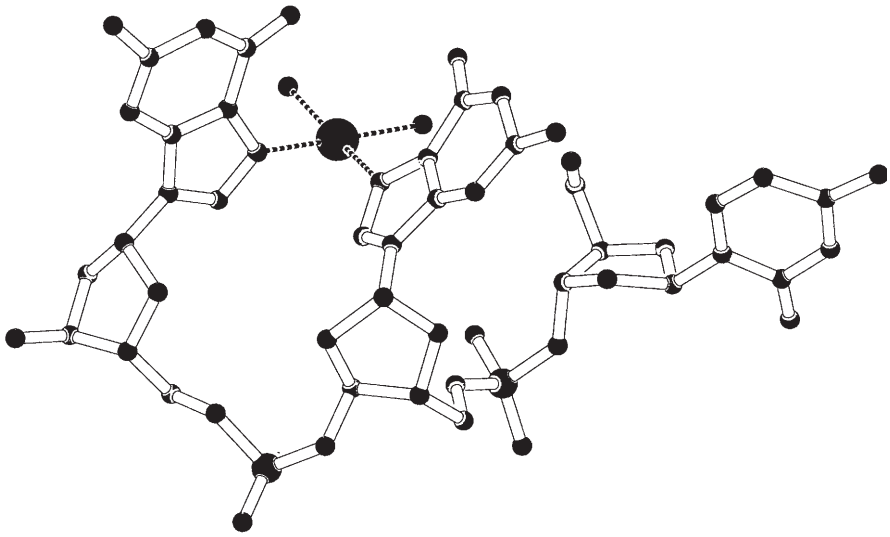

Fig. 8. *Molecular structure of the* cis-*[Pt(NH$_3$)$_2${d(CpGpG)}] complex* [43]. Courtesy of Prof. *Jan Reedijk*, University of Leiden.

Pt-DNA Dodecamer Complex

An experiment involving the diffusion of cisplatin into pre-grown crystals of the self-complementary double helical dodecamer CGCGAATTCGCG, resulting in a Pt-dodecamer adduct, was reported by *Wing*, *Dickerson* and co-workers [44]. Interestingly, it was found that only three of the eight potential guanine sites could be platinated.The three platinated sites, located close to the center of the molecule (*Fig. 9*), were only partially occupied (61%, 30%, 22%). Attempts to increase the degree of platination resulted in degradation of the crystals.

The structure of the dodecamer retained the normal B-DNA form with very little distortion, indicating that, at these low levels of Pt-binding, the basic double-helical structure of DNA is not greatly disrupted. The only detectable distortion was a movement of the platinated guanine rings slightly outward, by about 1 Å, towards the Pt-site into the major groove. The three

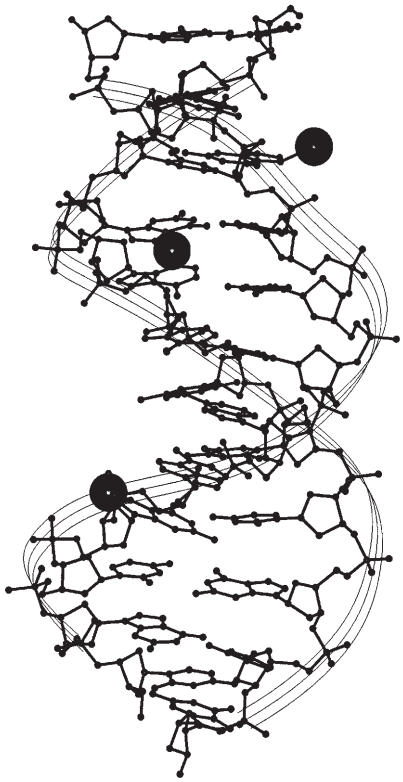

Fig. 9. *View of the partially platinated dodecamer duplex [dCGCGAATTCGCG]$_2$.* Pt-Binding sites are indicated by circles [44]. Coordinates taken from the Protein Data Bank [49].

Pt-atoms are all bound in a monodentate fashion to N(7) and are hydrogen-bonded, through their ammine hydrogens, to the O(6)-atom of guanine.

Perhaps the most significant finding of this paper is the clear and unambiguous demonstration of the overwhelming preference of Pt for guanine: even when all four bases are available for binding in the same target molecule, platinum still chooses exclusively the N(7) atom of guanine. Another significant feature of this paper is the fact that attempts to increase platination led to the degradation of the crystals, which strongly suggests that higher levels of platination would lead to a drastic and fundamental breakdown of the double helical structure of DNA.

Pt-tRNA Interactions

Even though it was discovered quite early that DNA is the primary target of the cisplatin binding, there have also been some attempts to bind the drug to crystalline $tRNA^{Phe}$, as a model for possible interactions with DNA. In one such study [45], crystals of the orthorhombic form of $tRNA^{Phe}$ were soaked for ten days in a saturated solution of *cis*-$Pt(NH_3)_2Cl_2$. X-ray data collected at 5.5-Å resolution allowed the identification of two major binding sites. These sites are the N(7)-atoms of guanine bases G15 and G18, located in the dihydrouridine loop. It should be noted that the platinum coordination occurs at the second base of the sequence A14-G15 with an N(7)(A)–N(7)(G) distance of 3.1 Å. The other binding takes place at the beginning of a sequence of seven purine bases. However, the N(7)G18–N(7)G19 distance of 8.1 Å is clearly too far for intrastrand cross-linking between G18 and G19.

In another study, the monoclinic form of the $tRNA^{Phe}$ crystals was employed [46]. Soaking these crystals for one week in a solution of *cis*-$[Pt(NH_3)_2Cl_2$ resulted in the drug binding in the vicinity of residues G3-G4, C25-m^2G26, G42-G43-A44-G45, and A64-G65. A 6-Å resolution difference-*Fourier* map used in this study showed a larger-than-usual amount of noise, attributed to a partial destruction of the crystal upon drug binding. As in the case of the DNA dodecamer, these two studies confirmed a preference for Pt-binding to N(7) of guanines. Both experiments also suggested that Pt-binding occurs preferentially at AG rather than GA sequences.

Pt-Duplex DNA Structure

The largest and most realistic Pt–DNA structure to date, reported recently by *Takahara*, *Lippard* and co-workers [47], is that of the *cis*-$(NH_3)_{2-}$

Pt^{II}-complex with d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG), where G*G* represents the positions of two guanine groups bound to platinum. This analysis provided, for the first time, a view of a fully-platinated duplex DNA at high resolution. The complex was prepared by synthesizing the two complementary strands individually, reacting the first strand (CCTCTGGTCTCC) with *cis*-$[Pt(NH_3)(H_2O)_2]^{2+}$, and then carefully annealing the complementary strand (GGAGACCAGAGG) onto it. The structure consists of a double helix distinctly bent at the platinated site (*Fig. 10*). Surprisingly, all the complementary base-pairing interactions, even the G-C base pairs involving the two platinated guanines, are still intact.

The two platinated guanine rings in the Pt-dodecamer duplex were found to be tilted with a dihedral angle of 26°, much less than the nearly perpendicular orientation of the guanine rings (~ 80°) in *cis*-$Pt(NH_3)_2$[d(pGpG)] (*Fig. 7*) [41]. The Pt-atom is displaced by about 1 Å out of the planes of the guanine rings. This is in contrast to the situation in *cis*-$Pt(NH_3)_2$[d(pGpG)],

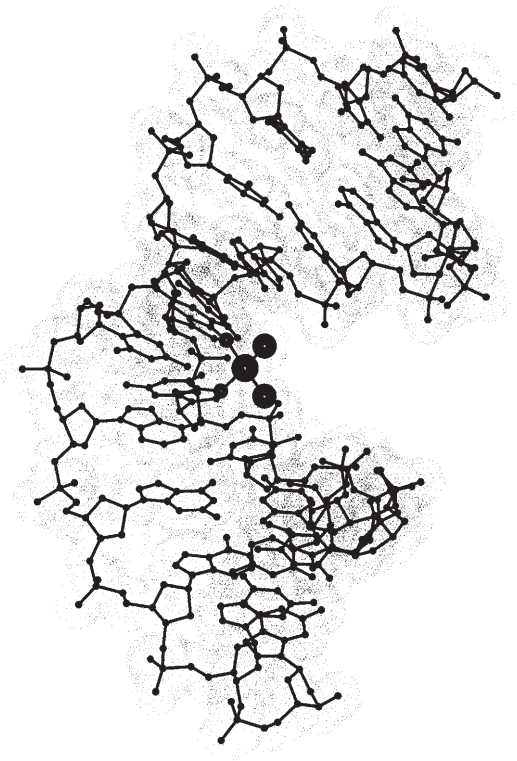

Fig. 10. *View of the fully-platinated duplex DNA d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG)* [47]. The Pt-coordination site is marked by a circle. Coordinates taken from the Protein Data Bank [49].

in which Pt and the guanine base are much more coplanar. Both features suggest that the dodecameric duplex, even though it becomes distinctly bent upon complexation to Pt, is much less prone to the severe distortions found in the dinucleotide complex because of the steric contraints imposed by the double-helical structure itself. In other words, a bulky dodecameric duplex is less easily 'distortable' than a single-stranded fragment.

The extent of bending was found to be around 35–40°, although this angle could not be estimated accurately because of the difficulty of defining the exact positions of the helical axes passing through the two halves of the molecule. Curiously, these two halves have different conformations: the 3′-end of the helix resembles B-DNA whereas the other half is more like A-DNA.

The Pt-DNA dodecamer structure represents the most detailed look at Pt-DNA interaction available thus far. It shows that Pt-complexation induces a DNA bend which spreads out over the adjacent base pairs. However, even though distorted, the *Watson-Crick* base pairing is not disrupted. Furthermore, platination causes a large positive roll between base pairs, which compresses the major groove and opens up the minor groove. A wider minor groove could be a good site for protein binding. In fact, it was noticed [47] that this feature resembles the conformation of the DNA portion in a complex of the human-testis-determining factor (SRY) bound to DNA through its high-mobility-group (HMG) domains [48].

Concluding Remarks

Intrastand d(GpG) and d(ApG) crosslinks constitute *ca.* 65% and 25%, respectively, of the cisplatin induced adducts *in vitro* [10]. Thus, the biological action of cisplatin is based on the interaction between Pt and the purine bases. Numerous studies have been devoted to the characterization of structural aspects of this interaction. In early years of the research, several model compounds mimicking Pt-binding sites in DNA were synthesized and investigated. Progress in DNA synthesis and crystal growing inspired several studies of larger Pt-DNA-complexes. The studies on model compounds and on large Pt-containing DNA fragments indicate an overwhelming preference of Pt for binding to the N(7) sites of the guanine bases. A detailed geometry of the dominant GpG crosslink has been established both in the model compound *cis*-$Pt(NH_3)_2$[d{pGpG)] and in the Pt-complex with a DNA dodecamer. Interestingly, the dihedral angle between the guanine bases is much smaller in the DNA dodecamer complex (26°) than that in the model compound (80°). On the other hand, the hydrogen-bonding network involving the Pt-coordination sphere in the model compound exists also in the macromolecular system.

So far, the second most abundant Pt-induced crosslink (ApG) has received much less attention. Two model compounds of the type *cis*-$[Pt(NH_3)_2AG]$ show a *head-to-head* orientation of the bases. A detailed structural study of a complex between Pt and the ApG dinucleotide, or of a larger fragment containing the AG sequence, is needed.

In order to understand all aspects of the action of cisplatin as an anticancer agent, further structural research on the complexes between HMG-domain proteins and platinated DNA fragments will be necessary. On the other hand, further studies of small model compounds may furnish several new details of the chemistry controlling cisplatin-DNA interactions.

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Platinum-Sulfur Interactions Involved in Antitumor Drugs, Rescue Agents and Biomolecules

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This chapter describes an overview of Pt-S interactions relevant for the mechanism of action of cisplatin and related Pt-antitumor drugs. There is little doubt that DNA platination is the ultimate event in the mechanism of action of platinum anticancer drugs, and the major adduct formed by attack of cisplatin on DNA is the intrastrand cross-link between N(7)-atoms of two adjacent guanine (G) residues. On its way to the ultimate destination, however, platinum complexes do also interact with many other biomolecules, especially those containing methionine and cysteine residues. In the blood and in the tissues several S-donor ligands are available for kinetic and thermodynamic competition, and so-called 'rescue agents' have been developed to overcome or reduce binding to such groups, thereby reducing the toxic side effects. These rescue agents are assumed to prevent binding of cisplatin to proteins and in tissues to DNA, and in some cases even can revert Pt-protein binding, thereby reducing the toxic side effects, such as kidney toxicity. The most frequently used reagents are discussed. An ideal rescue agent should protect against toxicity and at the same time will not reduce the antitumor activity. Inside the cells, molecules like methionine and glutathione (reduced GSH; oxidized G–S–S–G; in fact the most dominant intracellular S-donor with concentrations typically ranging from 0.5 to 10 mM) do compete with nucleobase for cisplatin. Several recent studies are discussed which have shown that eventually the Pt-binding to Guanine-*N(7)*, but not to Adenine-*N(7)*, is thermodynamically favored. The use of these data as a lead for a strategy towards new cisplatin derivatives (analogs) that do react slower with biological S-donor ligands is finally presented.

Introduction

As clearly written in several chapters of this volume, the story of cisplatin is indeed a success story, given the numerous patients that have been completely cured after cisplatin (or carboplatin) treatment of cancer [1]. This chapter will deal with a special aspect of platinum chemistry, namely its re-

Cisplatin. Edited by Bernhard Lippert

activity with a group of ligands that is not present in the drug, and most probably also not in the final Pt-DNA lesion, but which is very important in the process of drug distribution in the body, in the mechanism of metabolism of the Pt-antitumor compounds, in the therapeutic effect and in the toxic side effects.

Two important *shortcomings* of cisplatin and carboplatin, but also for related drugs are:

1. *Toxicity*, which is thought to be related to (competitive) protein binding of the Pt compounds [2], and might be controlled by combining platinum therapy with so-called rescue agents (usually sulfur-containing ligands). A selection of more or less well-known examples of such rescue agents is given in *Fig. 1*. Due to a lack of understanding in this field, none of them is as yet routinely used in patient treatment. At this moment, the most promising rescue agent appears to be WR-2721 (see below; already registered in a number of European countries [3]). Other recently used agents include mesna (BNP-7787; $[–S–CH_2–CH_2–SO_3]_2$), diethyldithiocarbamate (ddtc), and thiosulfate (sts) [4]. Their chemistry will be discussed later in this chapter.

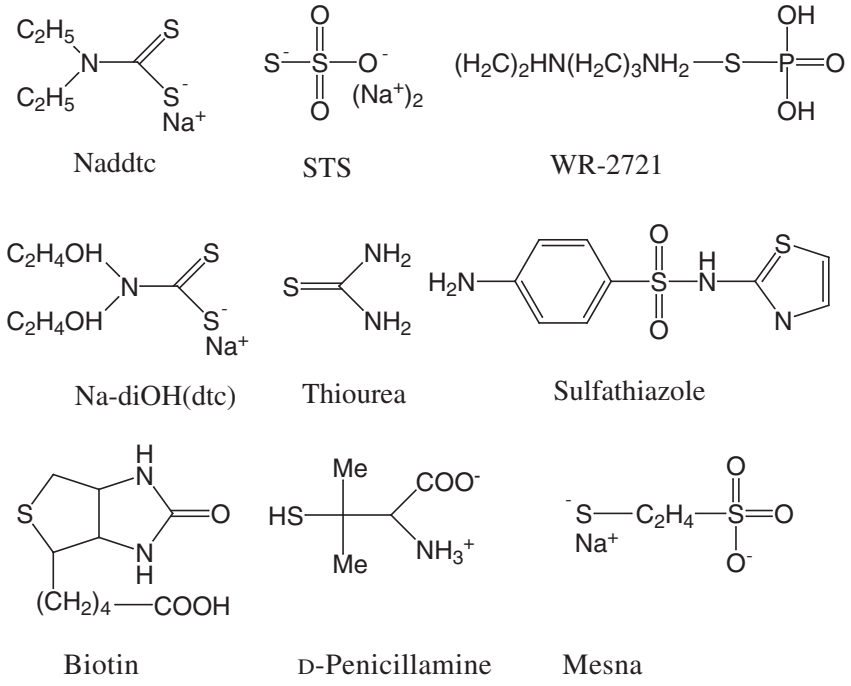


Fig. 1. *Structures of selected potential rescue agents*

2. An equally important challenge is to overcome the *development of resistance* of certain tumors to the first and second generation drugs [5]. This requires the development of a new generation of platinum compounds, lacking cross-resistance with cisplatin and/or carboplatin. Most recently a variety of other drugs, some of which are very promising, have become available and urgently require study; they are discussed in other chapters [6][7].

From the new compounds, in fact, the compound JM-216 (*cis,trans,cis*-$PtCl_2(OAc)_2(NH_3)(C_6H_{11}NH_2)$ is very special, because it can be given orally. In the gastrointestinal tract this drug is metabolized into several active compounds, probably after being reduced to Pt^{II}. Whether S-donor ligands have a key role in this reduction process, or whether other agents like ascorbate play a role, is likely but not yet sure in all cases [7].

Studies of the non-cross-resistant *trans*-compounds will play a crucial role in obtaining a better insight. In this respect the rather recently introduced [8] compound *cis*-$PtCl_2(NH_3)$(2-picoline), also called JM-473 (or AMD-473; or ZD-473) as described elsewhere in this book [7a], needs to be mentioned, as it has clearly been shown that it reduced the reactivity toward ligands, including glutathione [8b].

Even though the mechanism of action of cisplatin and its derivatives is only partly understood, overwhelming evidence strongly suggests that DNA is the ultimate target in cells [9][10], where Pt binds primarily to two adjacent guanine-N(7) sites. Many questions, however, remain and are expected to be the subject of research in the coming decade. To be mentioned are:

1) How does platinum reach DNA?
2) How do the Pt compounds react with rescue agents?

The transport through the cell membranes and possible intermediate binding to proteins both remain largely unknown [11]. Also still poorly understood are the deplatination reactions of DNA, and possible migration of Pt units along the DNA chain [12]. The process leading to cell killing and the role of apoptosis in these sequential events clearly require more study [13].

In this chapter we will mainly focus on questions related to the S-donor ligands. So, how can platinum reach the DNA after administration of the drug, after or despite its reactions with rescue agents, its transport through the cell membrane, and its possible binding to proteins and peptides as an intermediate?

As only the details of the structure of the cisplatin DNA-adduct are known [10][14–17], major challenges originate from the study of the other binding processes. The molecular chemistry of the other chemical reactions *in vivo*, namely those with proteins and peptides in the blood and in the cell,

and those with the rescue agents, is only just beginning to be understood and has hardly been studied at the molecular level.

So, in principle, we have to consider three types of species, all competing for cisplatin, namely, the rescue agents, the peptides and proteins, and the DNA. Although, at present, much highly relevant information is available about Pt-DNA binding, information of other aspects of *in vivo* platinum chemistry has become recently available [18–20]. A review devoted towards the interaction of (new, active, and some relevant inactive) platinum compounds (in model fluids; *in vitro* and *in vivo*) with cellular components (DNA; peptides) and additives (rescue agents) is highly relevant and timely, and the most important results available will be discussed below.

Early accepted key elements in the mechanism for cisplatin are [9][10][14]:

1) controlled hydrolysis, transport and binding to DNA;
2) a specific binding at neighboring guanine bases, and especially at guanine-N(7) positions;
3) a specific distortion of DNA, changing its interactions with proteins.

More recently added observations and conclusions can be summarized as follows:

Structure-activity relationships for Pt compounds have evolved, and it appears that the *cis*-geometry of amines (symmetric, asymmetric, chelating, or not), and the presence of at least one N–H group are necessary [10][14]. Newer platinum complexes have been developed which, in a few cases, deviate significantly from the classical ones. Some contain (tissue-specific) carrier molecules as ligands for achieving higher drug concentrations, or slower release, in (or at the surface of) certain tumor tissues. In other cases they are attached to other chemotherapeutic agents, such as intercalators as co-ligands, to obtain, *e.g.*, a possible synergistic effect [6–8]. Others contain more than one platinum atom connected by a bridge, and even some *trans* compounds [6] have been found to be active.

Many biological molecules may be targets for platinum compounds. Basic coordination chemistry knowledge predicts that S-donor ligands in proteins would rapidly bind and generate the most stable bonds. Also binding to lone-pairs of nitrogen atoms is known to be strong in the absence of S-ligands. Consequently, these types of binding would involve amino-acid side chains from cysteine, methionine, histidine, and also the solvent-exposed N(7) atoms of adenine and guanine in double-stranded DNA. In addition to these N(7) targets, which react with half-lives of a few hours, the N(3) of cytidine and N(1) of adenine would be accessible in single-stranded DNA. More than a decade ago, we proved that a macrochelate Pt(G-N(7))(G-N(7)) can be formed [15], and determined its 3-D structure.

Even double-stranded DNA structures have been determined and DNA has been found to be distorted and kinked at the Pt-binding site [17][21–24].

The textbook principle (HSAB theory) predicting a very strong (and rapid) interaction of Pt ions with S-donor ligands would leave no reactivity for N-donor ligands, with so many S-donors around *in vivo*! Nevertheless, the Pt-antitumor drugs do end up at N(7)-atoms of guanine. Why and how this process can happen will also be addressed in this chapter.

After administration, the drug circulates in the blood, primarily as the chloride (for cisplatin), or as another rather inert form (such as the biscarboxylate in carboplatin). In the blood, also reactions with proteins and rescue agents can take place. Upon passing through cell walls (either actively or passively), intracellular reactions with peptides and proteins may take place, presumably followed by transfer to nucleic acids. Given the strong (kinetic) preference of Pt compounds to react with class-B donor atoms (such as those from thiolates and thioethers), binding to nucleic-acid bases (a thermodynamic end product) must at least occur partially *via* labile intermediates.

Competition studies for Pt-amine compounds with nucleobases and S-donor ligands, such as in *S*-guanosyl-L-homocysteine, have shown that only a transfer from a thioether S ligand to a guanine-N(7) occurs, *i.e.*, for *S*-guanosyl-L-homocysteine and with the nucleopeptides Met-TpG and Met-TpGpG migration takes place to a guanine-N(7) site, showing that S-donor ligands (including rescue agents) may indeed act as intermediates [18][20].

Platinum Complexes and Rescue Agents

Introduction

In the general introductory section, we briefly discussed concentration-dependent toxicity as an important shortcoming of cisplatin. Toxicity has been associated with competitive protein binding of platinum compounds. Side effects of high-dose cisplatin treatment include nephrotoxicity, hematological toxicity, ototoxicity, neuropathy, and seizures [25]. Nephrotoxicity is a major problem in cisplatin treatment, and cisplatin could only become one of the most widely used anticancer agents after the severe nephrotoxicity had been reduced by pre- and post-hydration and mannitol-induced diuresis [26][27]. Nephrotoxicity can also be reduced by the use of the cisplatin analog carboplatin [28]. However, even for this second-generation compound with less nephrotoxicity than cisplatin, toxicity remains a major problem: in fact clinical use of carboplatin is limited by myelosuppression.

In spite of much effort that has been put into reducing the side-effects, toxicity remains a major limitation of the clinical use of platinum complexes in anticancer therapy [26][27][29][30] and several compounds termed 'rescue agents', or 'protective agents' have been investigated for co-administration with platinum compounds in order modulate these side effects of platinum therapy.

On the basis of a similarity in histopathology of the kidney after Hg^{II}- and Pt^{II}-exposure in the rat, it was suggested by *Borch* and *Pleasants* [31] that a similar mechanism might play a role in the nephrotoxicity of these metals (*i.e.*, inactivation of enzymes by the coordination of Hg^{II} and Pt^{II} to thiol residues). Supportive of this mechanism is that the total number of protein-bound thiol groups is depleted (by 14%) in kidneys after *cis*-Pt administration, especially in the mitochondrial fraction [32][33]. This mechanism is also in agreement with an early study by *Aull et al.* [34], who investigated the inhibition of the enzyme thymidylate synthetase by *trans*- and *cis*-Pt *in vitro*. They proved that both isomers do bind to thiol groups of the protein and showed that this interaction could be prevented, but not reversed, by addition of 2-mercaptoethanol. For nephrotoxicity the specific enzyme adenosine triphosphatase, which is critical for kidney function, has been proposed as the site of action [35], although the high concentrations necessary for inhibition are unlikely to be achieved *in vivo*.

The activity of sulfur towards platinum complexes has led to investigation of so-called 'rescue agents' to ameliorate the side effects of platinum therapy, without compromising its anti-tumor activity. These nucleophilic sulfur compounds include sodium thiosulfate (STS), sodium diethyldithiocarbamate (Naddtc), (*S*)-{2-[(3-aminopropyl)amino]ethyl}phosphorothioic acid (WR-2721, *Ethyol*®, amifostine), glutathione (GSH), methionine, thiourea, cysteine, *N*-acetylcysteine, penicillamine, biotin, sulfathiazole, sodium 2-mercaptoethanesulfonate (mesna), and its dimer (di)mesna (BNP-7787). The protective effect of these compounds is either due to prevention, or reversal of Pt-S adducts in proteins. Some of the more promising of the above-mentioned compounds (see *Fig. 1*) will be discussed below.

Short Overview of Investigated Rescue Agents

STS was shown to provide protection from nephrotoxicity when administered in a period between 1 h prior to and 0.5 h after cisplatin injection [36][37]. It has been shown that protein-bound cisplatin cannot be released by STS [38][39][40], although STS is able to break the Pt-thioether bond in methionine model systems [41]. A likely explanation for its protecting effect is that STS is known to be concentrated extensively in the kidney, where

it has been proven to react rapidly with cisplatin, thereby inactivating the drug locally [39][42].

Naddtc is effective in reducing several kinds of nephrotoxicity, as well as bone marrow toxicity, and, when administered 1 h to 4 h after cisplatin, it does not interfere with antitumor properties of *cis*-Pt [43]. This is in agreement with observations that Naddtc could not reverse Pt-DNA cross-links, except for the *cis*-Pt-adenosine 1:1 and 1:2 adducts and the *cis*-Pt-guanosine 1:1 complex; these three adducts have a low prevalence in cisplatin-treated DNA, and their relevance for antitumor activity is not clear [44][45]. Naddtc is capable of reversing the Pt-methionine bond, yet incapable of reversing the Pt-cysteine bond [41][46]. Together with thiourea [47], Naddtc is the only rescue agent that results in protection against nephrotoxicity when administered after cisplatin treatment, at a time when most of the reactive platinum species has already been taken up by cells or has been excreted through the urinary tract. This observation agrees with the hypothesis that Naddtc is actually one of the few real 'rescue agents', acting not only by prevention of protein inactivation, but also by repair of cisplatin-induced damage by dissociating Pt-protein adducts. This theory is also supported by the findings that the enzymes α_2-macroglobulin [48], γ-glutamyltransferase [40][44], and fumarase [38] are inactivated by cisplatin and can be reactivated by Naddtc. One study reports effective protection from kidney damage when Naddtc is administered 12 h before cisplatin treatment [49]. This can be, at least partly, attributed to Naddtc being a potent inducer of the synthesis of metallothionein (MT) [50]. In spite of promising results from animal models, it has not been very successful in the clinic, partly due to its toxicity to the central nervous system [51].

The reactive and rapidly excreted thiol mesna is commonly administered orally or intravenously as a uroprotective agent in the ifosfamide treatment [52], and its potential to reduce cisplatin-induced nephrotoxicity has been investigated. Results of early studies remain controversial and have not unambiguously illustrated its use in reducing nephrotoxicity in animal systems, possibly due to premature inactivation in the blood stream of cisplatin by mesna [53][54]. Recently, research has concentrated more on the disulfide (di)mesna (BNP-7787), which is administered as a prodrug for mesna. BNP-7787 was shown to have a very low toxicity [55], and to undergo an energy-dependent facilitated intracellular transport in the renal and intestinal epithelial cells [56–58]. In the epithelial cells, one molecule of BNP-7787 is believed to be reduced enzymatically to two molecules of mesna by glutathione reductase [57][59], which then locally inactivates cisplatin without hampering antitumor activity [60][61]. The use of BNP-7787 as protective agent is currently under investigation in a Phase I clinical trial [62].

The most important intracellular thiol GSH (glutathione) is present in varying concentrations (0.5 to 10 mM) in cells and has numerous cellular functions, including the detoxification of chemotherapeutic agents, and may play a role in modulating cisplatin cell sensitivity [25][63]. GSH has recently also been shown to protect against cisplatin-induced toxicity in animal models. GSH was administered before cisplatin [64], and another study applied GSH injections prior to and after cisplatin [65a]. A clinical phase I study of cisplatin and GSH has shown that toxicity is significantly reduced in the case of ovarian cancer treatment with cisplatin [65b]. No significant changes in anti-tumor efficiency were observed, but renal toxicity was markedly reduced.

Amifostine

By far the most promising and most frequently studied protective agent is the phosphorylated aminothiol amifostine, WR-2721 or *Ethyol*®. Amifostine has recently been registered in several European countries [3], and was originally developed as a radioprotective agent by the United States Army. It has shown protection of normal tissues from the cytotoxic effects of therapeutic radiation, as well as chemotherapy, with preservation of effect on the tumor. Clinical trials (including phase III trials) showed significant decreases in hematologic, renal, and neurologic toxicity without effecting the response rates to cisplatin treatment [66–68], and reduced duration of thrombocytopenia and hospitalization without interfering with the anti-tumor activity of carboplatin treatment in phase I and II clinical trials [69][70].

Much effort has been devoted to understanding the mechanism by which WR-2721 reduces nephrotoxicity. It is generally agreed that WR-2721 acts as a prodrug which is transformed into the active species WR-1065 when dephosphorylated by the membrane protein alkaline phosphatase (*Fig. 2*).

The so-formed uncharged free thiol species WR-1065 is generally accepted to be the actual species taken up by the cell and passes the membrane by passive diffusion [71]. The selective uptake of WR-1065 in normal cells might be related to the lower levels of alkaline phosphatase of tumor cells [72]. Additionally the neutral pH of normal cells compared with the slightly acidic pH of tumors could favor selective activation [73]. WR-1065 also can be oxidized further to form disulfides, *i.e.*, the dimerisation product WR-33278, or mixed disulfides of WR-1065 with endogenous thiols of peptides and proteins. These disulfides have been suggested to serve as an exchangeable pool of WR-1065 [3]. Experiments using model systems indicate that WR-1065 substitutes the Pt-methionine bond much slower than the more nucleophilic modulating agents STS and Naddtc [74], but is a very potent

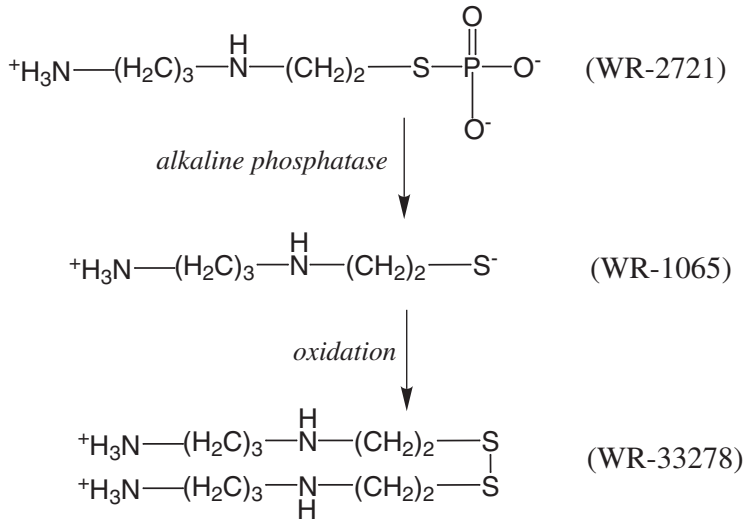


Fig. 2. *Generally accepted dephosphorylation and disulfide formation of WR-2721*

inhibitor of DNA platination [75]. This suggests that WR-1065 protects through direct interaction with cisplatin and in this way prevents toxicity. Pharmacokinetics of WR-2721 and its metabolites have been investigated in order to optimize the administration protocol. WR-2721 is rapidly cleared from the blood (half-life of 0.8 h) due to the fast conversion into WR-1065. This is in turn cleared from the plasma with a half-life of a few hours, which can be explained by the fast uptake in tissue and the formation of disulfides that are detectable for over 24 h [3].

WR-2721 is quite close to application although not yet used routinely in cisplatin treatment, as a standard protocol has not yet been agreed on. In a recent study, WR-2721 was initially administered 15 min before administration of the platinum complex and dramatically reduced the side effects. Two additional infusions were then given at 2 and 4 h thereafter [3]. As WR-2721 is generally also well tolerated by patients [76a], it is a promising modulating agent to ameliorate the side effects of platinum therapy; it not only allows a better patient tolerance of current regimes, but also potentially allows improved anti-tumor efficacy through possible dose escalation. Despite the promising outcomes of the above-mentioned clinical studies, as yet all studies were conducted on a limited number of patients and especially randomised clinical trials need to be expanded in order to establish the use of amifostine in standard cisplatin chemotherapy. In this respect it should be mentioned that, in mice, the antitumor effect of carboplatin is clearly enhanced by amifostine [76b].

Competition Studies for Pt-Amine Compounds with S-Donor Ligands and Nucleobases

Introduction and Early Indications of Importance of Pt-S Binding

Traditionally, interactions of platinum with sulfur-containing biomolecules have only been associated with negative phenomena as resistance and the above-discussed toxicity [77]. In this section we will discuss a possible beneficial effect of Pt-sulfur interaction on the anti-tumor activity of platinum compounds. The high affinity of platinum compounds for sulfur atoms, and the great abundance of sulfur-containing biomolecules in the cytosol and nucleus of the cell has raised the question whether Pt-sulfur interactions could serve as a drug reservoir for platination at DNA, thus affording an additional pathway towards platination of DNA [14]. Two reaction paths are possible, *i.e.*, the spontaneous release of platinum from the sulfur followed by a subsequent reaction with a DNA fragment, or the direct nucleophilic displacement of platinum from sulfur by the guanine-N(7) group. Several studies on model systems used to assess the viability of this idea are reviewed here. Competition has been studied intramolecularly in systems where the competing sulfur atom and the nitrogen donor are present in the same molecule. Other studies investigated intermolecular competition. Both types of study show that although Pt-sulfur interactions are kinetically preferred, the binding of Pt with guanine-N(7) is often thermodynamically favored.

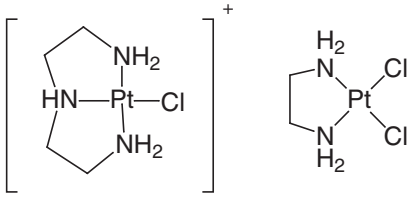
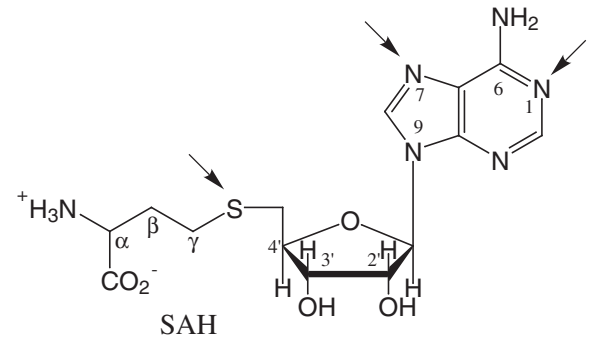


Fig. 3. *Structural formulae of* $[Pt(dien)Cl]^+$ *and of* $Pt(en)Cl_2$

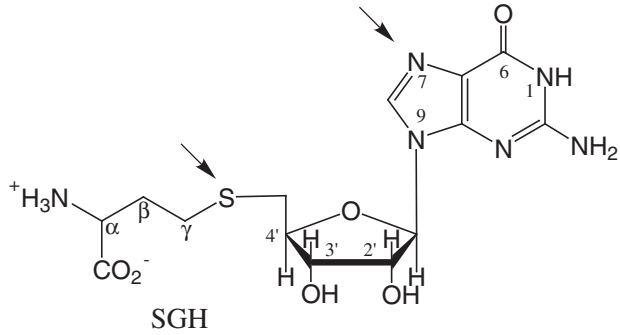
To study this process, a very suitable compound appeared to be [Pt(dien)Cl]Cl (dien = 1, 5-diamino-3-azapentane; *Fig. 3*). This compound is readily available, forms relatively stable complexes with S-donor ligands, has only one substitution step, and is not complicated by (often occurring) subsequent reactions caused by the *trans*-labilizing effects of S-donor ligands.

Intramolecular Competition Studies

S-*Adenosyl*-L-*Homocysteine*. As a first study in this field a relatively easily available ligand with two functionalities was selected by *Lempers* and *Reedijk* [78]. The molecule *S*-Adenosyl-L-homocysteine (SAH, *Fig. 4*) was employed to study the intramolecular competition between a sulfur-containing amino acid moiety and the nitrogen atom of a nucleobase. Although this first study showed no coordination to the adenine N(1)- or N(7)-atom, a pH-dependent migration was observed of the platinum atom from the sulfur atom to the amine group of the cysteine moiety and vice versa [78]. In a 1:1 reaction at pH < 7, SAH was platinated only at the sulfur atom to form [Pt(dien)(SAH-*S)*]$^{2+}$($t_{1/2}$=75 min for 5 mM concentrations). At pH > 7 this product spontaneously isomerizes rapidly ($t_{1/2}$= 10 min) to [Pt(dien)(SAH-*N)*]$^{+}$ in which platinum is coordinated to the amine of the cysteine group. Furthermore this pH-dependent migration was shown to be reversible at pH < 5 ($t_{1/2}$= 2 h). When SAH was reacted with two equivalents of [Pt(dien)Cl]Cl, the dinuclear complex [{Pt(dien)}$_2$(SAH-*S,N)*]$^{3+}$ was formed in which both the sulfur atom and the amine are platinated.



SAH



SGH

Fig. 4. *Structures of SAH and SGH*

S-*Guanosyl*-L-*Homocysteine*. Because no participation in coordination of the nucleobase could be observed when SAH was used as a model system for intramolecular competition, the molecule *S*-guanosyl-L-homocysteine (SGH, *Fig. 4*) was synthesized as a model compound to study intramolecular competition [18]. SGH was reacted with [Pt(dien)Cl]Cl to allow a direct, intramolecular comparison of the reactivity of the sulfur atom with N(7) of the more reactive guanine. The species $[Pt(dien)(GSH\text{-}S)]^{2+}$ formed upon reaction of SGH with one equivalent of [Pt(dien)Cl]Cl at $2 < pH < 6.5$ ($t_{1/2}$= 2 h, 319 K). This complex was found to isomerize slowly to $[Pt(dien)(SGH\text{-}N(7))]^{2+}$ with coordination at N(7) of guanine ($t_{1/2}$= 10 h, 310 K). Addition of a second equivalent of [Pt(dien)Cl]Cl yielded $[(Pt(dien))_2(SGH\text{-}S,N(7))]^{4+}$. Formation of these complexes is schematically depicted in *Fig. 5*. These complexes only formed in the range $2 > pH > 6.5$; at higher pH values the dehydronated amino group is able to coordinate to platinum as well, giving rise to additional complexes with NH_2 coordination. In this study [18] it was shown for the first time that the N(7) donor-atom can indeed intramolecularly replace the sulfur atom in a platinum-sulfur adduct. This was in fact the first evidence supporting the hypothesis that protein-bound platinum could potentially act as a drug reservoir.

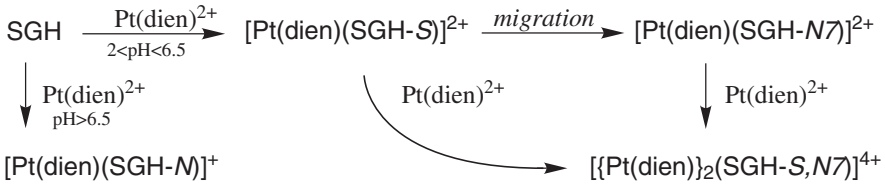


Fig. 5. *The formation of (dien)Pt^{II} complexes of SGH at pH < 6.5*

Nucleopeptide Models: Met-d(TpG)$^-$ and Met-d(TpGpG)$^{2-}$. To extend the above study and to diminish the influence of the pH on the competition studies, two nucleopeptide models Met-d(TpG)$^-$ and Met-d(TpGpG)$^{2-}$ (*Fig. 6*) were synthesized [79] as the next generation. These two models contain a methionine linked *via* its amino function to the 5′-end of a nucleotide moiety. Through this carbamate linkage coordination at the amino function is now avoided, making these nucleopeptide models suitable for pH-independent competition studies. In addition the thioether sulfur donor now mimics the methionine residues of proteins more closely. The nucleotide moiety of these nucleopeptide models consists of d(TpG) and d(TpGpG). The phosphodiester backbone affords closer resemblance to natural DNA than SGH and SAH, giving the model compounds a negative charge and also allowing possible additional hydrogen-bonding interactions with the DNA backbone.

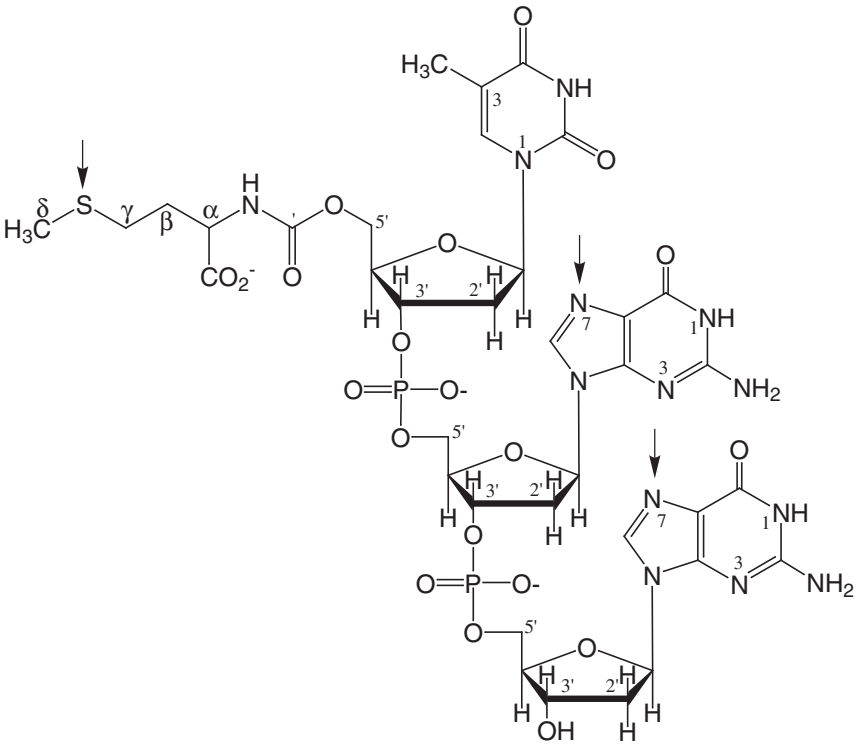


Fig. 6. *The structure of the nucleopeptide Met-d(TpGpG)*

The reactions of Met-d(TpG)$^-$ with the platinum complexes [Pt(dien)Cl]Cl and the cisplatin analogue Pt(en)Cl_2 (en = ethane-1,2-diamine, *Fig. 3*) were investigated at pH 7 and 310 K and monitored with ^{1}H- and ^{13}C-NMR [20]. The chemical shifts and relative intensities of the H(8) signal of guanine and the SCH_3 signals provided information on the course of these reactions. *Fig. 7* describes the formation of products of the reaction of Met-d(TpG)$^-$ with monofunctional [Pt(dien)Cl]Cl and bifunctional Pt(en)Cl_2. For this reaction initial coordination to the thioether function was clearly observed, and the formation of the complex [Pt(dien){Met-d(TpG)}-*S*]$^+$ was found to be complete within two hours. After prolonged incubation, however, this complex slowly isomerized, and after 6 days no Pt-S adducts could be detected, as all platinum was found to be coordinated to the N(7) of guanine, resulting in the complex [Pt(dien){Met-d(TpG)}-*N(7)*]$^+$. Repeating this experiment with two equivalents of [Pt(dien)Cl]Cl yielded [{Pt(dien)}$_2${Met-d(TpG)}-*N(7),S*]$^{3+}$, in which both the thioether and the N(7) are platinated. Within 2 h, platination of the sulfur was found to be completed, whereas subsequent platination of N(7) proceeded much slow-

er, and was found to be completed after 15 h, illustrating the kinetic preference of platinum for the sulfur donor atom over the N(7) of the purine base.

When the nucleopeptide Met-d(TpG)$^-$ was reacted with the bifunctional compound Pt(en)Cl_2, coordination of the platinum to the sulfur was slower than for [Pt(dien)Cl]Cl and appeared to be completed within 10 h ($t_{1/2}$= 100 min). Platination of the N(7) of the guanine was detected at the same rate. The nearly simultaneous decrease of signals corresponding to the free SCH_3 and H(8) in the ^{1}H-NMR spectrum indicates the platination of the thioether to be the rate-determining step, followed by a fast chelation step yielding a macrochelate between the sulfur and the N(7) of the guanine moiety, [Pt(en){Met-d(TpG)}-*N(7),S*]$^+$. This chelate is stable and no displacement of the S-bond thioether by N(7) was observed, even in the presence of unreacted nucleopeptide [82].

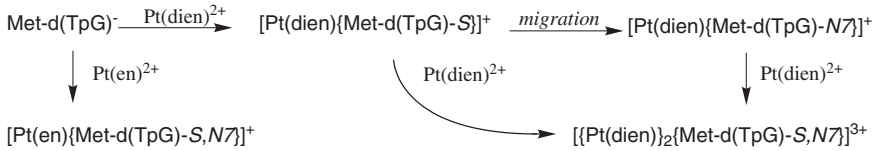


Fig. 7. *Product formation and interconversion in the reaction of Met-d(TpG) with Pt^{II}(dien) and Pt^{II}(en)*

The results of the competition experiments with Met-d(TpG)$^-$ and [Pt(dien)Cl]Cl were found to be in fair agreement with previous experiments using glutathione (GSH) [20], in that the thioether in a platinum-sulfur adduct can be substituted by the N(7) of guanine. The reaction with Pt^{II}(en), on the other hand, results in the formation of a stable *S,N(7)* chelate, even in the presence of free N(7). This seemingly contrasting finding, however, is in agreement with various reports that *S,N(7)* chelates are remarkably stable [80].

To investigate the competition between the sulfur atom and the highly reactive GpG sequence we extended this study and used the nucleopeptide model Met-d(TpGpG)$^{-2}$. Cisplatin is well known to preferentially bind to the N(7) atoms of two neighboring guanines in DNA [81].

Reacting Met-d(TpGpG)$^{2-}$ with [Pt(dien)Cl]Cl [82] initially yielded platination at the sulfur atom with a similar rate as was observed for Met-d(TpG), resulting in [Pt(dien){Met-d(TpGpG)}-*S*] . Platinum coordination was subsequently taken over by the N(7)'s of both guanines resulting in the formation of the monofunctional complexes [Pt(dien){Met-d(TpGpG)}-*N(7)(5′G)*] and [Pt(dien){Met-d(TpGpG)}-*N(7)(3′G)*] . When an extra equivalent of (dien)Pt^{II} was added, the final end product was the complex [{Pt(dien)}$_2${Met-d(TpGpG)}-*N(7)(5′G), N(7)(3′G)*]$^{2+}$, in which both guanines had been platinated (*Fig. 8*), but with a small preference for the 3′G. Studies with Pt^{II}(en) and this nucleopeptide are ongoing.

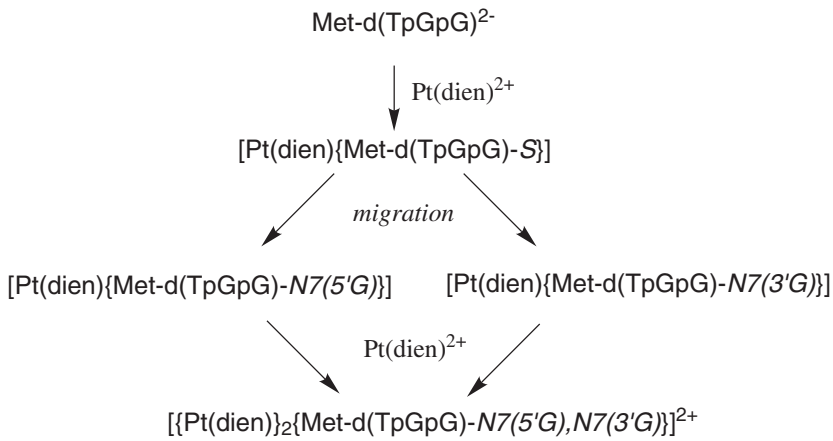


Fig. 8. *Product formation and interconversions in the reaction of Met-d(TpGpG) with* Pt^{II}*(dien)*

Intermolecular Competition Reactions

General considerations. The competition experiments described above have illustrated the possibility of N(7) platination of DNA *via* Pt-S intermediates. These studies also showed the great stability of *S*,*N* chelates of bifunctional platinum complexes. All of the above-discussed intramolecular competition studies were performed on models containing both the thioether function and the N(7) in the same molecule, thus in relatively close proximity.

In the next stage, intermolecular competition studies were conducted, using either methionine or methylated gluthathion (GSMe) as the sulfur-containing model and guanosine 5′-monophosphate (5′-GMP) and guanylyl (3′-5′)guanosine (dGpG) as N(7)-containing models for DNA (*Fig. 9*).

Displacement of Pt-S-Bound L-*Methionine by 5′-GMP.* In an effort to understand why cisplatin reacts with 5′-GMP even in the presence of methionine, *Barnham et al.* [83] performed a competitive reaction experiment between [Pt(dien)Cl]Cl, L-methionine, and 5′-GMP. They observed that, in the first 40 hours of the reaction, the methionine was platinated to yield $[Pt(dien)(Met\text{-}S)]^{2+}$, whereas only little of the 5′-GMP had reacted. In the latter stages of the reaction, this complex disappears as the platinum binds to 5′-GMP, resulting in the complex $[Pt(dien)(5'\text{-GMP}, N(7))]^{2+}$ and free methionine. The kinetics of the displacement reaction were studied and the half-life of the reaction was determined to be 167 h at 310 K. The calculated $H^{\ddagger}$ and $S^{\ddagger}$ values for this displacement reaction are indicative of a sub-

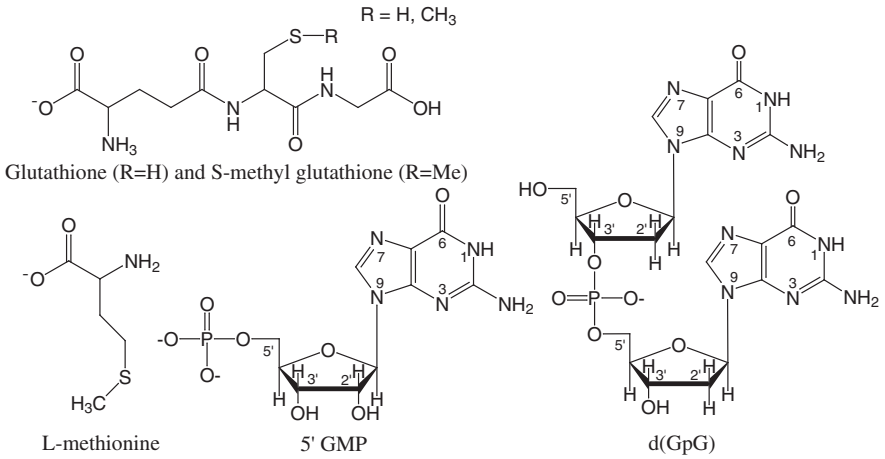


Fig. 9. *The structures of* L-*methionine, GSMe, GSH, 5′-GMP and dGpG used as model compounds in intermolecular competition reactions*

stitution mechanism at platinum *via* an associative mechanism (five-coordination transition state). The complex $[Pt(dien)(Met\text{-}S)]^{2+}$ was also reacted with adenosine 5′-monophosphate (5′-AMP), thymine 5′-monophosphate (5′-TMP), and cytosine 5′-monophosphate (5′-CMP), and no reaction was observed within 12.6 h. Also the N of the imidazole proved unable to displace platinum in the reaction between $[Pt(dien)(Met\text{-}S)]^{2+}$ and histidine [83].

Displacement of Pt-Bound GSMe by 5′-GMP. A similar study was undertaken to study the platination of 5′-GMP by the Pt-S adduct $[Pt(dien)(GSMe\text{-}S)]^{2+}$ [79]. This study employed the thioether-containing tripeptide GSMe (*Fig. 9*) and confirmed the intermolecular displacement of the thioether in a Pt-S adduct by 5′-GMP. The kinetics of this reaction were investigated, and the intermolecular rearrangement reaction of Pt^{II}(dien) to 5′-GMP proceeds slowly at 293 K ($t_{1/2}$ = 179 h). At 308 K the reaction proceeds faster ($t_{1/2}$ = 31 h). The arrangement was observed only with guanine, and not with adenine. Furthermore, the displacement of sulfur in the Pt-S adduct was only observed for the thioether-containing GSMe; when the reaction was performed with glutathione (GSH, *Fig. 9*), no displacement by the N(7) was observed. These findings confirm the possibility that platinum-sulfur adducts may serve as a drug reservoir; however, the existence of such a reservoir appears to be limited to Pt-thioether type adducts.

Reactions of N-*acetyl*-L-*Methionine Complexes of* Pt^{II}*(en) with 5′-GMP and dGpG.* Recently, intermolecular competition was studied in more de-

tail for the reactions of the bifunctional Pt^{II}(en) [19]. The complex [Pt(en)(MeCO-Met-*S*)Cl]NO_3 was prepared and reacted with 5′-GMP and dGpG. In the initial stages of these reactions an intermediate chelate complex [Pt(en){MeCO-Met-*S,N*)] is observed which reacts *via* a ring-opening reaction with 5′-GMP and dGpG to form the monofunctional mixed-ligand complexes [Pt(en){MeCO-Met-*S*}{5′-GMP-*N(7)*}]$^+$ and [Pt(en){MeCO-Met-*S*}{dGpG-*N(7)*}]$^+$, respectively, in which the chloride is replaced by N(7).

These monofunctional adducts were found to be very stable, but very slow displacement of the (MeCO-Met) was observed when the monofunctional 5′-GMP adduct [Pt(en){MeCO-Met-*S*}{5′-GMP-*N(7)*}]$^+$, was left to react with another molecule of 5′-GMP to form the *bis* complex [Pt(en)(5′-GMP-*N(7)*)$_2$]$^{2+}$. Displacement of ([Pt(en){MeCO-Met-*S*}) was also found to be slow for the monofunctional adducts of dGpG, [Pt(en){MeCO-Met-*S*}{dGpG-*N(7)*}]$^+$, in which either the 3′G or the 5′G was platinated. The stablility of these monofunctional adducts indicate that GpG chelate formation is not a driving force for this displacement reaction.

In this study the *bis*-complex [Pt(en)(MeCO-Met-*S*)$_2$][NO_3]$_2$ was also prepared, and the *S*-bound MeCO is more rapidly displaced from this complex by either 5′-GMP or dGpG, giving rise to the formation of the stable related monofunctional adduct. The reactions of the complexes [Pt(en)-(MeCO-Met-*S*)Cl]NO_3 and [Pt(en)(MeCO-Met-*S*)$_2$][NO_3]$_2$ with 5′-GMP are summarized in *Fig. 10*.

Competition in Methionine-Containing Di- and Tripeptides

Our knowledge of the interaction between platinum complexes and sulfur-containing peptides, and the competition between *S* and *N* donor-atoms in these systems, is still rather limited. *Siebert* and *Sheldrick* [84] investigated the pH-dependent competition between *N,S* and *N,N′* chelation in the reaction of [Pt(en)(H_2O)$_2$]$^{2+}$ with methionine containing di- and tripeptides. They showed that in met-Hgly and met-gly-Hgly, peptides in which the methionine is located at the amino terminus, the κ^2N (amino), *S* (thioether) chelation mode was found to dominate at pH < 8.6, whereas the κ^2N (amino), *N′* (amide) dominates at higher pH. For the peptides gly-Hmet and gly-met-Hgly the κ^2 *N′* (amide), *S* (thioether) chelate is observed, while at high pH (> 7.4) the κ^2N (amino), *N′* (amide) chelate dominates for these peptides as for the peptides with the N-terminal methionine. For the tripeptide gly-gly-Hmet only one major product is observed: the κ^2 *N″* (amide), *S* (thioether) chelate. This product is stable at pH < 10.6, and no κ^2N (amino), *N′* (amide) chelate was observed. This study illustrates the importance of *N,S*

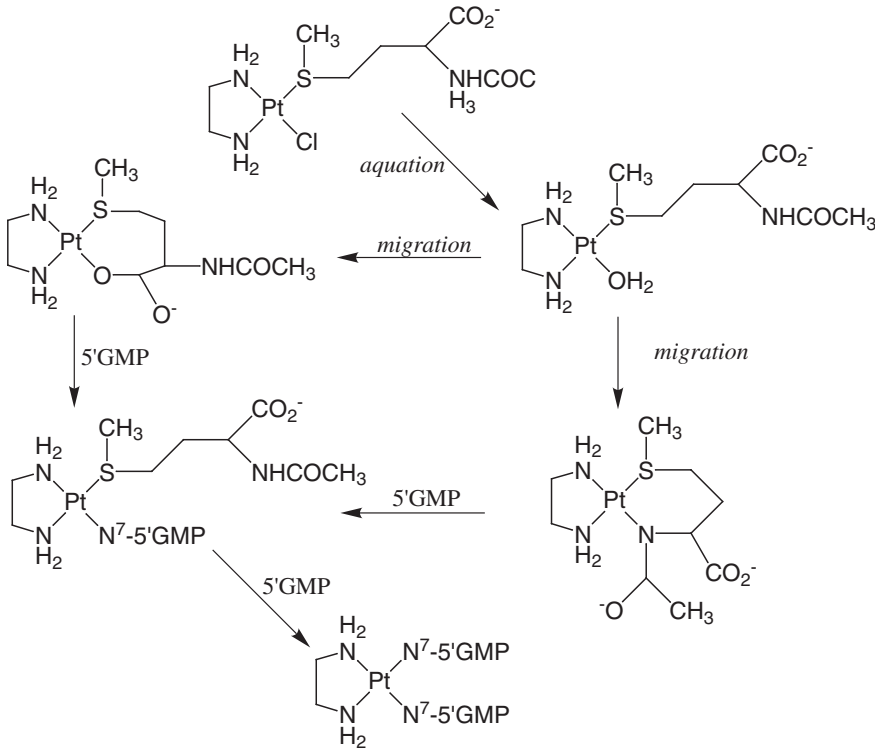


Fig. 10. *Reactions of [Pt(en)(MeCO-Met-S)Cl]NO_3 and [Pt(en)(MeCO-Met-S)$_2$][NO_3]$_2$ with 5′-GMP* (adapted from [19])

chelates and their sequence- and pH-dependent conversion to *N,N′* chelates for the reaction of platinum complexes with methionine-containing biomolecules.

Conclusions and Outlook

An exciting question that evolves from the above results deals with why thiols react so differently compared with thioethers. Clearly, the neutral thioether has a very high affinity for the square-planar Pt^{II} ion, although the thermodynamic stability is not very high, as the bond can be reverted and changed to guanine-N(7), which is formed more slowly, but which appears to have a higher thermodynamic stability, probably caused by the additional H-bonding interactions (which are absent in corresponding adenine-N(7) species).

That thiols present *in vivo* do not eliminate the antitumor activity is also not understood. Again a key role for the thioether binding may be the origin of this, when it is assumed that the rapid thioether binding protects (or at least slows down) the Pt species from the attack by thiols.

In this respect it should be mentioned that Pt-methionine species are generally not antitumor active! However, methionine is known to play an important role in the metabolism of cisplatin. The bischelate [Pt(Met-*S,N*)$_2$] has been isolated form the urine of patients treated with cisplatin [85]. On the other hand, intermolecular competition studies have shown that formation of a bifunctional G-N(7),G-N(7) adduct is possible for the cisplatin analog $PtCl_2$(en)[19][86].

From the studies reviewed above it has become evident that competion studies between thiols/thioethers and intact double-helical DNA are required, to find out whether or not the formation of the Pt-GG chelate is a driving force that can overcome the Pt-S interactions. Even then, one should realize that we are only dealing with a model, as in the cell other metals might also play a role in the disruption of Pt-S bonds. In this respect it should be mentioned that it was recently reported that addition of transition metals such as Zn^{II} or Cu^{II} can cleave even the Pt-S bond in thiolated terpyridine-platinum complexes at neutral pH [87].

Possible New Pt-Drugs Based on Knowledge of Pt-S Intercations

Given the knowledge of the subtle balance between Pt-S and Pt-N binding in biological systems, the possibility of drug design based on this knowledge may soon come closer. A critical process is the delivery of the Pt species at the DNA, where it must stay long enough to play its biological roles of preventing cell division and surviving DNA repair. On its route from injection/infusion (or oral absorbance) to the DNA, the Pt compound has to survive many attacks of S-donor ligands. In certain cases, as shown above, such an S-donor ligand can also be beneficial in preventing early toxic side effects.

So one could in general consider the following types of new, or improved drugs, *i.e.*,

a) new drugs that do not contain S-donor ligands but that do react – *in vivo* – slowly with competing S-donor ligands. Compounds of this type might be, *e.g.,* the earlier mentioned compound *cis*-$PtCl_2(NH_3)(C_6H_{11}NH_2)$, JM-473 [8][88][89]; also targetting, *e.g.*, with intercalators to target to DNA, might result in a more rapid DNA binding and less loss by S-donor binding [90],

b) new drugs which do contain S-donor ligands of optimal kinetic and thermodynamic stability, as discussed above [91–93],

c) new Pt^{IV} compounds that need to be activated *via* reduction, for instance using cellular thiol compounds [7][94].

In general terms, both steric effects and electronic factors are expected to play a role in determinating the reactivity of square-planar platinum complexes. The presence of planar amine ligands in *cis*- or *trans*-$Pt(anion)_2$ complexes and their orientation with respect to the coordination plane, as well as their substituents, can reduce the rates of DNA binding or thio binding compared to aliphatic ammine and amine complexes. Especially, substituents close to the coordination site should be expected to slow down axial substitution reactions at Pt. As there is now little doubt that DNA platination is a key event (or THE key event) in the mechanism of action of platinum anticancer drugs, attention to the process of formation of the major adduct (GG) as an intrastrand cross-link between N(7) atoms of two adjacent guanine (G) residues, will remain important.

Final Remarks

Research in the last decade has made clear that the toxic side effects of platinum compounds have an exciting molecular basis. It has stimulated the research activities dealing with Pt compounds and rescue agents (usually S-donor ligands) and especially the study of the reactions of these compounds in combination with other cellular components and their complicated cell-wall transport.

From the results summarized and discussed in this review chapter, it should be clear that many challenges do remain for the future. To be mentioned are answers to the following scientific questions:

1) Do direct chemical interactions occur between rescue agents and platinum compounds (such as the drugs cisplatin and carboplatin; transplatin), and between the relevant model compounds (such as Pt^{II}(dien), or perhaps the kinetically faster reacting Pd^{II} compounds)? Which interaction products are formed *in vivo* (structure, kinetics)? This topic has been largely neglected in the literature.

2) Assuming that such interactions do indeed occur, can these be influenced by the reaction conditions: pH, time, isomer (*cis*/*trans*), co-ligands, other ligands, buffer influence?

3) Do such Pt-rescue-agent interactions (and the resulting products) interfere with the binding of the Pt compounds in cells (especially with nucleic acids and/or proteins)?

4) Can rescue agents and sulfur-containing peptides cause *in vivo* deplatination of DNA and proteins? Do such interaction products still have antitumor activity on their own, and may this knowledge be used to the development of new drugs?

5) Could other physiologically relevant metal ions like Zn^{II} interfere with the processes of platination and deplatination of proteins and nucleic acids?

To answer these and related questions will require a highly original and innovative approach, and also input from the medicinal, biological and toxicological field. Research in the next 30 years undoubtedly will answer many of these questions, leading to further steps forward in the better understanding of reactions of metal-containing drugs in general, and at the same time leading to better drugs and improved drug administration.

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Diammine- and Diamineplatinum Complexes with Non-Sulfur-Containing Amino Acids and Peptides

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The most stable complexes between diammine- or diamine-platinum(II) entities and amino acids and peptides with no side chains, or with carboxylic acid side chains, involve five-membered *N,O*-chelate rings, while the most stable complexes of histidine and derivatives have six-membered chelate rings involving coordination of the amine (or amide) nitrogen atoms and imidazole N(3). Many other coordination modes are, however, observed as kinetic products, depending on the reaction conditions. Although free diammineplatinum complexes in plasma or cytoplasm would appear unlikely to bind to such molecules in preference to sulfur-containing nucleophiles or nucleobases, bridging by platinum compounds between DNA and proteins in close proximity to DNA may well be important biologically. Such bridging is modelled by 'ternary' diammine/amino acid/nucleobase complexes.

Abbreviations

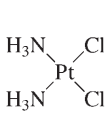
The acidic hydrogen atoms in the neutral molecules are italicised. The formulae give the usual zwitterion form. Atom numbering used in the text is indicated, where applicable.

H_2acgly	*N*-acetylglycine, $CH_3C(O)NHCH_2CO_2H$
H_3achis	*N*-acetylhistidine, $CH_3C(O)NHCH(CO_2^-)CH_2(C_3H_2N_2H_2^+)$
H_2asp	aspartic acid, $^+NH_3CH(CO_2^-)CH_2CO_2H$
dach	1,2-diaminocyclohexane
dien	diethylenetriamine, $HN(CH_2CH_2NH_2)_2$
H_2digly	*N*-glycylglycine, $^+N(1)H_3CH_2C(O(1))N(2)HCH_2CO(2)O(3)^-$
HdiglyOEt	*N*-glycylglycine ethyl ester, $N(1)H_2CH_2C(O)N(2)HCH_2C(O)OC_2H_5$
en	1,2-diaminoethane, $NH_2(CH_2)_2NH_2$
H_2glu	glutamic acid, $^+NH_3CH(CO_2^-)(CH_2)_2CO_2H$
Hglyam	glycinamide, $N(1)H_2CH_2C(O)N(2)H_2$
Hgly	glycine, $^+NH_3CH_2CO_2^-$
H_2his	histidine, $^+N_AH_3CH(CO_2^-)CH_2(C_3H_2N_2H)$
H_2mal	malonic acid, $H_2C(CO_2H)_2$
tpy	2,2′,6′,2″-terpyridyl
H_3trigly	*N*-(glycylglycyl)glycine, $^+NH_3CH_2C(O)NHCH_2C(O)NHCH_2CO_2^-$

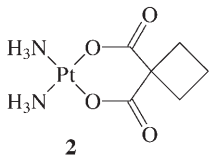
Cisplatin. Edited by Bernhard Lippert

Introduction

Biological fluids such as blood, plasma, and cytoplasm, contain many small molecules which are potential ligands for Pt^{II}, including free amino acids and small peptides. This review focuses on biomolecules of this type which are present *in vivo*, excepting those containing a thioether or thiolate group, which are dealt with elsewhere in this book. Most attention will be given to *cis*-diammineplatinum complexes, since the widely-used platinum drugs cisplatin (**1**) and carboplatin (**2**) are of this type.



1



2

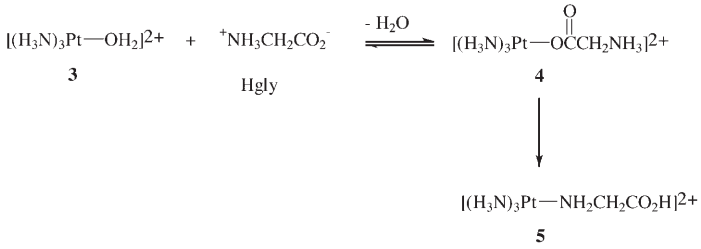
Some reviews related to aspects of this topic have been recently published [1][2].

Complexes with Glycine and Poly(glycine) Peptides

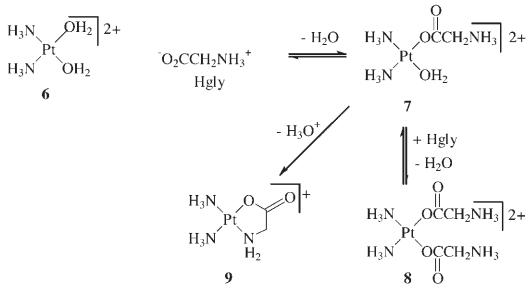
Even the simplest amino acid, glycine ($^{+}NH_3CH_2CO_2^{-}$, Hgly), can bind to a metal ion in several different ways: monodentate through either nitrogen or oxygen, chelating through both of these atoms, or bridging between metal ions, either through nitrogen and oxygen, or through the two carboxylate oxygen atoms. When only one coordination site is available at platinum, as in $[Pt(NH_3)_3(H_2O)]^+$ (**3**), the final product from reaction with glycine under any conditions is one in which glycine is nitrogen-bound, $[Pt(NH_3)_3(Hgly\text{-}N)]^{2+}$ (**5**). If glycine is added to **3** in weakly acidic solution, the initial product contains oxygen-bound glycine, $[Pt(NH_3)_3(Hgly\text{-}O)]^{2+}$ (**4**) (*Scheme 1*). This occurs because glycine nitrogen (pK_a 9.8) remains protonated in acid solution, while the carboxyl group (pK_a 2.35) is deprotonated. However, even in acid solution, there is a slow irreversible isomerization from *O*- to *N*-bound glycine complex (*Scheme 1*) [3]. The isomerization is faster at higher pH.

The reaction of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**), a hydrolysis product of cisplatin, with glycine under mildly acidic conditions, likewise leads to complexes **7** and **8** in which glycine is bound monodentate through oxygen only (*Scheme 2*). An isomerization occurs, slowly in acidic solution, faster at higher pH, to give the thermodynamically more stable chelate complex **9** [3–5].

Scheme 1

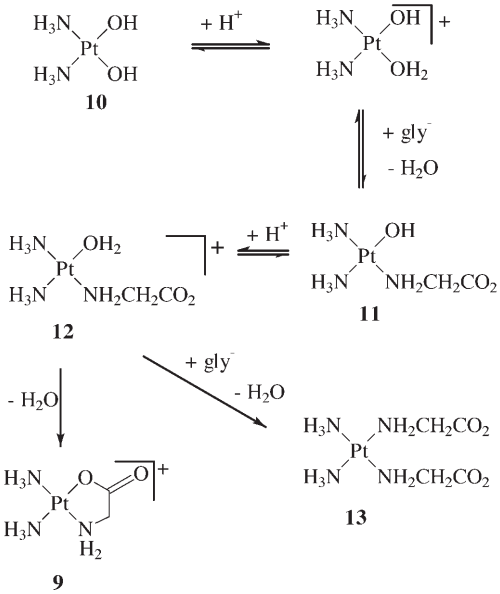


Scheme 2



cis-$Pt(NH_3)_2(OH)_2$ (**10**) does not react with glycinate at pH 12.8. The Pt-OH bond is inert toward reaction with N- or O-donor ligands. Slow reaction does occur at pH 9–11, presumably through traces of *cis*-$[Pt(NH_3)_2(OH)(H_2O)]^+$ in equilibrium with **10** (*Scheme 3*), to give initially

Scheme 3

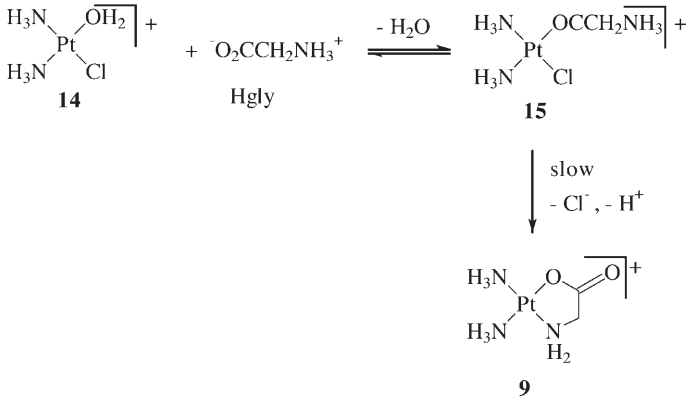


cis-[Pt(NH_3)$_2$(gly-*N*)(OH)] (**11**). Ring closure to [Pt(NH_3)$_2$(gly-*N,O*)]$^+$ (**9**) is slow under these conditions, proceeding through traces of *cis*-[Pt(NH_3)$_2$(gly-*N*)(H_2O)]$^+$ (**12**), but is rapid if acid is added to decrease the pH to 6. The major product of the reaction at pH 9–11 is ultimately *cis*-Pt(NH_3)$_2$(gly-*N*)$_2$ (**13**), produced by reaction of excess glycinate with both **9** and the traces of **12** present [5].

It has long been known that the products of the reaction of cisplatin (**1**) with glycinate are the chloride salt of **9**, *cis*-[Pt(NH_3)$_2$(gly-*N,O*)]Cl, and, with excess glycinate, *cis*-Pt(NH_3)$_2$(gly-*N*)$_2$ (**13**) [6][7]. *Pivcová et al.* [8][9] have shown that these products are obtained when **1** reacts with glycine under physiological conditions. The crystal structure of the *N,O*-chelate complex **9** has been determined [10].

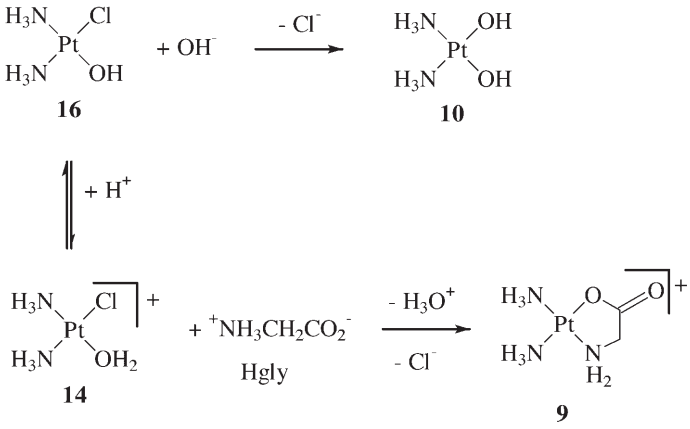
The reaction of cisplatin (**1**) with glycine is expected to proceed *via* the hydrolysis product *cis*-[Pt(NH_3)$_2$Cl(H_2O)]$^+$ (**14**). In acid solution, the complex *cis*-[Pt(NH_3)$_2$Cl(Hgly-*O*)]$^+$ (**15**) may be obtained in solution by addition of glycine to a solution of **14**. Slow ring closure to **9** then occurs (*Scheme 4*). However, **15** has not been observed as an intermediate in the reaction of **1** with glycine in acid, because the ring closure reaction of **15** is fast compared with the hydrolysis of **1** to **14** [3]. Under physiological conditions, **14** is expected to be largely deprotonated to *cis*-Pt(NH_3)$_2$Cl(OH) (**16**) [11][12]. *Miller* and *House* [13] studied the reaction of **16** with glycine at pH 7.4 by spectrophotometry. Two competing reactions occurred, leading to the chelate complex **9** and to the dihydroxo complex **10** (*Scheme 5*).

Scheme 4



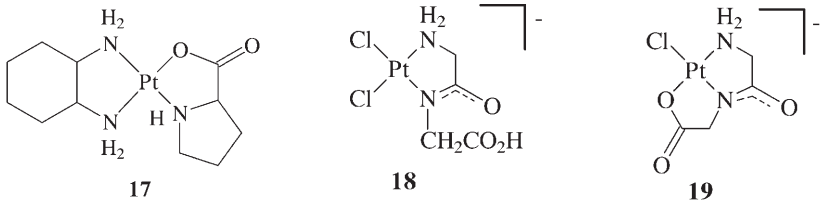
They concluded that the reaction with glycine proceeded through traces of the aqua complex **14** in equilibrium with **16**. Zwitterionic glycine was a less effective nucleophile than either carboxylate ion (in the form of malonate, $Hmal^-$) or chloride ion.

Scheme 5



Reactions of cisplatin and its hydrolytes with longer-chain amino acids $^{+}NH_3(CH_2)_nCO_2^{-}$ are less relevant to the biological properties of platinum because these compounds are less abundant *in vivo*. The six- and seven-membered chelate rings formed with β-alanine (n = 2) and γ-aminobutyric acid (n = 3) are progressively less stable kinetically and thermodynamically than the five-membered *N,O*-chelate ring formed with glycine [3].

Other amino acids with no side-chains bearing potential donor atoms also tend to form complexes having five-membered chelate rings with diamine- or diammine-platinum(II). An example is the complex [Pt(dach)(prolinate-*N,O*)]$^{+}$ (**17**) (dach = 1,2-diaminocyclohexane), whose crystal structure shows a *N,O*-chelate ring [14].

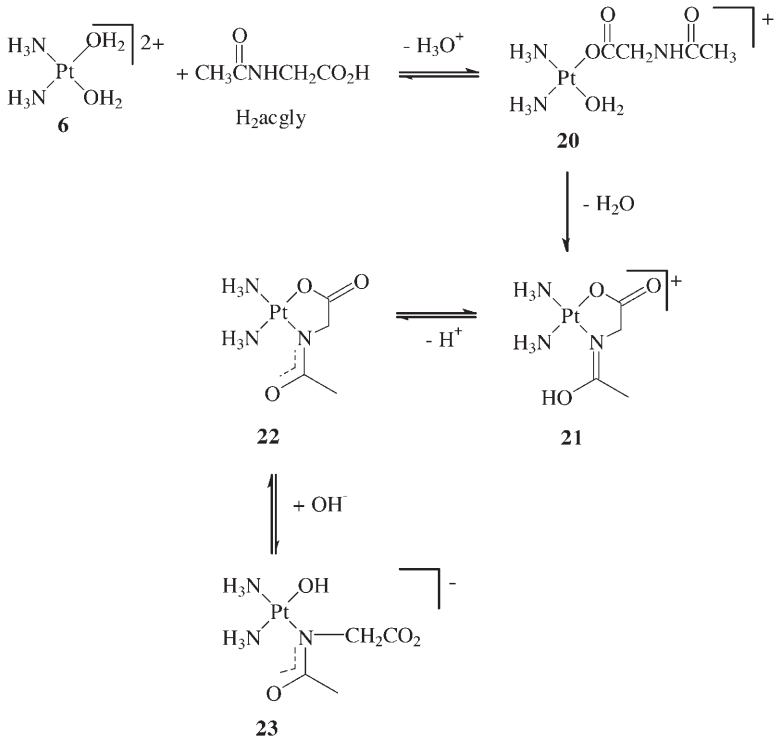


Reactions of $[PtCl_4]^{2-}$ with poly(glycine) oligopeptides produce complexes in which the peptides bind to the metal through terminal nitrogen. Among the complexes of this type that have been characterized are *trans*-$[PtCl_2(H_2digly\text{-}N(1))_2]$ (H_2digly = *N*-glycylglycine) [15] and *cis*-$[PtCl_2(HdiglyOEt\text{-}N(1))_2]$ (HdiglyOEt = *N*-glycylglycine ethyl ester) [16]. The slow reactions of some ^{15}N-labelled poly(glycine) oligopeptides with $[PtCl_4]^{2-}$ have been followed by ^{195}Pt-NMR spectroscopy. Terminal nitrogen atoms coordinate first, then the ligand progressively 'wraps itself'

around the metal ion as chelation occurs through deprotonated peptide nitrogen atoms [17]. *Watabe* and co-workers [18][19] prepared the complexes $K[Pt(Hdigly)Cl_2]$, $H[Pt(Hdigly)Cl_2]$ (which contain the anion **18**, as shown by X-ray crystal-structure determination), and K[Pt(digly)Cl] (**19**). Reactions of this type do not occur with diammineplatinum(II) complexes, as polyglycine peptides are unable to displace coordinated ammonia, (at least under reaction conditions that do not involve prolonged heating) [20].

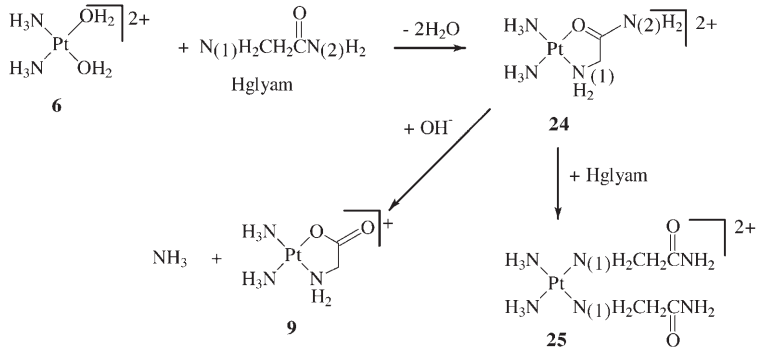
N-Acetylglycine (Hacgly) [21] and glycinamide (Hglyam) [20] have been used as simpler analogues of compounds containing a peptide bond. The initial product of the reaction of *N*-acetylglycine with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) is a complex *cis*-$[Pt(NH_3)_2(Hacgly\text{-}O)(H_2O)]^{2+}$ (**20**) in which the ligand is bound through carboxylate alone (*Scheme 6*). With standing in acid solution an *N,O*-chelate complex, $[Pt(NH_3)_2(Hacgly\text{-}N,O)]^{2+}$ (**21**) is formed, which may be deprotonated to $[Pt(NH_3)_2(acgly\text{-}N,O)]^{+}$ (**22**) by addition of alkali [21]. The acid dissociation constant for this deprotonation is 2.6. At high pH (> 9), the platinum-carboxylate bond is cleaved, to give a complex **23** in which the ligand is bound monodentate through the deprotonated amide nitrogen.

Scheme 6



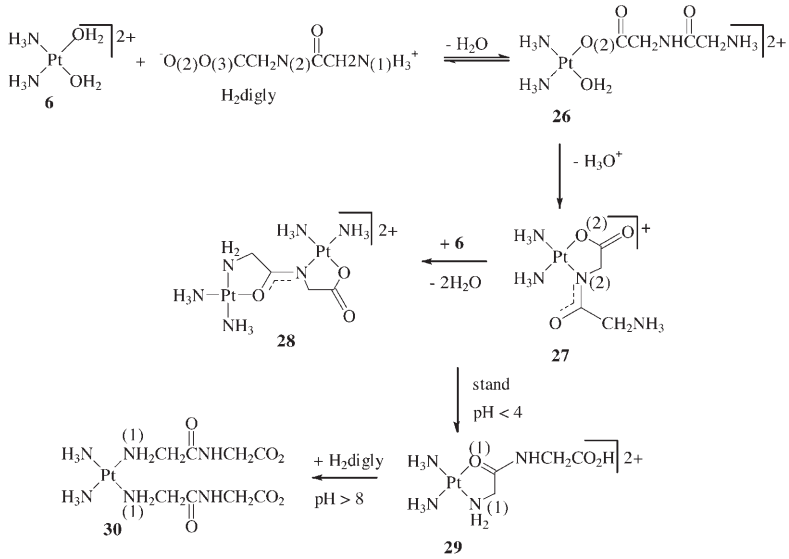
The reaction of glycinamide with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) at pH 5 gives the complex $[Pt(NH_3)_2$(Hglyam-*N(1),O*)$]^{2+}$ (**24**), in which the ligand chelates through the amine nitrogen atom (N(1)) and the amide oxygen atom. With excess glycinamide at pH 7, *cis*-$[Pt(NH_3)_2$(Hglyam-*N(1)*$)_2]^{2+}$ (**23**) forms. If the pH is increased to 8–10, in an attempt to form the *N(1),N(2)*-chelate complex, rapid hydrolysis of coordinated glycinamide occurs, to give the glycinate complex $[Pt(NH_3)_2$(gly-*N,O*$)]^{+}$ (**9**) (*Scheme 7*) [20]. The palladium analogue, [Pd(en)(glyam-*N(1),N(2)*$)]^{+}$ (en = 1,2-diaminoethane) is quite stable at pH > 3.5 [22][23]. It is, therefore, likely that the nonexistence of the *N(1),N(2)*-complex with diammineplatinum(II) is not due to low thermodynamic stability of this complex, but to a high kinetic barrier to isomerization from the *N(1),O*-complex **24**.

Scheme 7



The major reactions of *N*-glycylglycine (H_2digly) with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) [20] are summarized in *Scheme 8*. As would be expected from the results described above with glycine and *N*-acetylglycine, the initial coordination is through carboxylate oxygen O(2) to give *cis*-$[Pt(NH_3)_2$(H_2digly-*O(2)*$)(H_2O)]^{2+}$ (**26**), which then undergoes chelate ring closure involving the peptide nitrogen atom N(2), to form **27**. A second diammineplatinum unit then reacts with the bound glycylglycinate to give the dinuclear complex **28**. This is the dominant species ultimately present at pH < 7, even if the Pt/H_2digly ratio in the reactants is much less than 2:1. The crystal structure of the sulfate salt of **28** was determined [20]. Analogous dinuclear complexes were reported earlier [24] from the reaction of *Zeise*'s anion, $[PtCl_3(C_2H_4)]^-$ with dipeptides. The deprotonation of the peptide nitrogen (N(2)), and its coordination to platinum, appears to activate the adjacent peptide oxygen atom O(1) to facilitate formation of **28**. In a solution containing **27** and **28**, allowed to stand at pH < 4, significant amounts of the complex with H_2digly bound through terminal nitrogen (N(1)) and the pep-

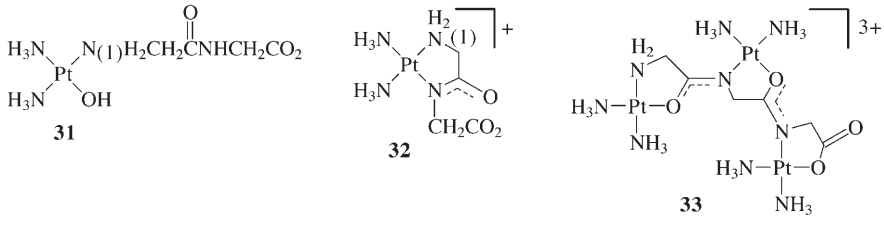
Scheme 8



tide oxygen (O(1)) (**29**) form. Addition of alkali in the presence of excess glycylglycine converts **29** into the complex containing two ligands bound monodentate through the terminal nitrogen, **30** [20].

Reaction of *cis*-$[Pt(NH_3)_2(OH)_2]$ (**10**) with glycylglycine at pH 9–11 gives *cis*-$[Pt(NH_3)_2$(Hdigly-*N(1)*)(OH)] (**31**) as the major product, with *cis*-$[Pt(NH_3)_2$(Hdigly-*N(1)*$)_2]$ (**30**) a minor species only, unless excess glycylglycine is present [20]. *Schwederski et al.* [17] did observe minor NMR peaks from $[Pt(NH_3)_2$(digly-*N(1),N(2)*)] (**32**) from the reaction of *cis*-$[PtCl_2(NH_3)_2]$ (**1**) with glycylglycine at pH > 11. By contrast, the palladium analogue of **32**, [Pd(en)(digly-*N(1),N(2)*)], is the dominant species in solution at pH > 2 [22][23]. As with the glycinamide complexes, the difficulty in obtaining the *N(1),N(2)*-chelate complex appears to be related to a high kinetic barrier against its formation, rather than low thermodynamic stability.

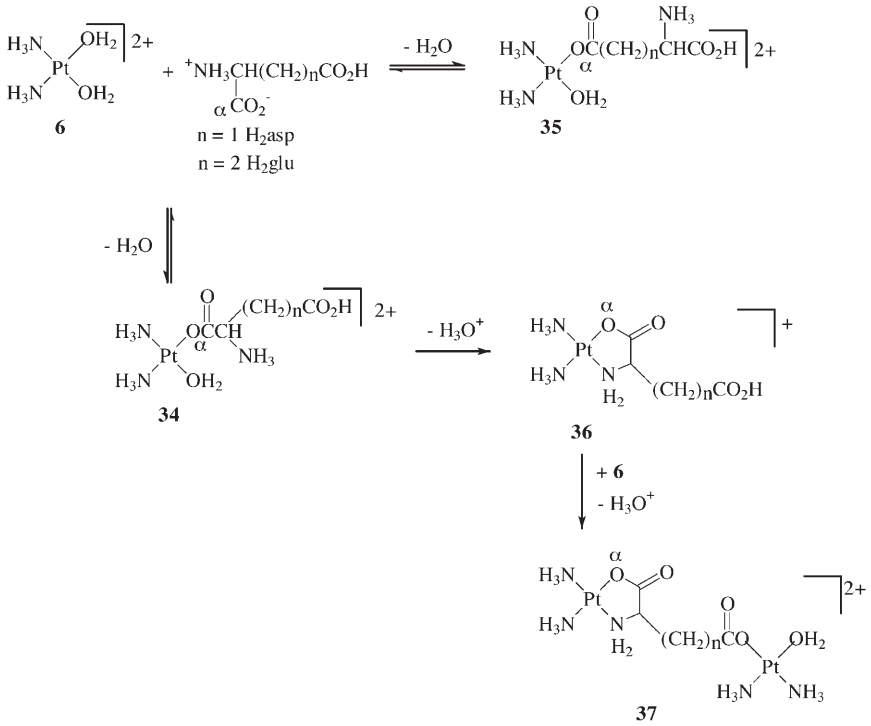
The reaction of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) with *N*-(glycylglycyl)-glycine (H_3trigly) gives a trinuclear complex $[(trigly)\{Pt(NH_3)_2\}_3]^{3+}$ (**33**) [20].



Complexes with Amino Acids with Acid Side Chains

The naturally-occurring amino acids with carboxylic acid side-chains are aspartic (H_2asp) and glutamic (H_2glu) acids. In square-planar complexes, the common coordination mode of these ligands is through nitrogen and the α-carboxylate group, to form a five-membered chelate ring, as in $[PtCl_2(Hasp\text{-}N,\alpha O)]^-$ and $[PtCl_2(Hglu\text{-}N,\alpha O)]^-$ [25]. In the reactions of these ligands with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) (*Scheme 9*), the α-carboxylate group coordinates preferentially at low pH (1–2) to give **34**, because the acid dissociation constant is higher for this group than for the β- (asp) or γ- (glu) carboxylate. Near pH 4, both isomers (**34** and **35**) with one carboxylate-bound form, but there is no evidence for a large chelate ring with both carboxylates bound. Slow formation of an *N,αO*-chelate ring (**36**) occurs, but no isomer with the larger chelate ring involving the other carboxylate is formed. With excess **6** present, the free carboxylate group of *36* coordinates to platinum to form **37** [26]. An *N,αO*-chelate complex analogous to **36** has been obtained with diaminocyclohexane replacing diammine [27].

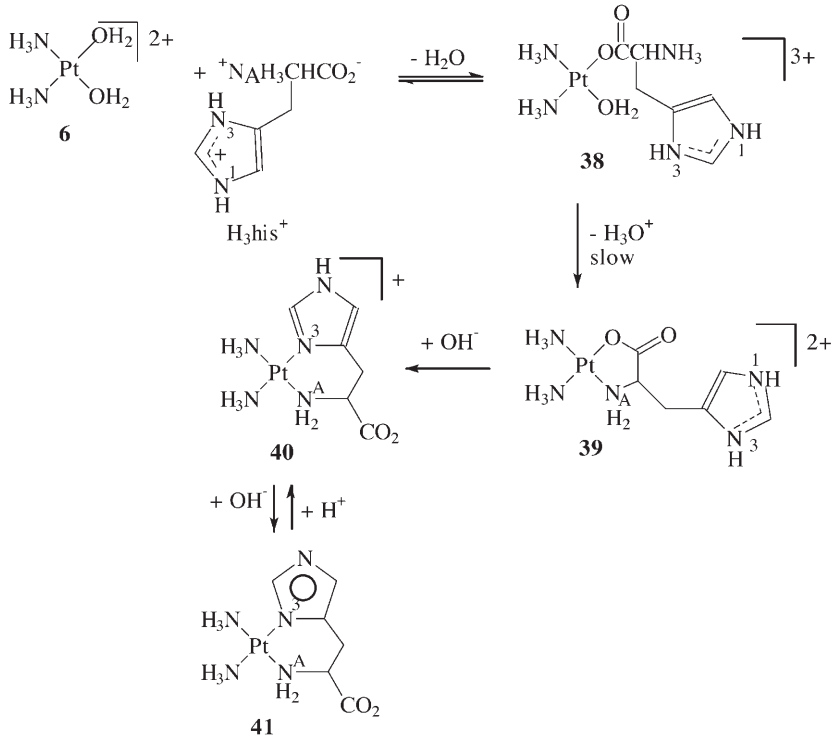
Scheme 9



Complexes with Histidine Derivatives

Kostic and co-workers [28][29] showed that [Pt(tpy)Cl]Cl (tpy = 2,2′:6′,2″-terpyridine) reacts selectively with the imidazole rings of histidyl residues in peptides and proteins, provided that cysteine groups have been blocked. Coordination to methionine residues is sterically hindered by the tpy ligand. In alkaline solution, histidine (H_2his) and *N*-acetylhistidine (H_3achis) each reacts with $[Pt(dien)(H_2O)]^{2+}$ (dien = diethylenetriamine) to give a mixture of linkage isomers, with either the imidazole N(1)- or N(3)-atom bound to the metal. Similar linkage isomers are formed when *N*-acetylhistidine reacts with [Pt(tpy)Cl]Cl [30]. Where two coordination sites are available on Pt^{II}, the common coordination mode for histidine is chelation through the amine nitrogen atom N_A and imidazole N(3), as in the crystal structure of [Pt(Hhis-N_A,$N(3)$)$_2$] [31]. *Saudek et al.* [32] studied the reaction of *cis*-$PtCl_2(NH_3)_2$ (**1**) with histidine at 100 °C, pH 7.3. The major product identified was $[Pt(NH_3)_2$-(Hhis-N_A,$N3$)$]^+$ (**40**), with some minor products formulated as containing two monodentate ligands bound through either N_A or N(3).

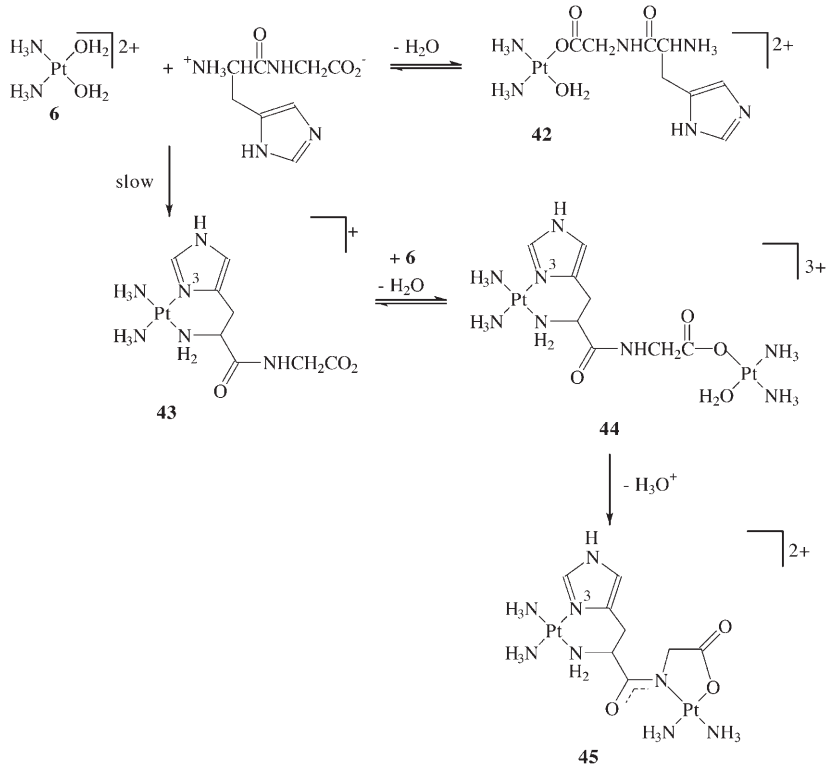
Scheme 10



The reaction of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**6**) with histidine at pH 2–3 (*Scheme 10*) [33] gives initially the complex **38**, with the ligand bound through carboxylate. A slow chelation reaction then occurs to give **39**, in which the amine nitrogen N_A and carboxylate oxygen are bound. This complex **39** is stable indefinitely in acid solution, but on addition of alkali, to increase the pH to 8–9, rapid irreversible isomerization occurs to give the thermodynamically more stable N_A,*N(3)*-chelate complex **40**. With further addition of alkali, the remaining imidazole proton is removed (pK_a 11.0, *cf.,* 14.4 for free histidine) to give **41** [33].

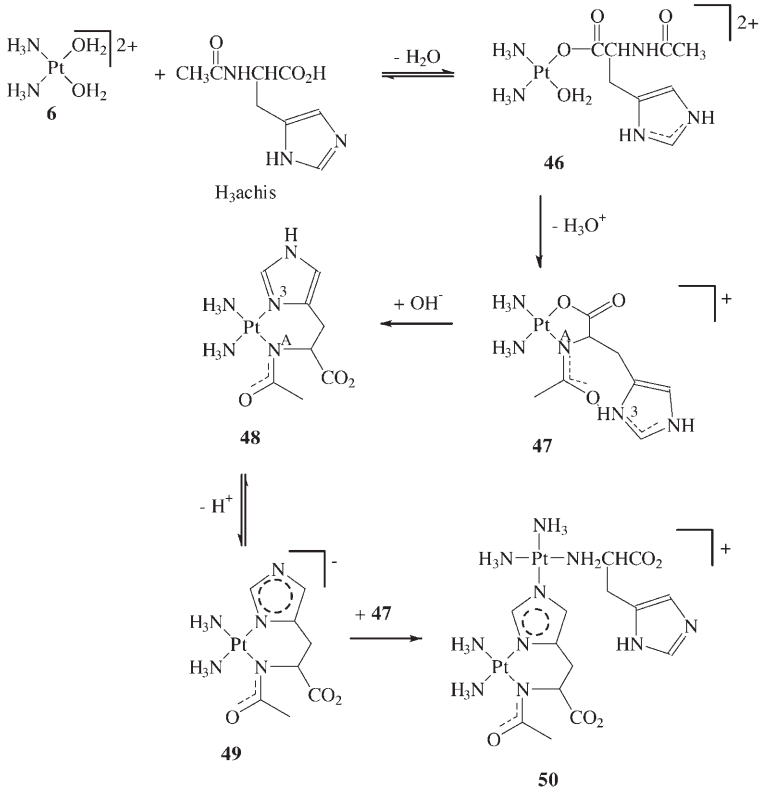
For histidine methyl ester and histidinamide, the carboxylic acid group is blocked. The N_A,*N(3)*-chelate complex forms slowly when these molecules react with a solution of **6**. In the reaction of **6** with histidylglycine (*Scheme 11*), the carboxylate group binds first to give **42**, then there is a slow reaction to form the N_A,*N(3)*-chelate complex **43**. If there is an excess of **6** present, the free carboxylate binds to another diammineplatinum unit (**44**), followed by ring closure to **45** [33].

Scheme 11



With *N*-acetylhistidine (*Scheme 12*), carboxylate coordinates initially (**46**), followed by chelate ring closure involving the amide nitrogen atom, N_A, to form **47**. The pK_a value for dissociation of an imidazole proton from **47** is much higher than for the histidine analogue **39**, perhaps because of the hydrogen bonding depicted in structure **47**. Little reaction of **47** occurs at pH < 10. At pH 10, the major product after standing is the dinuclear complex **50**. Once some of the N_A,*N(3)*-chelate complex **48** forms, it can readily deprotonate, then attack the Pt-O bond of remaining N_A,*O*-chelate complex **47**, to form **50** [33].

Scheme 12

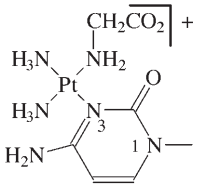


Conclusions – the Potential Importance of DNA-Protein Linkages

The results summarized above indicate that cisplatin and its hydrolysis products react readily with amino acids and peptides containing only nitrogen- and oxygen-donor atoms when there are no reagents present containing more effective nucleophilic groups. However, as pointed out by *Miller*

and *House* [13], the relatively slow kinetics of reaction of the platinum compounds with N- and O-donors present in amino acids and peptides makes it unlikely that, in plasma or cytoplasm, they will compete effectively with nucleobases or with sulfur-containing nucleophiles. The available results also show, however, that the tendency for platinum to bind with such donor atoms is greatly enhanced when they are held in proximity to the metal atom. Such circumstances can occur when platinum compounds bound to DNA come into close contact with proteins closely associated with DNA, including histones and regulatory proteins. It has long been recognized [34] that DNA-protein cross-links can play an important role in determining the biological effects of platinum drugs.

Lippert, *Hadjiliadis* and co-workers have prepared and characterized a number of 'ternary' complexes containing ammine, amino acid or peptide, and nucleobase, for example, the complex **51** containing 1-methylcytosine and glycine [35][36] and **52** containing 9-methylguanine and glycine [35][36]. These complexes may be prepared either by reaction of $[Pt(NH_3)_2(gly\text{-}N,O)]^+$ (**9**) with the nucleobase B (in which case the amino acid was sometimes completely displaced by the base molecules [37]), or by reaction of *cis*-$[Pt(NH_3)_2(B)(H_2O)]^{2+}$ with the amino acid (in which case the complex with *O*-bound amino acid was observed initially as an intermediate [35]).



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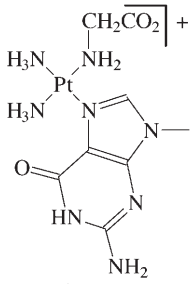

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Part 5. Inorganic Chemistry Revived or Initiated by Cisplatin

Platinum Blues: On the Way toward Unraveling a Mystery

Bernhard Lippert

Heteronuclear Pt^{II} Complexes with Pyrimidine Nucleobases

Lucio Randaccio and *Ennio Zangrando*

Diplatinum(III) Complexes: Chemical Species More Widely Spread Than Suspected

Giovanni Natile, Francesco P. Intini, and *Concetta Pacifico*

Inorganic and Organometallic Chemistry of Cisplatin-Derived Diplatinum(III) Complexes

Kazuko Matsumoto

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Platinum Blues: On the Way toward Unraveling a Mystery

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Several blue tetra- and octanuclear Pt complexes, prepared upon reaction of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with open and cyclic amides, as well as cyclic imides and a uracil nucleobase, and comprised of binuclear building blocks interacting through Pt-Pt bond formation, have been isolated and structurally characterized in recent years. Without exception, the average Pt oxidation state in these compounds is 2.25. Nevertheless, the structure and mode of action as antitumor agents of the 'Platinum Pyrimidine Blues', as prepared by *Rosenberg* in the early 70's, remain elusive. This account represents a summary of our present knowledge on cationic 'Platinum Blues', with a focus on those 'blues' obtained from *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and pyrimidine nucleobases, and presents speculations on reasonable alternative structures.

Introduction

Terms from the realm of inorganic chemistry rarely make it to the title of novels. This is true in particular for stories dealing exclusively with chemistry between people rather than chemistry between elements. NO (nitric oxide) by *Carl Djerassi* does not conform to this category, but 'Platinum Blues' by *William Deverell* [1] surely does. To the unbiased reader the relationship between 'platinum' and the 'blues' (singular) in the music business may not be an obvious one, and consequently one wonders if the author, at some stage, may have had an encounter with inorganic chemistry. After all, comprehensive inorganic chemistry textbooks frequently refer to this class of 'blues' (plural), which still provides an aura of fascination.

In general, intensely-colored Pt compounds owe this feature to intervalence CT transitions, hence to the presence of Pt in different oxidation states. Provided the solid state structure is favorable, a tiny deviation (< 1 ‰) from an integral oxidation number can be sufficient to cause the phenomenon of color. For example, the famous *Magnus Green salt*, usually formulated as

Cisplatin. Edited by Bernhard Lippert

the Pt^{II} complex salt $[Pt(NH_3)_4][PtCl_4]$, is not faint red, as might have been expected from combination of a colorless cation and a red $[PtCl_4]^{2-}$ anion, but rather green due to an impurity, formally equivalent to Pt^{III} centers in a long chain of Pt^{II} ions. Examples of deeply colored Pt complexes have been known since the first half of the last century, even though the nature of these compounds has not been understood until the sixth decade of this century [2]. Frequently, these materials were/are named after their discoverers (*e.g.*, *Wolfram's Red Salt*, *Reihlen's Green*) or their investigators (*e.g.*, *Krogmann's salts*).

Today the term 'platinum blues' is used as a generic one, describing Pt compounds that absorb in the yellow or near-IR spectral regions. Frequently no strict distinction is made with purple and green species if they are believed to be of similar structure. It appears that 'blues' can vary considerably as far as charge and ligands are concerned. The following account will largely concentrate on 'blues' derived from *cis*-$(NH_3)_2Pt^{II}$ and a variety of different ligands, but it will start out with some thoughts about 'Platinblau'.

Pt Acetamidate Blues

Among the colored platinum compounds, those containing amide and/or amidate ligands have received attention for at least 100 years. In 1895 *Nikolai Kurnakow*, in a paper on metal complexes of thioamides, briefly mentioned the formation of blue and purple solids during the reaction of K_2PtCl_4 with acetamide or other aliphatic amides [3]. In 1908, the group of *Karl Andreas Hofmann*, then at the Royal Academy of Sciences in Munich, Germany, reported on 'Platinblau' [4], a compound obtained upon reaction of $PtCl_2(CH_3CN)_2$ with $AgNO_3$ in water. 'Platinblau' was formulated as a bis(acetamidato) complex of Pt^{II}, $Pt(CH_3CONH)_2 \cdot 1\ H_2O$, based on elemental analysis data, its molecular mass as determined by freezing point depression, and its reactivity toward HCl, which produced $[PtCl_4]^{2-}$ in over 90 % yield. Formation of 'Platinblau' included hydrolysis of two acetonitrile ligands to acetamidato ligands, hence the following reaction (*Eqn. 1*):

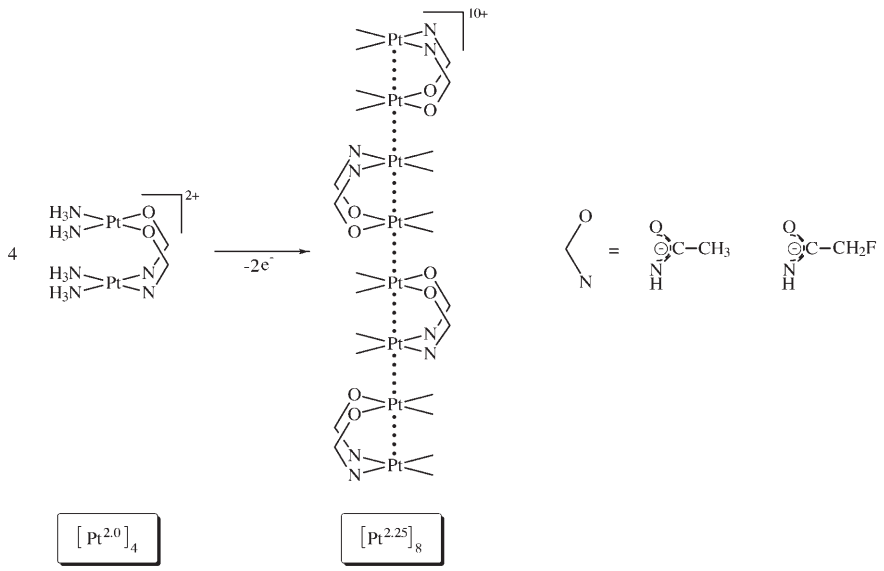
$$PtCl_2(CH_3CN)_2 + 2\ AgNO_3 + 3\ H_2O \rightarrow Pt(CH_3CONH)_2 \cdot H_2O + 2\ AgCl + 2\ HNO_3 \quad (1)$$

This reaction sequence, although consistent with the observed drop in pH [5], does not provide an explanation for the unusual color of the product. Careful monitoring of pH and development of color during reaction of $[PtCl_4]^{2-}$ with acetonitrile later clearly revealed that release of H^+ is complete long before the 'blue' is fully formed [6]. Obviously, formation of

'Platinblau' involves an induction period, suggesting that an autocatalytic process might be operative. Over the years there have been various attempts [5–10] to assign a specific structure to the original 'Platinblau' and to provide a convincing explanation for the origin of the blue color of this material, but an unambiguous answer concerning structure and Pt oxidation state could not be reached.

A major reason for this uncertainty, also relevant to substituted acetamides (C-methyl- and C-fluoro derivatives), is the versatility of the acetamide/acetamidato molecule/ion as a ligand for metal ions in general and for Pt in particular [11–22]. Thus, a large variety of mono- and bidentate binding patterns has been established, which includes various monodentate binding modes (*N*- or *O*-linkage isomers), different protonation states (neutral or anionic), different tautomers (amide or iminol form of neutral molecule), as well as the possibility of acting in a chelating or in a bridging fashion. There is at least one example of a dinuclear Pt^{III} compound, $[Pt_2(en)_2(CH_3CONH)_4]^{2+}$ (en = 1,2-diaminoethane) [15], which displays simultaneously two different binding modes, *N*,*O*-bridging and *N*-monodentate coordination. As far as the possibility of bridge formation is concerned, the existence of distinct binuclear species is well established, both for Pt [13], other transition-metal ions [18], and heteronuclear complexes [20]. Mixed acetamidate/hydroxide-bridging is likewise documented [22]. It has been demonstrated by *Matsumoto et al.* [13] that dinuclear entities of composition *cis*-$[Pt_2(NH_3)_4(CH_3CONH)_2]^{2+}$ can associate, through partial Pt oxidation and intercationic H-bond formation, to a tetrad of dimers (*Scheme 1*).

Scheme 1



In the octanuclear array, the average Pt oxidation state is 2.25, with a formal description as $[Pt^{2.0}]_2\ [Pt^{2.5}]_4\ [Pt^{2.0}]_2$ justified on the basis of Pt-Pt distances (*vide infra*). Partial oxidation is brought about by air and requires strongly acidic reaction conditions (starting pH 2.0 [13]). Thus, stacking of dinuclear Pt units – favorably with *head-to-head*-oriented bridging ligands, since this situation permits a maximum of intermolecular H-bonds between dimeric entities – appears to be *a* structural motif for 'platinum blues' derived from *cis*-$(NH_3)_2Pt^{II}$.

A challenging alternative to this octanuclear 'Pt acetamide blue' could be a truly polymeric one, if connectivity properties as seen in two mixed Pt/Ag complexes of acetamidate were to be applied: In both *cis*-$[Pt(NH_3)_2(CH_3CONH)_2Ag]NO_3 \cdot 4\,H_2O$ and *trans*-$[Pt(NH_3)_2(CH_3CONH)_2Ag]NO_3 \cdot 1.5\,H_2O$ [21], infinite Pt/Ag chains with reasonably short Pt-Ag separations of 2.897(1) Å (*cis*-complex) and 2.903(1) Å (*trans*-complex) and bridging acetamidato ligands are observed (*Fig.*). Although these heteronuclear compounds are not blue, it is possible to imagine a 'blue' on the basis of these structures: If both $(NH_3)_2Pt^{II}$ *and* Ag^{I} entities were to be replaced by either Pt(acetamidate-*N*,*O*) chelates (*cis*-isomer) or Pt(acetamidate-*N*)(H_2O) moieties (*cis*- or *trans*-isomer), the bridging acetamidate ligands retained, and partial Pt oxidation allowed, a polymeric 'Platinblau' of composition $[Pt(CH_3CONH)_2](NO_3)_x \cdot y\,H_2O$ or $[Pt(CH_3CONH)_2(H_2O)](NO_3)_x \cdot (y-1)\,H_2O$ ($x \ll 1$) would be feasible. The actual structure could be further complicated by different orientations of the bridging acetamidates (*head-to-head*, *h-h* or *head-to-tail*, *h-t*) and acid/base equilibria between co-

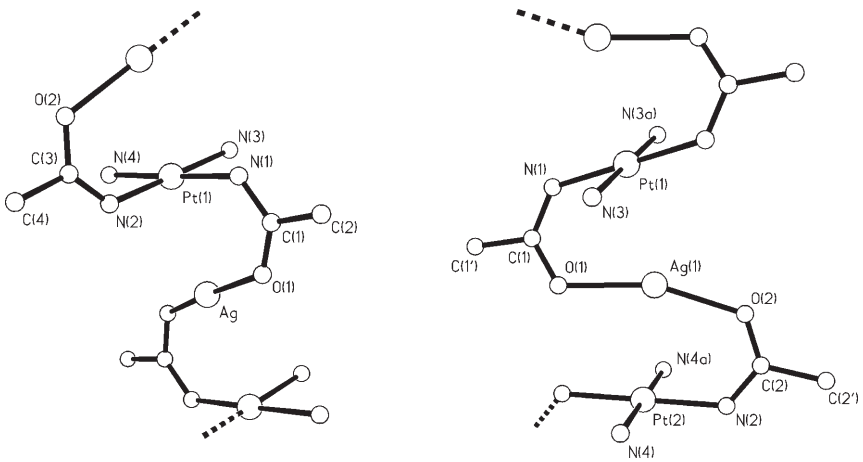


Figure. *Sections of polymeric structures of* cis-*$[(NH_3)_2Pt(CH_3CONH)_2Ag]NO_3 \cdot 4\,H_2O$* (left) *and of* trans-*$[(NH_3)_2Pt(CH_3CONH)_2Ag]NO_3 \cdot 1.5\,H_2O$* (right)

ordinated aqua/acetamidato and hydroxo/acetamide pairs. It should be kept in mind that Pt-O bonds generally are much more labile than Pt-N bonds, and that consequently any poly- (or oligo)meric array probably is prone to (concentration-dependent) solution equilibria.

'Platinum Pyrimidine Blues'

Discovery and Early Studies

Testing of so called 'platinum pyrimidine blues' for antitumor activity began in the laboratory of *Rosenberg* in around 1972. These materials were obtained upon reaction of the diaqua species of cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, with uracil and thymine as well as related cyclic and open-chain amide ligands [23]. Interest in these 'blues' had arisen from yet another serendipitous finding in *Rosenberg*'s group: postdoctoral associate *Samir Mansy*, while studying the interaction of cisplatin hydrolysis products with polynucleotides by laser-*Raman* spectroscopy, had observed formation of a curious blue color when poly U (polyuridine) was applied. Subsequently, it was shown that isolated uridine, thymidine, uracil and thymine nucleobases likewise produced blue materials when reacted with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. The relationship of these 'platinum pyrimidine blues' with 'Platinblau' was recognized at an early stage, and, in addition to 'blues' derived from *cis*-$(NH_3)_2Pt^{II}$ (class I [23]), also the classic 'Platinblau' complexes derived from $Pt(RCN)_2Cl_2$ (R = CH_3, C_2H_5) and $AgNO_3$ (class II), as well as from $Pt(CH_3CN)_2Cl_2$ and amides (class III), were prepared and tested. The unexpected activity of these compounds against the Ascites S-180 tumor system – ILS (*I*ncrease in *L*ife *S*pan) values of up to 100 % were obtained with animals treated with the 'blues' – and their low toxicity (concentrations applied were rather high, on the order of several hundred mg/kg bodyweight, compared with a few mg/kg for cisplatin) made these compounds prime candidates for second generation Pt drugs at that time [23][24].

A major problem, that of reproducibility, soon became evident. Preparation of a class I 'blue', to which this discussion is restricted, typically involved incubation of an aqueous solution of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ (prepared from cisplatin and $AgNO_3$) with a pyrimidine-2,4-dione or a cyclic amide over a period of 3 – 5 days in air, 37 °C, with the pH kept constant (at 7) by repeated addition of NaOH. Various fractions of products were then obtained upon cooling the solution and/or after addition of ethanol. Consequently, these fractions differed in color (dark blue, light blue, green, purple), as did elemental analysis data. If the pH was not kept at 7 but rather allowed to drop, products were likewise different. In particular,

no correlation with a specific structure could be made. In around 1976 a procedure was agreed upon between industrial laboratories and *Rosenberg*'s laboratory to make preparations of the various 'blues' reproducible, but this did not, of course, solve the problem of composition.

Early on it was evident that atmospheric oxygen was associated with the formation of the blue color, pointing toward some oxidation process. However, it had later also been shown [25][26] that a blue color developed even in the absence of O_2, provided the concentration of the diaqua species was sufficiently high. Whether this was due to Pt^{IV} impurities in the starting material, or was the consequence of a disproportionation of Pt^{II}, remained unclear.

Physicochemical Properties of Class I 'Blues'

At the end of 1974 the cationic nature of 'platinum pyrimidine blues' had been recognized. The presence of NO_3^- counter ions was concluded from IR spectroscopy, quantitative NO_3^- analysis, anion exchange reactions (*e.g.*, precipitation of 'blues' by large anions such as $[B(C_6H_5)_4]^-$, pyrophosphate *etc.*). Final proof came from electrophoresis experiments, carried out by *James H. Burness* in *Rosenberg*'s laboratory. The results of these experiments, specifically the pronounced tailing of the moving bands, their separation into components of varying shades of purple, blue and green, and the similar behavior of eluted fractions (believed to be homogeneous) that had been subjected to a second electrophoresis, lent strong support to the idea that the 'blues' were complicated mixtures in slow equilibrium. Applying HPLC, it was later shown that the 'blues' contained both distinct diamagnetic species, which were colorless, and the actual blue and green components [27] (see also below). Consequently, absorption maxima (typically around 500–720 nm) could be determined for the various fractions, yet not extinction coefficients. ^{1}H- and ^{13}C-NMR spectra were ambiguous in that sometimes sharp sets of resonances were observable [26], sometimes adjacent to unstructured broad 'bands', whereas in other cases a steady signal broadening and eventually the complete disappearance of the resonances was observed [28]. Recording ^{1}H-NMR and EPR spectra in a parallel way finally provided an answer [28]: it was convincingly demonstrated that formation of a paramagnetic Pt species, suggested to be Pt^{III}, and its interaction with *several* adjacent Pt^{II} centers in an aggregate of approximate axial symmetry, was responsible for the features seen in the EPR (It is noteworthy that *Inorg. Chim. Acta*, which at the time included a separate section on bioinorganic chemistry, used the EPR spectrum of a typical sample of a 'blue' on its front cover). Fast relaxation of the NMR nuclei, brought about

by the paramagnetic Pt^{III} centers, caused broadening of the resonances, eventually beyond resolution. More thorough EPR studies on a variety of different 'blues' [29] and, in particular, on 'blues' characterized by X-ray crystallography (see below) essentially confirmed this picture.

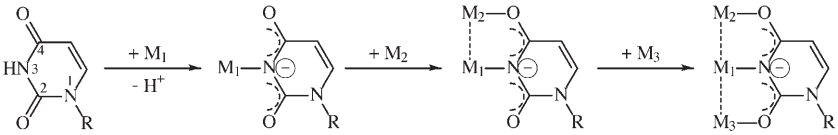
Using EPR spectroscopy, unpaired electrons were quantified for a large series of 'platinum pyrimidine blues' and gave rather low values of few percents (5.7% at most) of Pt^{III} [28]. This explained why magnetic susceptibility measurements on these 'blues' had provided no clear indication of their mixed valency. Treatment of the 'blues' with HCl (with heat, under N_2) leads to a complete disproportionation of Pt^{III} into Pt^{II} and Pt^{IV}. $[PtCl_6]^{2-}$ had been assayed (by reduction with Cu^{I}) and found to give somewhat higher values than expected if only Pt^{III} were the origin of Pt^{IV} [28]. This points either to the existence of distinct Pt^{IV} species or of (EPR silent) diamagnetic diplatinum(III) complexes in the blues. Overall, these results are consistent with the assumption that the average Pt oxidation state in 'platinum pyrimidine blues' as prepared by the *Rosenberg* protocol is only marginally higher than 2.0, *e.g.*, between 2.01 and 2.06. This is in marked contrast to the situation for $Pt^{2.25}$ compounds structurally characterized by X-ray (see below). Preparation of the latter materials appears to require always strongly acidic reaction conditions. While it may be argued that the only difference between tetranuclear $Pt^{2.25}$ 'blues' and the original 'Pt pyrimidine blues' is a lower content of the former in the latter, this assumption is inconsistent with a large body of evidence from other investigations (*vide infra*).

Nucleobase-Binding Modes

Even before X-ray crystallography had firmly established N,O bridge formation in the 'α-pyridone blue' and eventually also in a '1-methyluracil blue', it had been suspected that this binding pattern might be relevant to the 'platinum pyrimidine blues', since such a situation would explain the short contacts between neighboring Pt entities believed to be a prerequisite for the formation of 'blues'. *Tobias* and co-workers, in an excellent paper of the 'necessary conditions for formation of platinum uridine blues' [30], not only had firmly established binding of *cis*-$(NH_3)_2Pt^{II}$ to N(3) of uridine, but at the same time had also recognized that Pt-binding to N(3) of uridine promotes an increase in negative charge on the two adjacent carbonyl oxygens that should lead to 'some donor character' of these sites. The first crystallographic proof for this binding pattern – *N(3),O(4)*-bridging of 1-methylthymine nucleobases by *cis*-$(NH_3)_2Pt^{II}$ – was provided by *Lock et al.* in 1978 [31]. Subsequently, it was shown, primarily by work of *Guay* and *Beauchamp* [32] as well as own work [33][34], that *N,O*-bridging is not at all un-

usual in metal complexes of these ligands but rather almost the rule. It was later demonstrated that even the second exocyclic oxygen of uracil bases had donor properties [33], thereby producing compounds with arrays of three metals in a row (*Scheme 2*).

Scheme 2



DNA-Binding Properties of 'Pt Pyrimidine Blues'

There have been only very few studies concerned with the interaction of 'Pt pyrimidine blues' with DNA. The first published one, conducted in *Rosenberg*'s laboratory by *Burness et al.* [35], unequivocally proved an interaction between cationic 'Pt uracil blue' and DNA while ruling out an interaction between anionic 'Pt oxamate blue' and DNA. This work had been initiated by findings of *Harish C. Pant* in 1973, who clearly had demonstrated a salt-dependent affinity of 'Pt pyrimidine blues' with DNA (strong binding in low salt conditions, weak binding in high salt conditions) and subsequent observations of *Aggarwal et al.* [36] on the usefulness of the 'blues' as stains for electron microscopy of fine structural studies of cells. Specifically, it had been shown that *i*) the 'blues' penetrate cell membranes, and *ii*) stain the nuclear components such as chromatin and the nucleolus as well as ribosomes, while generally not reacting with proteins. The appearance of additional electron-dense cell-surface patches on tumor cells [36] caused great excitement for a while, but was subsequently not confirmed to be specific for tumor cells [37]. Slow cell-membrane penetration and a build-up of 'blues' at the surface was later invoked as a possible explanation.

A detailed study on the binding of 'Pt uracil blue' to closed and nicked circular DNA, reported in 1978 [38], confirmed some of the salient features of the earlier study. However, it was also shown that presumably low-molecular-weight Pt entities *not* carrying a uracil nucleobase had formed covalent adducts with DNA. Whether or not these Pt species were solvolysis products of the 'blue' used or simply part of the complex mixture, was not obvious. Hints for a hydrolytic decomposition of another 'blue' obtained from 1-methyluracil rather than uracil during reaction with DNA were later found [39], but there appears to be no consensus about the nature of the DNA-binding Pt species.

Antitumor Activity

The initial excitement about the antitumor activity of the 'platinum pyrimidine blues' has already been mentioned. The idea that the (presumably polymeric) 'blues' may have a depot function, releasing low molecular weight Pt species with time, had been forwarded at an early stage [40]. The proposal was based on experiments involving soaking with crystals of two proteins, lysozyme, and prealbumin, which had shown that the protein crystal remained colorless during this procedure and revealed binding of Pt to sites where normally monomeric *anionic* Pt^{II} salts bind. As far as binding to DNA is concerned, slow release of *cationic* Pt^{II} species from a typical (cationic) 'Pt pyrimidine blue' would seem more logical. Testing of potential decomposition products of the 'blues', *e.g.*, mono- or dinuclear nucleobase or cyclic amide complexes with Pt in the (reduced) oxidation state of +2 [39][41][42], as well as testing of several X-ray structurally characterized 'blues' [41] gave a somewhat conflicting picture: While *Matsumoto* and co-workers [41] emphasize that the breakdown of *head-to-head* dinuclear species and release of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ is the most likely reason for activity – a mechanism previously shown to take place in NaCl solution [43] – whereas 1 : 1 complexes, as derived from breakdown of *head-to-tail* dimers, are inactive, *cis*-$(NH_3)_2$Pt(1-MeU)Cl has been reported [39] to be surprisingly active. Activity of these types of compounds (with 1-MeU replaced by other heterocyclic ligands, including the nucleobase cytosine) had later also been reported by *Hollis et al.* [44], and is unexpected in that it contradicts an important 'rule of thumb' for antitumor activity of Pt coordination compounds: that of having two good leaving groups in *cis* positions. From binding studies it appears that the heterocyclic ligand is not replaced during reaction with DNA, and that therefore these compounds bind only monofunctionally to DNA [45]. An interesting aspect concerning the antitumor activity of dinuclear, amidate-bridged Pt^{II} compounds (*h-h*), has been made by *Okuno* and co-workers [42]: Accordingly, optimal hydrophobicity of the charged compound, important for cell permeation, and sufficient 'electrophilicity', necessary to accomplish cleavage of the Pt-O bonds in the *head-to-head* dimer, hence its breakdown in biological medium, are equally important for activity of these dinuclear charged species. Thus for those '-blues' composed of dinuclear, *head-to-head*-bridged *cis*-$(NH_3)_2$Pt entities, the mixed valency appears to be irrelevant with regard to the mode of action as an antitumor agent, since intracellular reduction is rapid, leading to diplatinum(II) entities. Whether this indeed is the complete story needs to be seen.

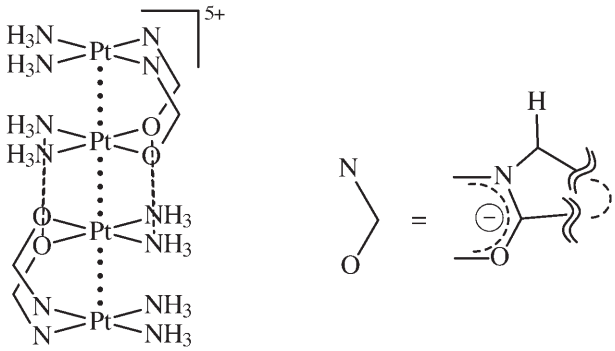
In 1986, *Okuno et al.* reported a modified procedure of preparing 'blues' and 'greens' from *cis*-$[Pt(NH_3)_2(H_2O)_2]SO_4$ and uridine, and a work-up that included gel filtration [46] and isolation of a green fraction which

displayed remarkable activity against L1210-tumor-bearing mice. Characterization of these green materials indicated oligomeric molecular weights with 8–16 Pt atoms, and microanalysis data suggested that the 'greens', unlike the 'blues', had lost NH_3 ligands in part [47].

X-Ray Crystallography: 'Blues' Containing Cyclic Amides

In early 1977, *Barton et al.* [48] published the X-ray crystal-structure analysis of a 'Pt blue' derived from *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and α-pyridone. The compound was shown to consist of two dinuclear entities containing two *head-to-head*-oriented α-pyridonato ligands and two *cis*-diammineplatinum residues, associated by an unsupported (H-bonds excluded) Pt-Pt bond between the two *O*,*O*-coordinated Pt ions. The average Pt oxidation state of this 'blue' is +2.25. Formally, the Pt_4 chain thus consists of three Pt^{II} ions and one Pt^{III} ion, with the unpaired electron delocalized over the Pt_4 chain (*Scheme 3*). The choice of the cyclic amide α-pyridone (1-hydroxypyridine) by *Lippard* and co-workers had been a fortunate one in that it reduced the

Scheme 3



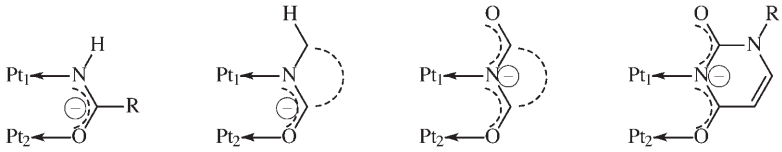
number of potential binding sites to two, namely to the endocyclic N-atom and the exocyclic O-atom of the anionic oxygen. Crystallization of this 'blue' was achieved from a strongly acidic (pH 1) aqueous HNO_3 solution, to which $NaNO_3$ had been added to facilitate precipitation. Pt-Pt distances are 2.779 Å (within dimers) and 2.885 Å (between dimers). The 'α-pyridone blue' has been extensively studied by a variety of physico-chemical techniques such as X-ray photoelectron spectroscopy (XPS) [49], visible spectroscopy [50], redox titration [50], EPR spectroscopy [51], magnetic susceptibility measurements [51], and resonance *Raman* spectroscopy [52]. The origin of the blue color has been assigned essentially to two transitions within the tetranuclear metal chain [53].

Until recently, there have been only three additional examples of tetranuclear $Pt^{2.25}$ 'blues' of analogous structures: That of a '1-methyluracil blue' of *cis*-$(NH_3)_2Pt$ [54][55], that of an 'α-pyridone' blue derived from Pt^{II}en (en = 1,2-diaminoethane) [55] and a '3,3-dimethylglutarimidate blue' derived from *cis*-$(NH_3)_2Pt^{II}$ [41]. Very recently, two 'α-pyrrolidinonato $Pt^{2.25}$ blues' prepared from *cis*-$(NH_3)_2Pt^{II}$ have been characterized by X-ray crystal structure analysis [56]. They differ with respect to their counterions, but are otherwise structurally very similar. The same authors were also able to crystallize a stoichiometric 1 : 1 mixture of the tetranuclear $Pt^{2.25}$ 'blue' and its next oxidation product, the tetranuclear $Pt^{2.5}$ 'tan', which in the solid state gives rise to a green compound [56]. Its average Pt oxidation state thus is 2.375. The $Pt^{2.5}$ 'tan', composed like the $Pt^{2.25}$ 'blue', yet differing from the latter in its Pt-Pt distances and charge, had originally been described by *Matsumoto et al.* [57]. These authors also reported on non-stoichiometric compounds with violet [58] and green colors [59] and general compositions $[Pt_2(NH_3)_4L_4]X_n \cdot m\ H_2O$ (L = α-pyrrolidonate) and average Pt oxidation states of 2.14 (violet; $X_n = (PF_6)_2$, $(NO_3)_{2.56}$; $m = 5$) and 2.37 (green; $X_n = (NO_3)_{5.48}$; $m = 3$). In all of these compounds, the *identical* principle of two stacked *head-to-head*-bridged dinuclear entities is realized. It thus appears that dimer-of-dimers formation is a common feature for cyclic amides. With aliphatic amides further aggregation (tetrad-of-dimers) is possible as a consequence of reduced steric constraints of the bridging ligands (see above).

'Blues' Derived from 1-Substituted Uracils and Imides

A comparison of aliphatic amides, cyclic amides, cyclic imides and 2,4-dioxopyrimidines (uracils) in their deprotonated and diplatinated form (*Scheme 4*) reveals an increasing steric shielding of the *N*-bonded Pt ion (Pt_1). With respect to formation of stacked and partially oxidized dinuclear species, it is evident that application of the binding principles seen in the 'blues' of cyclic amides to the uracils and imides allows for tetranuclear species only. On the other hand, the presence of an additional O-donor in the imides and uracils (and likewise the cytosines, *vide infra*) provides an

Scheme 4



opportunity for association patterns different from those of the aliphatic and cyclic amides.

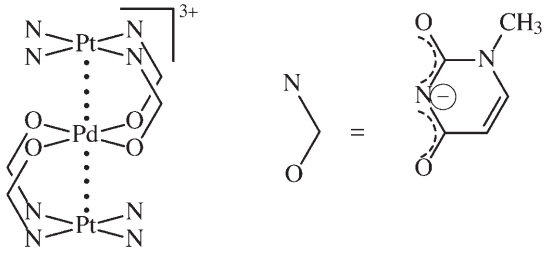
The existence of dinuclear, *N*,*O*-bridged Pt^{II} complexes with pairs of anions of 1-methyluracil [60] and 1-methylthymine [61], as well as other imides [42], oriented *head-to-head*, is well established. Therefore, it is not surprising that analogues of the 'α-pyridone blue' exist for 1-methyluracil [54][55] and 3,3-dimethylglutarimide [41]. Isolation of these $[Pt^{2.25}]_4$ 'blues' likewise requires strongly acidic reaction conditions, unless other potential oxidants (Ag^+, other transition metal ions [62][63]) are present, which appear to form heteronuclear species prior to the redox process. Interestingly, these heteronuclear precursor complexes provide a rationale for the formation of the partially oxidized species. Thus, in a trinuclear complex of 1-methyluracil, containing two *head-to-head* oriented nucleobases, two *cis*-$(NH_3)_2Pt^{II}$ entities (at N(3) and O(4)) and in addition a Ag^+ bound to O(2), the shortening of the Pt-Pt distance from 2.937(1) Å (in Pt_2 precursor) to 2.885(1) Å in Pt_2Ag almost suggests that an electron from the Pt_2 core is already on its way to Ag^+, to give eventually elemental silver and an oxidized Pt species [63].

It is also possible to prepare, in aqueous acidic solution, '1-methyluracil blues' of the $[Pt^{2.25}]_4$ type having different am(m)ine ligands at the two Pt ions within the dinuclear unit [64][65]. Formation of these 'blues' is influenced by a variety of factors such as steric and electronic properties of the am(m)ine ligand as well as stacking properties, if heterocyclic diamine ligands are used. Isolation of tetranuclear 'blues' from strongly HNO_3-acidic solution can lead to incorporation of HNO_3 in the product, either as an impurity or in stoichiometric amounts [65]. Unless a careful analysis is carried out, which does not rely exclusively on elemental analysis data but also includes potentiometric titration, this feature can lead to the erroneous impression of a Pt oxidation state higher than +2.25 in these materials.

'Pt blues' of the $[Pt^{2.25}]_4$ type generally undergo rapid reduction when dissolved in water. Similarly, any increase in pH of an acidic aqueous solution of a $[Pt^{2.25}]_4$ 'blue' causes loss of blue color and eventually leads to complete decolorization, presumably because the redox potential favors oxidative reactions such as water oxidation, for example [64]. This phenomenon has previously been reported [66] for structurally related diplatinum(III) complexes containing α-pyrrolidone. As a consequence, conventional NMR techniques (^{195}Pt, ^1H, ^{13}C) are usually applicable to the study of subsequent reactions. With glutarimide and 3,3-dimethylglutarimide 'blues', *Matsumoto* and co-workers have demonstrated that reduction to the Pt^{II} state is virtually instantaneous [41]. Frequently, secondary isomerization and/or hydrolysis reactions take place. Again, this behavior appears to contrast that of a typical 'Pt pyrimidine blue' prepared according to the *Rosenberg* protocol.

A '1-methyluracil blue' of distinctly different composition, containing (formally) two Pt^{II} and a heterometal ion, Pd^{III}, has likewise been prepared and structurally characterized byX-ray analysis (*Scheme 5*) [67]. It can be (reversibly) further oxidized to a $Pt^{II}Pd^{IV}Pt^{II}$ state and also reduced to $Pt^{II}Pd^{I}Pt^{II}$. There is no reason why Pt instead of Pd should not behave similarly, although this would require the loss of two NH_3 ligands from a *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ entity.

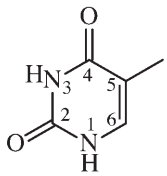
Scheme 5



'Blues' from Unsubstituted Pyrimidine Nucleobases

Although 'blues' prepared from unsubstituted uracil, thymine and related bases (*e.g.*, 6-methyluracil, 5,6-dihydrouracil *etc.*) were the first to be prepared and tested, their composition is the least clear. The author suspects that there is still long way to go to fully understand the nature of these 'blues'. It is possible that there are even 'blues' built on different principles. A main obstacle to the elucidation of 'Pt blues' derived from the unsubstituted pyrimidine nucleobases lies in their versatility as ligands. Not only is there the possibility that these ligands bind to metal ions, specifically Pt, *via* N(1) *or* N(3) *or* (only with uracil) C(5), but also many possible combinations of two or more binding sites, *e.g.*, N(1),O(2); N(3),O(2); N(3),O(4); N(1),N(3); N(3),O(2),O(4); N(1),O(2),N(3),O(4) *etc.* (*Scheme 6*). A series of these binding patterns has been established by X-ray crystal-structure analyses [68–70], and others are likely on the basis of spectroscopic studies [71][72] or from comparison with results obtained for *N(1)*-substituted derivatives. The possibility of different tautomers of platinated forms being

Scheme 6



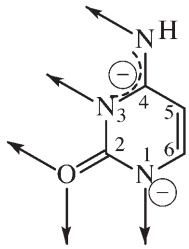
present [71][73], pH-dependent differences in stabilities of these linkage isomers [71], and rearrangement reactions lead to further complications. Finally, the formation of cyclic oligomers [69][70] and different rotamers within these cyclic species [74] needs to be mentioned. For example, in the cyclic, octanuclear complex of the type $[\{Pt(en)\}_4\{cis\text{-}(NH_3)_2Pt\}_4U_4]^{4+}$ (U = uracil dianion) the four uracil nucleobases bridge the metal entities both *via* N(1),O(2) and N(3),O(4) [70]. The corresponding species comprised exclusively of $cis\text{-}(NH_3)_2Pt^{II}$ entities is extremely air-sensitive, undergoing easy oxidation to a 'blue', and it has not yet been obtained in pure form.

Much previous work on 'Pt blues' of unsubstituted nucleobases did not explicitly pay attention to any of these crucial aspects and therefore should be treated with caution.

'Cytosine Blues'

Unlike uracil and thymine, which provide three N,O combinations of two adjacent metal-binding sites – N(1),O(2); N(3),O(2); N(3),O(4) – the situation with cytosine is different in that, in addition to two N,O combinations – N(1),O(2) (following N(1) deprotonation) and N(3),O(2) – there exists also the possibility of *N(3),N(4)*-bridge formation (*Scheme 7*), with N(4)

Scheme 7



being deprotonated. An additional possibility – C(5),O(4) in uracil and C(5),N(4) in cytosine – is not considered here, even though it is a viable option [75]. With *N(1)*-substituted cytosine bases, to which the following discussion shall be restricted, it appears that the Pt-binding sequence is N(3)>N(4) ≫ O(2), hence that the second Pt entity binds preferentially to N(4) (with deprotonation of the amino group) rather than O(2) [76]. In this way, the cytosinato ligand is similar to the amidinato ligand (*Scheme 8*) or its substituted derivatives, which are well known for their ability to bridge two metal centers [77]. Nevertheless, participation of O(2) in metal-binding within heteronuclear Pt,M complexes is also established [76][78][79], either in a N(3),O(2) or a N(4),N(3),O(2) fashion.

Scheme 8

The binding situation in '1-methylcytosine blues' is not clear, although there is strong indication that base deprotonation (hence *N(3),N(4)*-bridging) is involved and that a 'blue' (or actually 'purple') product forms from the mononuclear precursor *cis*-$[Pt(NH_3)_2(1\text{-MeC-}N3)(OH)]^+$ (1-MeC = 1-methylcytosine) [80] (see also below).

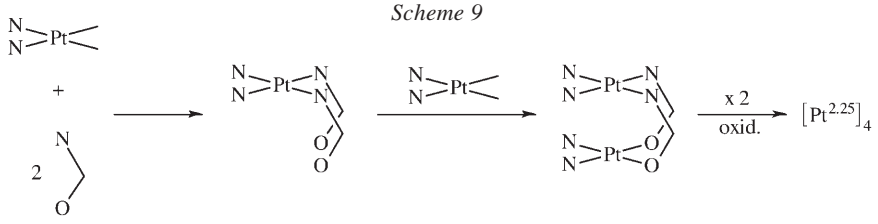
Creatinine, which has a close structural similarity with cytosine as far as potential metal-binding sites are concerned, has likewise shown to form 'blues', with both K_2PtCl_4 and *cis*-$(NH_3)_2Pt^{II}$ as reactants [81]. These 'blues' can be positively charged, neutral or negatively charged, but structural data are not available.

Dimers-of-Dimers *vs*. Oligo- and Polymers

Bridging Nucleobases

In all X-ray structurally characterized tetranuclear $Pt^{2.25}$ 'blues' containing a cyclic amide (α-pyridone; α-pyrrolidone), an imide (3,3-dimethylglutarimide), or a pyrimidine-2,4-dione (1-methyluracil), an identical structural principle is realized – that of *i*) dinuclear building blocks, containing *ii*) two *head-to-head*-oriented bridging ligands, *iii*) held together by partial Pt-Pt bond formation, and *iv*) by H-bonding between the two dinuclear entities (*Scheme 9*).

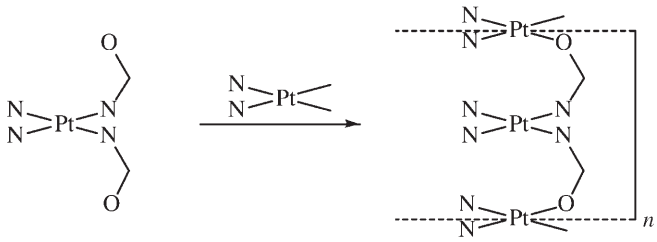
Scheme 9



There are, however, various feasible alternatives, all of which also take advantage of N,O bridge formation, yet utilize different modes for oligomer formation. For example, if in the 1 : 2 complex the two bases were to adopt a *head-to-tail* rather than a *head-to-head* orientation, formation of a

polymeric rather than a dinuclear species, again of 2 : 2 stoichiometry, could take place (*Scheme 10*) (*c.f.* also comment on heteronuclear Pt,Ag acetamide complexes).

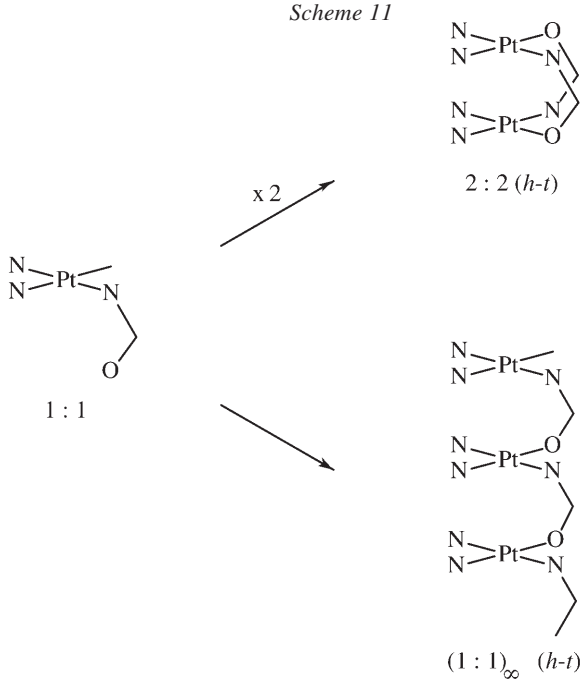
Scheme 10



1 : 2 (*h-t*) 2 : 2 (*h-t*)

Moreover, if a 1 : 1 complex is considered as a starting material (*Scheme 11*) it can dimerize to a *head-to-tail*, 2 : 2 species or form an oligo- or polymer. Although the 2 : 2 (*h-t*) species can further associate *via* H-bond formation in the solid state [43], intercationic Pt-Pt distances (3.9–4 Å) are then much too long to permit metal-metal bond formation on oxidation. By contrast, there is obviously no problem for the polymeric structure to achieve short Pt-Pt contacts.

Scheme 11



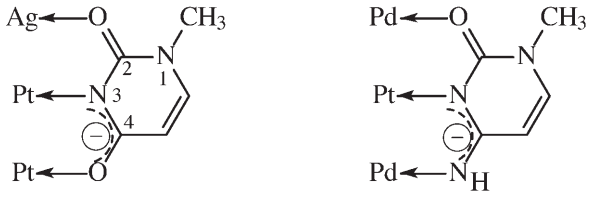
2 : 2 (*h-t*)

1 : 1

$(1 : 1)_\infty$ (*h-t*)

These options apply to all three mentioned types of ligands. For the pyrimidine nucleobases (N(1) position blocked for simplification), additional variations emerge if Pt-binding is assumed to also take place via the third exocyclic group, *e.g.*, O(2) in *N(3),O(4)*-bridged 1-methyluracilate or O(2) in *N(3),N(4)*-bridged 1-methylcytosinate ligands. In heteronuclear compounds, containing Pt and another metal ion, these features have been observed in a number of cases [62][63][76][82] (*Scheme 12*). If realized in systems containing exclusively *cis*-$(NH_3)_2Pt^{II}$ and the nucleobase, this could mean that even 2 : 2 (*h-t*) complexes could be joined to oligo- or polymeric structures if *cis*-$(NH_3)_2Pt^{II}$ entities were to bind to the still available O(2) sites. It is to be expected that as the number of bonded metal entities increases, association becomes weaker.

Scheme 12

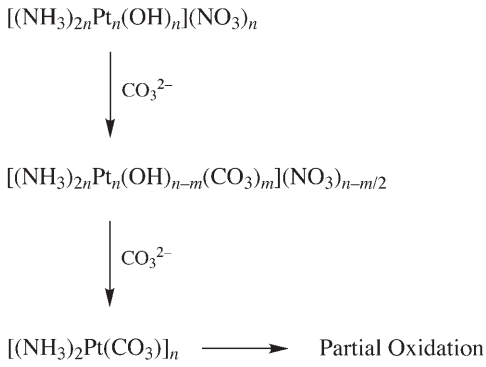


Simple Anion Bridging: OH^- and HCO_3^-/CO_3^{2-}

It is well documented that what is frequently termed the 'diaqua species' of cisplatin, *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$, in fact is a complicated mixture, containing also μ-OH di-, tri- and possibly even higher nuclearity species [83][84]. The μ-OH dimer *cis*-$[(NH_3)_2Pt(OH)_2Pt(NH_3)_2]^{2+}$ reacts with neutral 1-methyluracil (and other uracils), even at weakly acidic pH (despite the rather high pK_a value of 1-MeUH), because the OH group can act as a base which deprotonates the uracil. Nucleobase deprotonation and N-binding could be preceded by attack of an exocyclic oxygen at one of the two Pt atoms, possibly leading to a species that is either singly bridged by OH^- or doubly bridged by OH^- and 1-MeU.

If a 'diaqua species' is kept in an open flask at pH 7 for several days, with the pH readjusted regularly, a blue soluble species forms, which varies in its composition depending on concentration and other reaction conditions. The materials that can be isolated upon concentrating the solution followed by precipitation definitely contain carbonate (or bicarbonate), according to elemental analysis and IR spectroscopy. From the latter it appears that these anions are coordinated (bridging?) rather than existing as simple counter ions. If HCO_3^- is intentionally added, intensely colored materials (brown, blue, black) of an empirical composition close to $Pt(NH_3)_2CO_3$ can

Scheme 13



be isolated (*Scheme 13*), which display EPR spectra similar to those of the typical 'Pt pyrimidine blues'.

The structures of these materials are as yet unknown. Interestingly, a tetranuclear complex containing four *cis*-$(NH_3)_2Pt^{II}$ units and two μ_4-carbonato ligands, $[Pt_4(NH_3)_8(CO_3)_2](NO_3)_4$, has been isolated from a preparation of a 'Pt deoxyuridine blue' and characterized by X-ray crystal structure analysis [85]. The four Pt ions in this complex form roughly a square of sides of *ca*. 3.18–3.28 Å and consequently do not form metal-metal bonds.

These findings strongly suggest that μ-CO_3 or μ-CO_3H complex formation for 'Pt blues' prepared near neutral pH in open air is possible. Because of this, it is questionable if stoichiometries of 'Pt blues' (*e.g.*, Pt/nucleobase ratio) can be properly estimated from elemental analysis data. Aside from that, there is yet another uncertainty, that of whether the NH_3 ligands remain intact (see next paragraph).

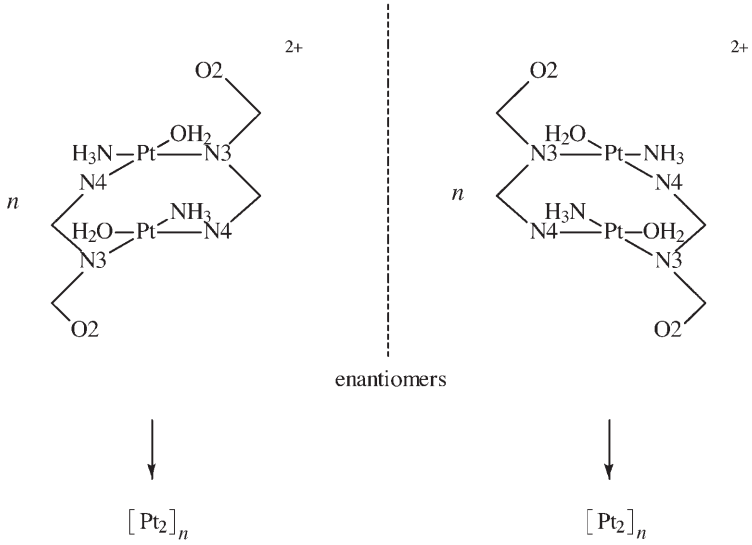
NH_3 Ligands: Really Always Retained?

All X-ray structurally characterized $[Pt^{2.25}]_4$ compounds derived from cisplatin have been shown to retain the two ammonia ligands in *cis*-arrangement. On the other hand, 'Platinblau' by *Hofmann* and *Bugge* did not contain any NH_3 ligand, and neither do the 'blues' prepared from K_2PtCl_4. It is obvious that removal of a NH_3 ligand from a cisplatin-derived 'blue', either in a precursor or in the final, oxidized product, substantially increases the possibilities for ligand-bridge formation, and hence offers patterns of association of building blocks that are different from those seen in the tetranuclear 'blues'. There were suggestions initially [86] that the instability of the 'blues' might be related to loss of NH_3 ligands, based on the elemental analysis data of *Rosenberg's* 'blues' which indicated a generally lower N

content than expected (even though the presence of carbonate might explain it as well, see above), and because an excess of NH_4NO_3 led to an enhanced stability of 'α-pyridone blue' in solution. *Okuno et al.* likewise have formulated their 'pyrimidine greens' as NH_3-deficient materials [46][47], and a proposed structure for a 'phthalimide blue' included partial replacement of ammonia ligands as well [87]. We have indeed verified [25] that the workup of a typical preparation of a 'Pt pyrimidine blue' according to *Rosenberg*'s protocol, yields NH_4^+ salts in the final, most soluble fraction. It is as yet unclear, at what stage and why NH_3 is released. Of the two ways by which we have previously seen loss of NH_3 from a *cis*-$(NH_3)_2Pt$ entity – *via trans*-effect of a Cl^- ligand [88] and by mild HCl treatment of a diplatinum(III) complex [89] – the second one may be important in the present case. With *cis*-$(cpa)_2PtCl_2$ (cpa = cyclopropylamine), which forms a 'purple' with 1-methylthymine [90], we have clear evidence for significant loss of the amine ligand, in particular when heated [91].

In order to further pursue this aspect, we have started to prepare and characterize pyrimidine nucleobases containing a single NH_3 group bound to Pt^{II} only. Starting from *trans*-$[Pt(H_2O)_2(NH_3)$(1-MeC-*N(3)*$)]^{2+}$ it was observed that there is rapid formation of a dinuclear *head-to-tail*-species with *N(3),N(4)*-bridging 1-methylcytosinato ligands and, in addition, formation of a purple material which displays all the characteristics of paramagnetism and intense color of the 'blues' [92]. It is tempting to speculate that the dinuclear entities might oligomerize, simply by loss of water ligands and utilization of the O(2) atoms as bridging groups. The oligomers formed could be stabilized by H-bonding between O(2) and NH_3 of adjacent dimer building blocks and by partial oxidation of Pt. Clearly, a single Pt^{III} might be delocalized over more than four Pt centers, thereby leading to an average oxidation state lower than +2.25. There are two more points worth mentioning in this context. First, it has to be realized that due to the chirality of the *head-to-tail* dinuclear complex, only *identical* enantiomers can combine, and therefore the proposed oligomerization pattern would lead to two types of oligomers that differ in their helix sense (*Scheme 14*). Second, we noticed that the *head-to-tail* dinuclear complex, in the presence of a chelating ligand (glycine or L-alanine) undergoes easy oxidation (pH 3, in air) to diplatinum(III) complexes [92]. This behavior contrasts with that of most other diplatinum(II) complexes containing two ammonia ligands at either Pt-atom, which usually require strongly acidic reaction conditions for oxidation to diplatinum(III).

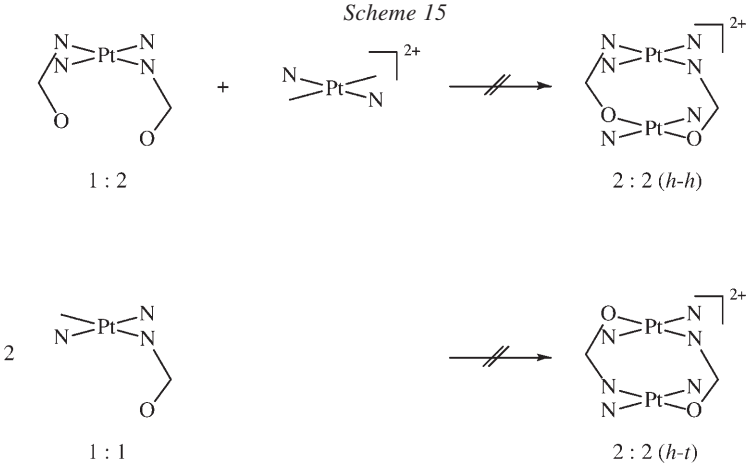
Scheme 14



trans-$(NH_3)_2$Pt(II): Why no 'Blues'?

'Pt pyrimidine blues' derived from *trans*-$(NH_3)_2Pt^{II}$ appear not to be known. This statement undoubtedly is true for the tetranuclear $Pt^{2.25}$ analogue, simply because a dinuclear precursor, *trans*-$[Pt_2(NH_3)_4L_2]^{2+}$ (with L = amidate, imidate or amidinate) cannot be formed. This is a consequence of the steric hindrance [93] between the parallel-oriented am(m)ine ligands in a doubly bridged species containing two ligands with short N,O, or N,N bite distances, as present in these ligands L (*Scheme 15*). This holds up both

Scheme 15



for (hypothetical) *head-to-head* and *head-to-tail* dinuclear species. In open-chain oligomers (derived from 1 : 2 or from 1 : 1 complex) the steric interference between am(m)ine ligands of adjacent Pt^{II} centers may be less severe due to the possibility of tilting of coordination planes (and in fact such compounds may exist), but as soon as Pt-Pt bond formation is to occur, this problem would recur. It is of interest, in this context, to recall how the analogous *trans*-$(NH_3)_2Pd^{II}$ circumvents this problem: In the case of a dinuclear *head-to-tail* complex, *trans*-$(NH_3)_2Pd^{II}$ escapes the steric clash of ammonia ligands by isomerization to the *cis*-isomer [94]. In the mixed *trans*-$(NH_3)_2Pt^{II}$/*trans*-$(NH_3)_2Pd^{II}$ system, the Pd^{II} simply gets rid of an ammonia and instead chooses Pt^{II} as its ligand [93].

Short Pt-Pt contacts and the possibility of Pt-Pt bond formation is definitely feasible if, for example, occasional loss of both am(m)ine ligands from a *trans*-a_2Pt^{II} entity is considered. After all, a Pt_2Cu analogue of a hypothetical *trans*-$[\{a_2PtL_2\}_2Pt]^{2+}$ species (L = α-pyridonate) has indeed been synthesized and structurally characterized [95]. It appears that other scenarios leading to polymeric arrangements and reasonably short Pt-Pt contacts are feasible for *trans*-a_2Pt (in combination with am(m)ine deficient Pt species), but a definite judgement is possible only after more work has been performed.

As a final point, an interesting difference in the principles of oligomer-formation between Ag^+ on one hand and *cis*- and *trans*-$(NH_3)_2PtL_2$ on the other should be mentioned: whereas in Pt_xAg_y compounds derived from the *cis*-isomer basic structural features as seen in the 'blues' are retained, *viz*. bridging heterocyclic ligands that are essentially perpendicular to the Pt planes, in a heteronuclear Pt_2AgNa complex derived from *trans*-$Pt(NH_3)_2$(1-MeU-*N(3)*)$_2$ a different principle – stacking between adjacent *trans*-$Pt(NH_3)_2$(1-MeU)$_2$ entities – is observed [96]. It precludes any direct Pt-Pt long-range interactions in a putative oligomer built on this feature.

Summary

Despite some indisputable progress in the understanding of the nature and composition of 'platinum blues' derived from *cis*-$(NH_3)_2Pt^{II}$ which has been achieved within the last twenty years, in particular thanks to X-ray crystallography, there is still no comprehensive picture available on the '-blues'. This is true in particular for the 'platinum pyrimidine blues', on which this review had focused. The amorphous nature of these materials has restricted the methods of investigation to questions such as metal-metal separations [97] or delocalization of spins due to the presence of Pt^{III} centers in a Pt chain [29][51]. These methods have, however, not provided insight

into the association pattern on a molecular level in materials larger than a tetramer. While model nucleobase chemistry can offer a large number of answers potentially relevant to this question, they need to be proven. Clearly, the mystery of the 'platinum blues' is beginning to be clarified, but there is as yet a long way ahead until it is fully understood.

I am grateful to my coworkers and collaborators, the names of which are found in the individual references, for their contributions to the research. The financial support of the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie* are acknowledged. My special thanks go to Prof. *James H. Burness*, Pennsylvania State University York, and Dr. *Gabriele Trötscher-Kaus* for carefully reading the manuscript and for making many suggestions for its improvement, and to Dipl.-Chem. *Jens Müller* for preparation of the schemes.

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Heteronuclear Pt^{II} Complexes with Pyrimidine Nucleobases

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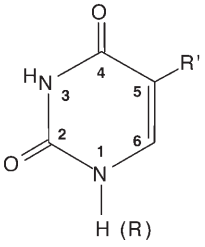
Pyrimidine nucleobases, particularly in their anionic forms, are versatile ligands for metal ions. In fact, their several potential donor sites enable them to form homo- and heteropolynuclear complexes. These compounds exhibit different metal/ligand stereochemistries, as well as metal-metal interactions, ranging from non-bonding to bonding ones, as a function of the nature of the metal centers and of the particular arrangement of the nucleobases. The interactions in these complexes are rather variable and can be interpreted applying semiempirical MO-methods. Generally, the increase in number of the deprotonated sites leads to an increase in polynuclearity of the complexes, which can be considered as being built up of simple mononuclear blocks. However, monoanionic ligands may favor the formation of high nuclearity compounds when appropriate geometrical conditions allow the match of the mononuclear building blocks. Two kinds of pyrimidine-bridged polynuclear species have been found, the first characterized by approximately linear chains of metal ions, and the second one by tri- or polycyclic arrangements of metal ions including 'molecular squares and boxes'. In the cyclic arrangements, the long distances between the metal centers exclude any metal-metal interaction, whereas dinuclear and linear polynuclear chains are characterized by a wide range of interactions, from metal-metal bonding to *van-der-Waals* interactions.

Introduction

Following the discovery of the antitumor activity of *cis*-$[(NH_3)_2PtCl_2]$ (cisplatin) by *Rosenberg et al.* [1], studies on the interaction of Pt ions and Pt coordination complexes with DNA have demonstrated that the cellular DNA is the major target of cisplatin [2]. From *in vitro* studies, a number of binding patterns of cisplatin to DNA in intra- and interstrand fashion have been established [3], which revealed that the cisplatin-DNA adducts are essentially formed, at a low level of platination, by binding of Pt to the N(7) donor sites of guanine and, to a lesser extent, of adenine. Therefore, a great deal of work in the area of Pt-DNA modelling is centered on the intra- and

Cisplatin. Edited by Bernhard Lippert

interstrand purine adducts. However, there is evidence that additional base sites can be available and minor cross-links are also formed, which might include pyrimidine nucleobases. The pyrimidine bases, which commonly occur in nucleic acids, are uracil (UrH_2), thymine (TH_2) and cytosine (CyH_3), although DNA and tRNA may contain other rare pyrimidine bases as well. CyH_3 and TH_2 are ordinarily present in DNA, while CyH_3 and UrH_2 are RNA bases. The formulae of the pyrimidine bases together with the atom numbering scheme, are shown in *Fig. 1*. Appropriate model studies have shown the possible involvement of the cytosine N(3) donor site [4], and the guanine-cytosine cross-link has been observed in tRNA [5]. While binding to N(3) of thymine is not a major reaction, this in principle may occur since the product is expected to be the most thermodynamically stable of all the Pt-nucleobase complexes involving endocyclic N-donors [6]. At the dinucleotide level (d(TpG)), chelate formation with the N(3) site of thymine and N(7) of guanine involved in Pt-binding has clearly been established [7]. Furthermore, among the most intriguing classes of Pt antitumor agents are the 'Pt pyrimidine blues' [8] obtained by oxidation of Pt^{II} complexes containing aliphatic or heterocyclic amide ligands [9]. The difficulty in the characterization and the complex chemistry of the 'blues' have limited their clinical trials. However, substantial progress has been made in understanding their chemistry since then, to justify further studies, particularly concerning their polymeric nature, the fractional Pt oxidation state and the intermetallic bond. The findings on the synergistic effect of joint application of cisplatin and deoxynucleotides of thymine and uracil were relevant in order



R' = H

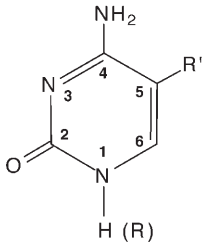
UrH_2 = uracil

1-MeUrH = 1-methyluracil, R = Me

R' = Me

TH_2 = thymine

1-MeTH = 1-methylthymine, R = Me



R' = H

CyH_3 = cytosine

1-MeCyH_2 = 1-methylcytosine, R = Me

R = R' = Me

1,5-Me_2CyH_2 = 1,5-dimethylcytosine

Fig. 1. *Pyrimidine nucleobases with the atom-numbering scheme*

to ascertain the role played by the latter bases in the antitumor activity [10]. Therefore, the interest in platinum pyrimidine nucleobase complexes originates from several considerations relevant to the Pt antitumor agent as well as to their fascinating chemistry, based on the versatile binding patterns of these ligands, including supramolecular assemblies [11] and on Pt-metal interaction.

Pyrimidine Nucleobases as Metal Ligands in Neutral and Anionic Forms

Tautomers of Pyrimidine Nucleobases

The predominant tautomeric structures of uracil (R′ = H, X = O), thymine (R′ = Me, X = O), and cytosine (R′ = H, X = NH), together with the atom numbering scheme, are shown in *Fig. 1*. The shorthand, such as UrH_2, TH_2 and CyH_3 (*Table*), makes evident the number of H atoms bonded to the pyrimidine nitrogens (*Fig. 2*) and the notation will be useful when deprotonated species, such as UrH^-, CyH^{2-}, *etc.* are to be considered. When used as model of the respective nucleotide binding metals, the nucleobases are often alkylated at N(1) to avoid coordination at this site, giving for example the 1-MeUrH, 1-MeTH, and 1-$MeCyH_2$ derivatives. Still, the parent bases have been also used in metal binding studies. Several reviews [3][6][12][13] have appeared covering the literature up to 1990 and, more recently, a survey of the structural properties of the nucleobase complexes was published [14]. The most important properties of the ligands are summarized in this section.

In aqueous solution, the parent bases can exist as neutral, mono- and dicationic species as well as mono- and poly-anionic ones, depending on pH. Formation of these species implies protonation and deprotonation at the

Table. *Shorthands for Neutral, Mono-, and Dianionic Forms of Pyrimidine Nucleobases* [a])

Neutral	Monoanion	Dianion
CyH_3	CyH_2^-	CyH^{2-}
UrH_2	UrH^-, Ur^-H_2	Ur^{2-}
TH_2	TH^-	T^{2-}
1-$MeCyH_2$	1-$MeCyH^-$, 1-$MeCy^-H_2$	1-$MeCy^{2-}$, 1-$MeCy^-H^-$
1-MeUrH	1-$MeUr^-$	
1-MeTH	1-MeT^-	

[a]) Only H-atoms bonded to nitrogens are shown. Species deprotonated at the ring C(5) atom are reported in italics with the negative charge given before the H-atoms.

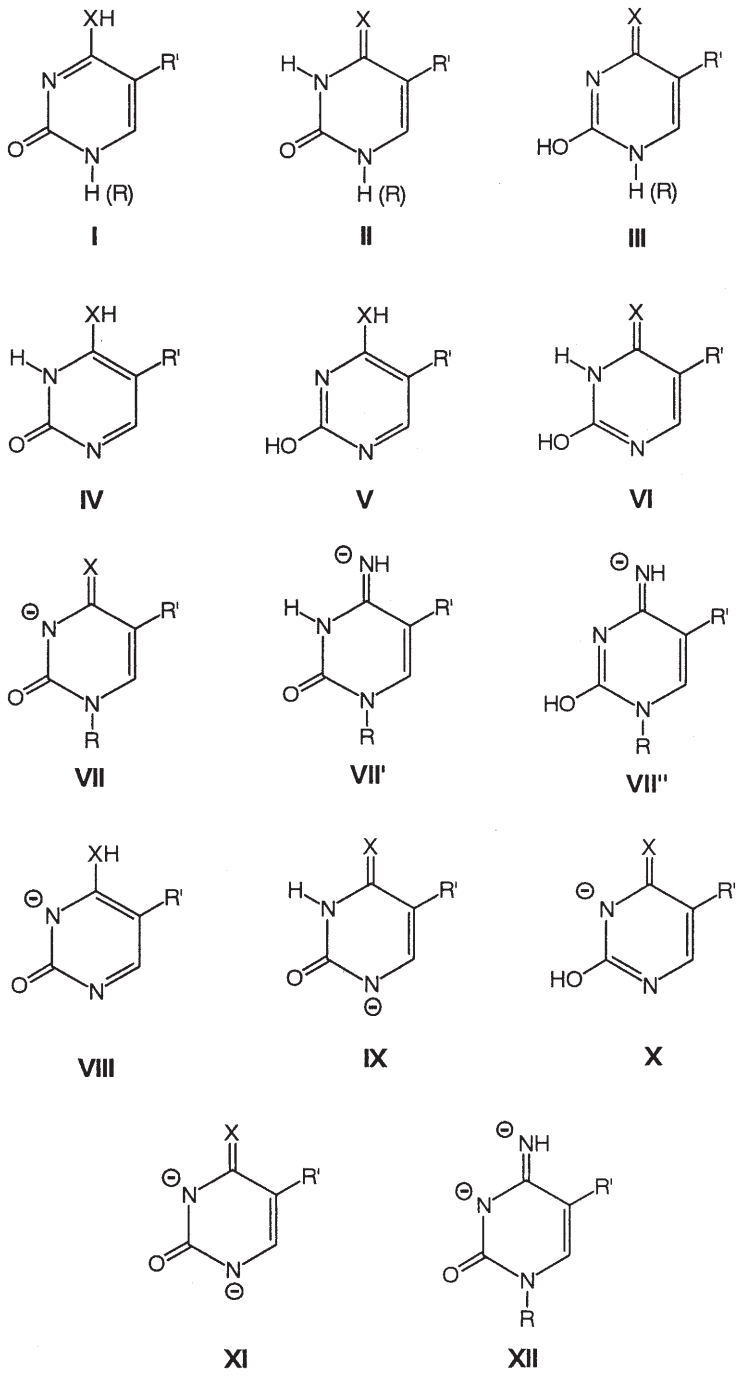


Fig. 2. *Tautomers of uracil* (R′ = H, X = O)*, thymine* (R′ = Me, X = O)*, cytosine* (R′ = H, X = NH) *and their* N(1)*-alkylated derivatives in neutral and in the most common anionic species*. Only one of the resonance structures is shown.

pyrimidine N atoms. Unsubstituted bases in their neutral form can exist in six tautomeric forms (**I**–**VI** of *Fig. 2*). However, the tautomer **II** (for UrH_2 and TH_2) and the tautomer **I** (for CyH_3) exceed the other five forms as indicated by structural, spectroscopic and theoretical studies [15][16]. Alkylation at N(1) reduces the number of possible tautomers to three (**I**–**III**, *Fig. 2*). Monodeprotonated species can form four tautomers (**VII**–**X**, *Fig. 2*) and UV spectroscopic studies suggest that forms **VII** and **IX** of uracilate are predominant in water solution [17]. The tautomer **VII** (*Fig. 2*) is only possible for *N(1)*-substituted monoanions of uracil and thymine, whereas **VII**, **VII′**, and **VII″** are the tautomers for the monoanion 1-$MeCyH^-$.

The dianion of the parent CyH_3 may form three tautomers (not shown in *Fig. 2*), whereas that of UrH_2 and TH_2 has only one tautomer (**XI**, *Fig. 2*), as well as the dianion of 1-Me substituted cytosine (**XII**, *Fig. 2*).

On the other hand, electrophilic metal binding at C(5) with consequent deprotonation of this site was observed [12]. Mono- (at C(5)) [18–20] and di-deprotonated species (C(5),N(4)) [21] have been reported. The shorthand used to represent deprotonation at C(5) for uracils and cytosines bears the negative charge before the shown *N*-bonded H-atoms. In the *Table*, the shorthand for these anions is reported in italics.

Rare tautomeric forms of the neutral (or monoanionic) base may be stabilized by coordination to metal ions [13][22–24], especially with metals in high oxidation states such as Pt^{III} and Pt^{IV}, but also with Pt^{II} [25].

Ligand Coordination Modes

The neutral nucleobases act as monodentate ligands and their anions either as mono- or poly-dentate ones, often bridging two or more metal centers. The monoanion 1-$MeCyH^-$ has been found to chelate Pt^{IV} species through N(3) and N(4) [26].

The binding mode of uracils and thymines in neutral and deprotonated forms has been reviewed up to 1987 [13]. They coordinate hard, and relatively few soft metal ions, through O(4) (preferentially) and O(2). Uracil (thymine) behaves as a weak dibasic acid in alkaline media with the more basic site N(3) at $pK_a \approx 9.69$ (10.16), as compared to N(1) at $pK_a \approx 14.2$. At high pH the monoanions of uracil and thymine bind the metal ions preferentially *via* N(1). However, the N(3) linkage isomer of the Pt^{II} complex has also been obtained [24]. The relatively few examples of complexes with soft metal ions, containing monodentate uracilate anions, are due to the high tendency of the ligand to bind additional metal ions to form polynuclear species [13].

Neutral cytosine and its alkylated derivatives prefer metal binding through N(3), which represents the more basic site ($pK_a \approx 4.6$) as compared

to N(1) ($pK_a \approx 12.2$) and N(4) ($pK_a \approx 16.7$) [12]. However, binding of 1-$MeCyH_2$ through N(4) has been reported both with Pt^{IV} [27] and Pt^{II} [25], which implies stabilization of the rare imino-oxo tautomer **II** (*Fig. 2*). The O(2) site in cystosine is in no instance involved in Pt-binding in contrast to other transition metals, such as Ni^{II} and Mn^{II}, as well as Mg^{II} [28]. 1-$MeCyH^-$ has been found to coordinate through N(4) metal centers in high oxidation states such as Pt^{IV} [29] and Ru^{III} [30]. Deprotonation of cytosine at C(5) (1-$MeCy^-H_2$) leads to the generation of rare monoanions and facilitates subsequent coordination at this site [12]. However, no crystallographic evidence of monodentate *N(1)*-coordinated cytosinate has been reported as yet.

Pyrimidine nucleobases in monoanionic form act as bidentate ligands coordinating either two metal centers as doubly bridging ligands or, more rarely, the same metal ion in a chelating fashion [13][14][26]. The monodeprotonated uracil, alkylated at N(1), acts as bridging ligand in homo- and heterodinuclear complexes *via* N(3),O(4), but not *via* N(3),O(2) [14]. Deprotonated 1-$MeCyH^-$ and 1,5-Me_2CyH^- have been found to act as bridging ligands *via* N(3), N(4) in di- or trinuclear species [14][31][32].

Two mixed doubly bridged (1-$MeCyH_2$, 1-$MeUrH^-$) complexes have been reported where the cytosinate monoanion acts as bridging ligand *via* N(3),O(2) [33][34]. However, UrH^- and 1-$MeCyH^-$ have been found to act as singly bridging ligands, through N(1),N(3) and N(3),N(4), respectively, in only a few tri- and tetranuclear cations [24][35][36].

A number of polynuclear Pt,Ag complexes containing the methyluracilate monoanion have been reviewed [13]. The base acts as tridentate ligand *via* O(2),N(3),O(4) and, in one case, as a tetradentate ligand through an additional binding to O(4) (*Fig. 3,a*) [37]. When the uracil is deprotonated at the N(1) and N(3) sites, the resulting dianion (**XI**, *Fig. 2*) behaves as a tetradentate N(1),O(2),N(3),O(4) ligand in the octanuclear Pt_8 and Pt_4M_4 (M = Ni, Cu, Pd, Ag) complexes [21] leading to so-called 'molecular boxes' (see below).

Cytosinate anions have been shown to act as O(2),N(3),N(4) tridentate ligands (*Fig. 3,b*) in trinuclear Pt,Pd_2 [32] and Pt,Ag_2 [31] complexes. 1-Methylcytosine, deprotonated at N(4) and C(5) (1-$MeCy^-H^-$), binds Pt^{II} through N(3) and two different Hg^{II} ions through N(4) and C(5) in a Pt_2Hg_6 polynuclear species (*Fig. 3,c*) [21]. The structural analysis of the $[(MeHg)_3(1\text{-}MeCy^{2-})](NO_3)$ complex [38] revealed the only example in which the NH_2 group of the pyrimidine base is fully deprotonated and substituted by two $MeHg^+$ groups, with a third $MeHg^+$ entity being coordinated to N(3). This tridentate binding pattern of the 1-$MeCy^{2-}$ dianion is shown in *Fig. 3,d*.

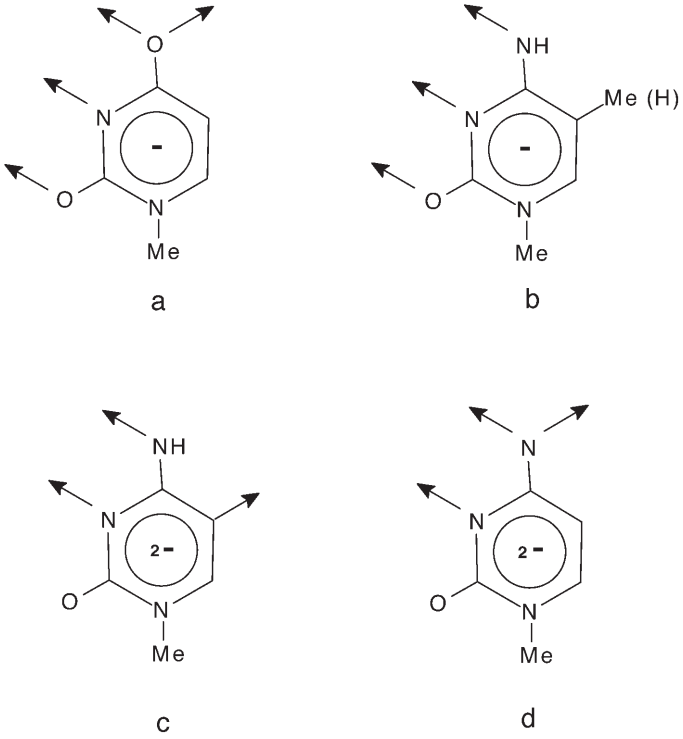


Fig. 3. *Pyrimidine nucleobase anions acting as tri- and tetradentate ligands*

Mononuclear $(pym)_2Pt^{II}$ Complexes

The mononuclear Pt complexes, particularly those having two coordinated nucleobases, can be considered as starting building blocks for the synthesis of polynuclear ones. For *cis*-$(pym)_2Pt^{II}$ complexes, two nucleobase orientations are feasible with respect to the coordination plane, the so-called *head-to-head* (*h-h*) orientation where the bases are related by a mirror (or pseudo mirror) plane, and *head-to-tail* (*h-t*) orientation where they are related by a C_2 (or pseudo C_2) axis. In the *trans*-$(pym)_2Pt^{II}$ complexes, the *h-h* nucleobases are related by a mirror (or pseudo mirror) plane perpendicular to the coordination plane, whereas the *h-t* pyrimidines are related by an inversion (or pseudo inversion) center [39]. Crystallographic studies widely proved that the plane of coordinated bases lies roughly perpendicular to the coordination plane, usually adopting a *h-t* conformation, regardless of the possible *cis* or *trans* geometry at the metal [14].

The nitrate salt of *cis*-$[(NH_3)_2Pt(1\text{-}MeCyH_2,N(3))_2]^{2+}$ crystallizes with an additional cytosine molecule hydrogen-bonded in the crystal lattice

[40][41]. The *h-t* orientation of the two bases permits the formation of weak interbase intramolecular H-bonds of type N(4)H···O(2).

The ^{1}H-NMR resonances in *cis*-[$(NH_3)_2$Pt$(pym)_2$] (with pym = 1-MeUr$^-$ [42] and 1-MeT$^-$ [43]) are shifted upfield relative to those of the neutral base. The spectrum does not give any indication of signal splitting at 30 °C that could indicate the presence of stable stereoisomers with a high barrier of rotation, as observed, for example, in [Pt(*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)(guanosine)$_2$]$^{2+}$ [44]. The formation of dinuclear *h-h* dimers (see below) proves that the rotation of the 1-MeUr$^-$ ligands about the Pt-N(3) bond is possible in solution. The mutual repulsions of the exocyclic oxygens in the *cis*-$(NH_3)_2$Pt(1-MeUr$^-$,*N(3)*)$_2$ complex should be comparable for the *h-h* and *h-t* arrangements, as compared with those in bis(1-MeCyH$_2$) [40] and bis(guanosine) [44] complexes, where unfavorable interligand interactions (missing H-bonding, repulsion between identical exocyclic groups) do not stabilize the *h-h* arrangement of the two ligands in *cis* positions.

As far as the *trans*-$(pym)_2Pt^{II}$ complexes are concerned, the ^{1}H NMR spectrum of *trans*-[A_2Pt(1-MeCyH$_2$,*N(3)*)$_2$]$^{2+}$ (A = NH_3 or amine) in aqueous solution is consistent with the existence of two rotamers with mutual *h-h* and *h-t* orientations of the two nucleobases with a distribution of 3.4 : 1, with the *h-t* form preferred. Interconversion of the two rotamers is slow on the NMR scale even at 85 °C, above which decomposition sets in [39][45].

The corresponding complex containing N(4) bound neutral 1-methylcytosine was obtained from the *trans*-[A_2Pt(1-MeCyH$_2$,*N(3)*)$_2$]$^{2+}$ *via* oxidation to a Pt^{IV} species, followed by metal migration to N(4) and subsequent reduction to Pt^{II}, a pathway which represents an excellent example for a redox-assisted metal migration at a heterocyclic ligand [25]. The crystal structure of *trans*-[A_2Pt(1-MeCyH$_2$,*N(4)*)$_2$]$^{2+}$ (with the model nucleobase in the rare imino-oxo tautomer of type **II**, *Fig. 2*) shows the complex arranged on a crystallographic symmetry center, and consequently a *h-t* arrangement of the bases, with Pt *syn* relative to N(3). This arrangement corresponds to the most abundant species present in solution [25].

From a structural point of view, the [Pt(1-MeCyH$_2$,*N(3)*)$_3$Cl]$^+$ complex cation exemplifies, in an impressive manner, how drastic a distortion of DNA is to be expected if binding of three bases to a single metal of square planar coordination geometry were to occur. While in bis(nucleobase) complexes of *cis*-A_2Pt^{II} some base 'overlap' can occur, due to the possibility of the bases to slightly tilt from the perpendicular positions relative to the metal coordination plane, in the tris(nucleobase) complex the bases are forced to be almost perpendicular to that plane in order to minimize the mutual repulsion. The ^{1}H-NMR spectra of [Pt(1-MeCyH$_2$,*N(3)*)$_3$Cl]$^+$ in D_2O display two sets of resonances in a 2:1 ratio, corresponding to the mutually *trans* positioned 1-MeCyH$_2$ ligands and to that *trans* to the chloro, respectively.

This is consistent with a complex containing *N(3)*-coordinated nucleobases in a *h-t-h* orientation, as detected in the solid state [46]. This conformation is stabilized, with respect to the *h-h-h* and *h-h-t* ones, by the formation of a more favorable H-bond pattern. However, consideration only of intramolecular hydrogen bonding in predicting rotamers of the tris(nucleobase) complex in solution is questionable, since the distribution of rotamers is strongly dependent on the solvent [47].

The four bases in the Pt tetrakis(cytosine) cation of $[Pt(1\text{-}MeCyH_2)_4]X_2$ (where $X = NO_3^-$, Cl^-) are arranged in a way that adjacent ones are orientated *head-to-tail* with respect to each other [46]. This cation provides two N_2O_2 donor sets, while the rare *h-h-h-h* rotamer, not observed in solution at detectable amount, provides two donor sets, O_4 and N_4 above and below the Pt coordination plane. Both rotamers represent interesting starting material for the preparation of heteronuclear complexes which display short intermetallic distances and intriguing magnetic properties [48] (see below). The ^{1}H-NMR spectrum of $[Pt(1\text{-}MeCyH_2)_4]^{2+}$ in D_2O displays one set of cytosine resonances, as expected for the equivalency of the four ligands in a *h-t-h-t* orientation. Assuming that rotation of the ligands is slow on the NMR time scale, which undoubtedly is reasonable, this conformation is retained in solution. Three additional orientations of the cytosine rings are possible, but none of these rotamers is observed in aqueous solution, neither upon prolonged standing of the sample nor upon heating it up to 90 °C [46].

Dinuclear Complexes

The systematic studies of polynuclear complexes based on *cis*- and *trans*-$Pt(pym)_2$ blocks have shown several examples of metal-metal interactions between Pt and the heterometals at close distance, making allowances for suitable geometrical conditions. Only a few examples of a single pyrimidine base, bridging two metals, have been reported, [21][24][35][36][49] in contrast to polynuclear species where doubly bridged complexes, both homo- and hetero-nuclear, have been synthesized. Schematic representations of di- and polynuclear species, formally obtained starting from a *cis*- and *trans*-$(pym)_2Pt^{II}$ fragment are reported in *Fig. 4* and *Fig. 5*, respectively.

As far as the arrangement is concerned, the *cis* complexes of type $Pt(pym)_2M$ are very common with a *h-h* bases arrangement (**I**, *Fig. 4*), whereas a *h-t* pattern is found in diplatinum complexes (**V**, *Fig. 4*). These compounds are prepared through a condensation reaction between mononuclear complexes, according to

$$2\ cis\text{-}[(NH_3)_2Pt(pym)H_2O]^+ \rightarrow cis\text{-}[(NH_3)_2Pt(pym)_2Pt(NH_3)_2]^{2+} + 2\ H_2O \quad (1)$$

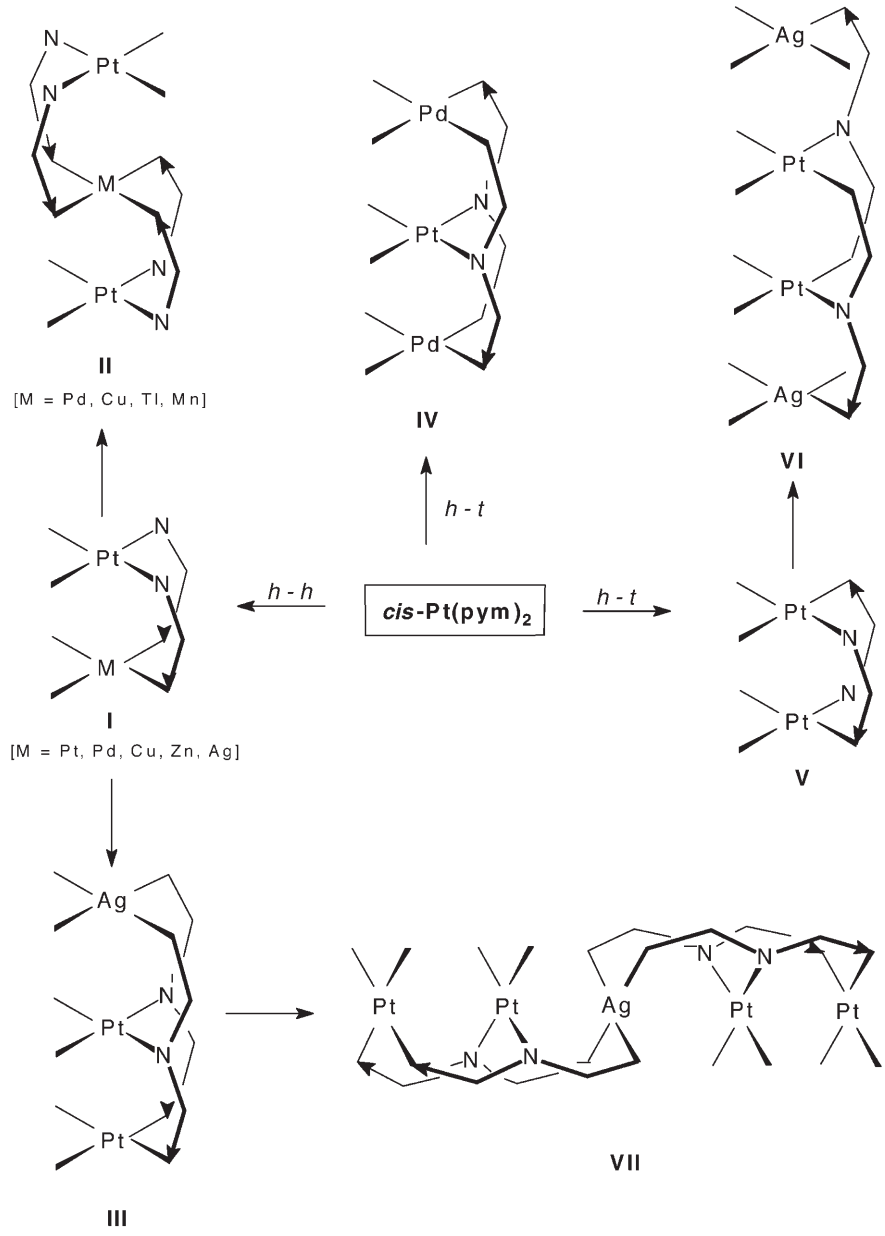


Fig. 4. *Concise scheme of di- and polynuclear species derived from* cis-$PtA_2(pym,N(3))_2$. The arrows indicate the arrangement of the bases, with N being the N(3) endocyclic donor atom.

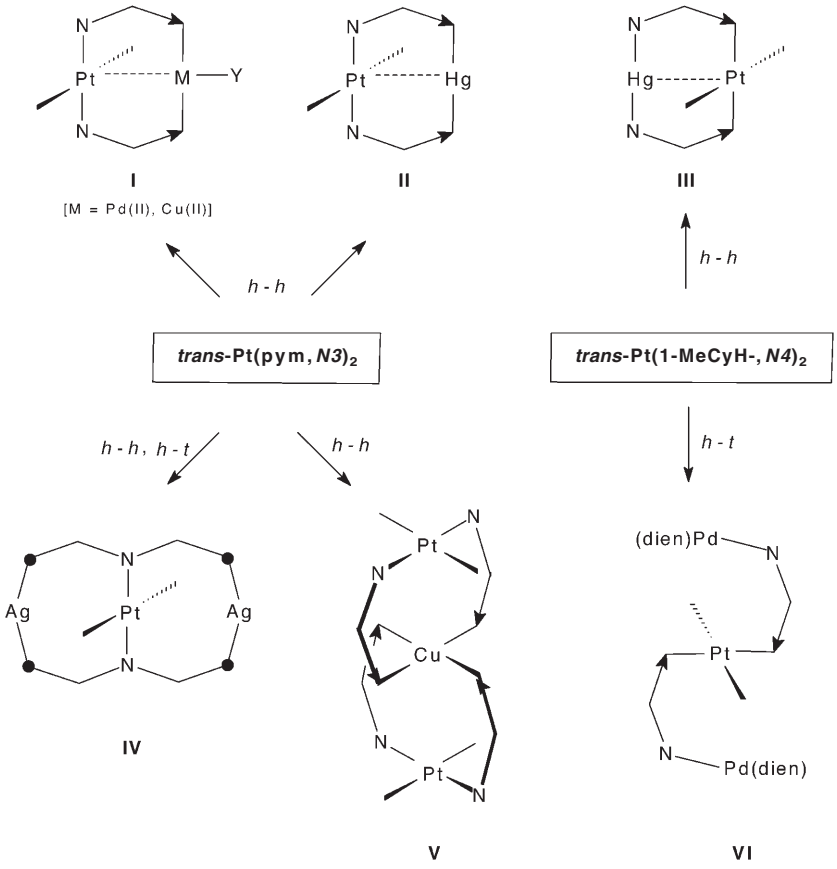


Fig. 5. *Concise scheme of bi- and trinuclear species derived from* trans-$PtA_2(pym)_2$. The arrows indicate the arrangement of the bases, with N being the N(3) endocyclic donor atom.

which affords dinuclear complexes with a *h-t* arrangement of the nucleobases. On the other hand, complexes with the pair of base ligands with a *h-h* configuration are obtained through a reaction of a neutral *cis*-$(pym)_2Pt(NH_3)_2$ with cations of type *cis*-$[A_2M(H_2O)_2]^{2+}$, (where A = NH_3 or A_2 = en, bipy) or with aquaspecies $[M(H_2O)_n]^{m+}$,

$$\begin{aligned} &cis\text{-}(NH_3)_2Pt(pym)_2 + cis\text{-}[A_2M(H_2O)_2]^{2+} \rightarrow [(NH_3)_2Pt(pym)_2MA_2]^{2+} \\ &cis\text{-}(NH_3)_2Pt(pym)_2 + [M(H_2O)_n]^{m+} \rightarrow [(NH_3)_2Pt(pym)_2M(H_2O)_n]^{m+} \end{aligned} \quad (2)$$

In these equations, pym is either a deprotonated or a neutral form of the pyrimidine base.

The uracilate and thyminate anions simultaneously bind the metals through N(3) and O(4), whereas the 1-methylcytosinate anions do so through N(3) and the deprotonated amino group N(4) [14]. The neutral cytosine was found also to act as bridging ligand through N(3) and O(2) [33].

The compounds *cis*-$(NH_3)_2Pt(1\text{-}MeUr^-,N(3))_2$ and *cis*-$(NH_3)_2Pt(1\text{-}MeT^-,N(3))_2$ show a remarkable tendency to bind additional metal ions to form di-, tri-, and pentanuclear complexes as well as even larger aggregates. This is likely due to an electronic effect (Pt, coordinating at deprotonated N(3) position, increases the electron density at the exocyclic oxygens as compared to the neutral base) and to the favorable coordination geometry of these oxygens. Regarding *Eqn. 2*, the starting Pt^{II} complexes are coordinatively saturated as far as the metal is concerned, and they require other metal ions to be present in order to form aggregates. On the other hand, the mononucleobase complexes of *Eqn. 1* have the ability to form di- or oligomeric structures without the presence of other metal ions. They can self-associate mainly in two fashions: either through *N*,*O*-bridging of $1\text{-}MeUr^-$ (*Fig. 6, a*) or through OH-bridge formation (*Fig. 6, b*) [50]. Analogous behavior is observed with $1\text{-}MeT^-$ [51].

Dinuclear complexes of type *cis*-$[A_2Pt(pym)_2MY_2]^{m+}$, obtained by *Eqn. 2*, are built up so that the metal coordination planes practically face each other (**I** and **V**, *Fig. 4*). Following the notation introduced by *Balch* and *Catalano* [52], they are represented by the symbolism 4:4, corresponding to the

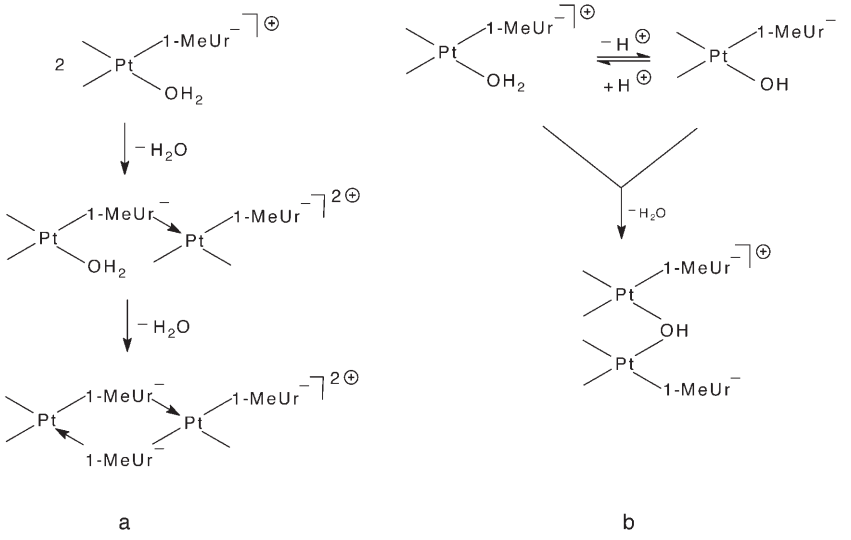


Fig. 6. *Formation of dinuclear Pt_2 complexes through double uracilate bridges* (a) *and through OH bridge* (b)

number of ligands around the two metals. In Pt_2 complexes the intermetallic distances vary in the range 2.86–3.20 Å, while in heterobimetallic complexes the Pt-M distances are within the range found for diplatinum species when M = Pd^{II} (2.927 Å), or Ag^{I} (2.907 Å), whereas they are significantly shorter when M = Cu^{II} (2.765 Å) or Zn^{II} (2.760 Å). The steric repulsion between the PtA_2 and MY_2 entities, which increases with the increasing ionic radius of M and with the bulk of ligands A and Y, may be modulated in some cases by a tilt of the coordination planes about the Pt-M vector [14]. Similar dimeric structures have been reported for a series of complexes of general formula *cis*-$[A_2X_nPt(pym)_2PtY_3]^{m+}$ (where A = NH_3 or amine; X,Y = monoanion or neutral species; n = 0 or 1) with Pt in +3 or in intermediate oxidation states between +2 and +3 [14].

The corresponding derivatives with bases in *trans*-configuration (*Fig. 7*) have never been isolated, possibly as a consequence of unfavorable steric interaction between the A and Y ligands at the adjacent metals, which prevents their formation. This hypothesis was also suggested by the observation that a *trans* → *cis* isomerization occurred when *trans*-$[(1\text{-}MeCyH_2,N(3))_2Pd(NH_3)_2]^{2+}$ reacted with *trans*-$[(NH_3)_2Pd(H_2O)_2]^{2+}$ to give the *h-t cis*-dipalladium species of type **V** (*Fig. 4*) [53].

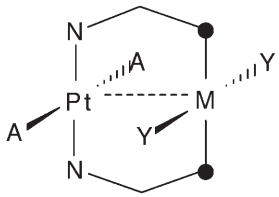


Fig. 7. *Hypothetical structure of a dinuclear complex with* trans-*arranged pyrimidine bases*

Therefore, it is not surprising that several examples of heterobimetallic complexes of general formula *trans*-$[A_2Pt(1\text{-}MeCyH^{-},N(3),N(4))_2MY]^{m+}$, (M = Pd^{II}, Cu^{II}, A = NH_3 or NH_2Me, Y = various N-, S-, O-donor ligands), with a *trans h-h* arrangement of the bridging 1-$MeCyH^{-}$ monoanions, have been prepared and structurally characterized [45][54][55]. The possible steric clash between the amines at Pt^{II} and ligands at M in the hypothetical 4:4 complex of *Fig. 7*, is avoided through the loss of a ligand Y on formation of the dinuclear complexes of type **I** of *Fig. 5*. In these complexes, the 1-$MeCyH^{-}$ ligands are approximately coplanar and the Pt-M distances (close to 2.5 Å) are shorter by *ca*. 0.40 (M = Pd) and *ca*. 0.25 Å (M = Cu), with respect to those found in the *cis* derivatives. Using the *Balch* notation and neglecting the metal-metal interaction, these complexes can be classified as 4:3 species. In complexes of the series

trans-[A_2Pt(1-MeCyH$^-$,*N(3)*,*N(4)*)$_2$PdY]$^{m+}$ (**I**, *Fig. 5*), the Pt–Pd distance does not appear to be significantly influenced by the electronic properties of the Y ligand, in contrast to ^{195}Pt-NMR chemical shifts that span a range of 500 ppm. These ^{195}Pt chemical shifts display a linear dependence with electronegativity of the halide Y, while no simple relationship is apparent with the nature of Y other than halides [45].

Intermetallic distances around 2.80 Å have been found in analogous complexes of formula *trans*-[A_2Pt(1-MeCyH$^-$,*N(3)*,*N(4)*)$_2$Hg]X_2 (X = Cl^-, NO_3^-) [56], where 1-MeCyH$^-$ binds Pt through N(3) and Hg through N(4) (**II**, *Fig. 5*). They will be indicated as 4:2 type complexes. The crystal structures reveal that pairs of dimers are held together by the counterions, Cl^- or NO_3^-, in such a way as to form tetranuclear species.

By reaction of *trans*-[$(NH_3)_2$Pt(1-MeCyH$_2$,*N(4)*)$_2$]$^{2+}$ with Hg^{2+} ions a novel dinuclear Pt,Hg compound has been recently reported, where the bases are reversed with respect to the Pt,Hg complex described above. The heterometal is bound through the N(3) sites of the two cytosine rings (**III**, *Fig. 5*) [35].

A heterometallic dinuclear species is also obtained (in very low yield) starting from the Pt(cytosine)$_4$ cation according to the reaction sketched in *Fig. 8,a*. The structural analysis for the 4:5 type complex revealed a short Pt–Co distance of 2.40 Å [48].

Metal-Metal Interaction in Dinuclear Species

The nature of the metal-metal interaction in bimetallic complexes has been recently examined using extended *Hückel*-MO calculations [57]. The molecular-orbital diagram for 4:4 dimers Pt_2 (d^8,d^8 system) is qualitatively depicted in *Fig. 9*, and shows that the main intermetallic interaction involves the two z^2 fragment molecular orbitals, FMO. The resulting four-electrons/two-orbitals interaction suggests no intermetallic bond, since the formal bond order is zero. However, due to the s, p_z and z^2 configuration mixing, with subsequent rehybridization in out-pointing direction of both the metal z^2 orbitals, the bond order becomes slightly greater than zero, suggesting a slightly bonding interaction [57]. The same scheme applies also to Pt-Pd dimers, making allowance for a shift in the FMO energies on one side of *Fig. 9*. Such kind of weak interaction corresponds to Pt–Pd distances around 2.90 Å, as found in several *cis*-[$A_2Pt^{II}(pym)_2M^{II}Y_2$]$^{m+}$ (M = Pt, Pd, A = NH_3, Y = NH_3 or monoanionic ligand species).

A similar correlation diagram applies to the 4:5 dimer [Pt^{II}(1-MeCyH$^-$*N(3),N(4)*)$_4$$Co^{III}(H_2O)]^+$ (d^8,d^6 system) of *Fig. 8, a*. In fact, the diagram of *Fig. 10* shows that the main interaction between the Pt square-pla-

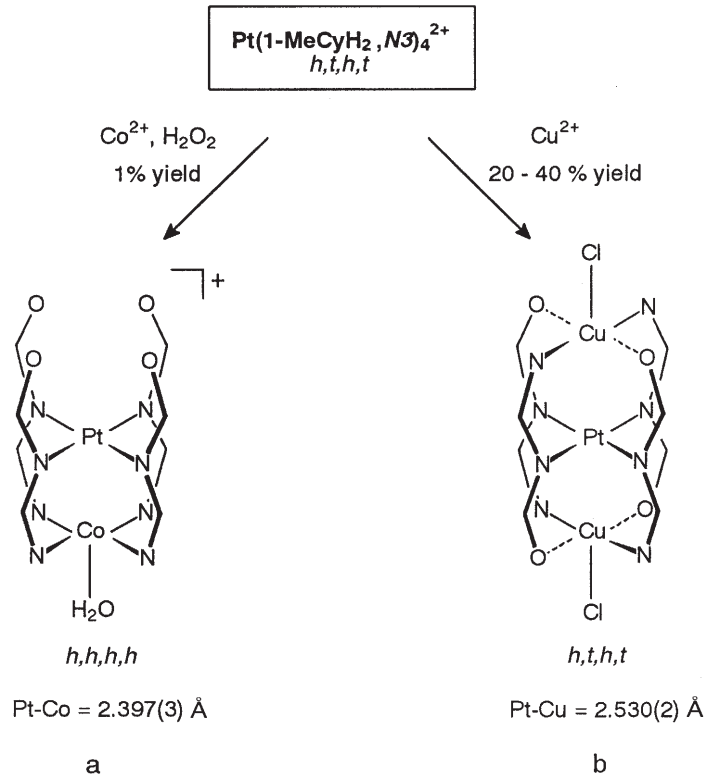


Fig. 8. *Scheme of Pt-Co and Cu-Pt-Cu species obtained from* $[Pt^{II}(1\text{-}MeCyH_2,N(3))_4]^{2+}$

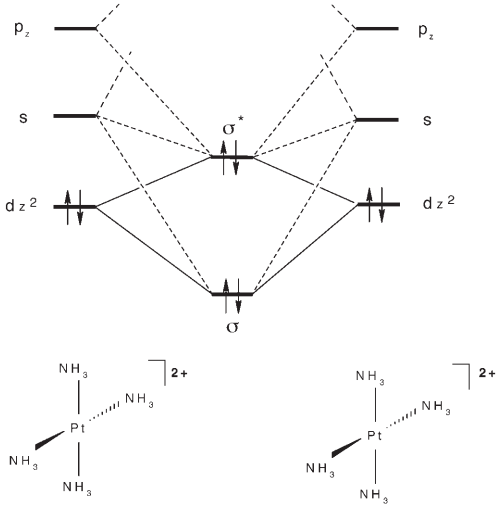


Fig. 9. *Molecular-orbital diagram for the* $[(NH_3)_4Pt^{II}–Pt^{II}(NH_3)_4]^{4+}$ *model compound* (4:4 type dimer). σ-Bonding MO is part of 6 (omitted for clarity) +1 filled d orbitals.

nar (*left*) and the square-pyramidal (*right*) fragments, is of type two-electrons/two-orbitals, suggesting a donor-acceptor (*i.e.*, dative) Pt→Co bond. The formal bond order of one corresponds to a Pt–Co distance of 2.40 Å [48]. This bond length should be compared (making allowance for the larger size of Pt) to that of 2.3178(9) Å, reported for a Co^{II}–Co^{II} complex, which corresponds to a bond order of 0.75 [58].

The interaction diagrams for the 5:5 *cis*-$[A_2XPt^{III}(pym)_2Pt^{III}Y_3]^{m+}$ (A = NH_3 or amine, X, Y = monoanionic ligand or amine, pym = pyrimidinate monoanion) and for the 4:5 *cis*-$[A_2Pt^{III}(pym)_2Pt^{III}Y_3]^{m+}$ are closely related to that of *Fig. 9* and suggest a bonding scheme caused by two-electrons/two-orbitals, with Pt–Pt distances of *ca.* 2.57 Å, and a formal bond-order of one [24]. In this case, the dative bond is operative from a formal Pt^{II} to a formal Pt^{IV} species.

The MO diagram of the $[(NH_3)_4Pt^{II}–Pd^{II}H_2(NH_3)]^{2+}$ model for the heterobimetallic complexes of type 4:3 (*Fig. 11*) shows a different pattern. The strongest Pt-Pd interaction between the square-planar Pt fragment (*left*) and the T-shaped $Pd(NH_3)H_2$ one (*right*) involves z^2- and $x^2–y^2$-type FMOs of Pt and Pd, respectively, resulting in a two-electrons/two-orbitals donor-acceptor bond (Pt→Pd) with a formal bond order of one. This is in agreement with the very short Pt–Pd distance of *ca.* 2.50 Å, observed in several *trans*-

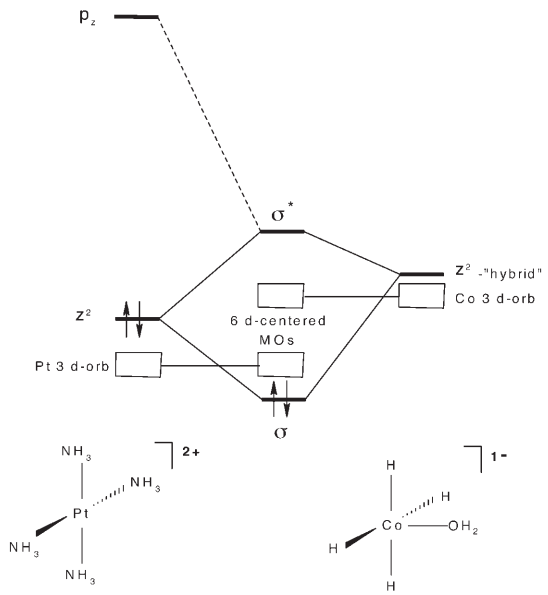


Fig. 10. *Molecular-orbital diagram for the* $[(NH_3)_4Pt^{II}–Co^{III}H_4(H_2O)]^+$ *model compound* (4:5 type dimer).

$[(NH_3)_2Pt^{II}(1\text{-}MeCyH^-,N(3),N(4))_2Pd^{II}X]^{m+}$ ions, with a shortening of *ca.* 0.40 Å with respect to the 4:4 *cis*-analogues (see above). Such a diagram suggests that substitution of the d^8 Pd^{II} with the d^9 Cu^{II} or d^{10} Hg^{II} ions causes the formal bond order to decrease to 0.5 and slightly above zero, respectively. Accordingly, in several 4:3 dimers, *trans*-$[(NH_3)_2Pt^{II}(1\text{-}MeCyH^-,N(3),N(4))_2Cu^{II}L]^{n+}$ (L = OH, H_2O, NH_3), [55] distances around 2.50 Å are observed, *ca.* 0.20 Å shorter than that of 2.765 Å found in the 4:4 analogue *cis*-$[(NH_3)_2Pt^{II}(1\text{-}MeUr^-,N(3),O(4))_2Cu^{II}(H_2O)_2]^{2+}$ [24], where the Pt-Cu interaction is essentially non-bonding. Therefore, theoretical calculations fairly well rationalize the observed metal-metal distances in term of electronic factors. Nevertheless, strong steric interactions among bulky ligands at Pt and M have been shown to be responsible for a significant lengthening of the Pt–M distances [24].

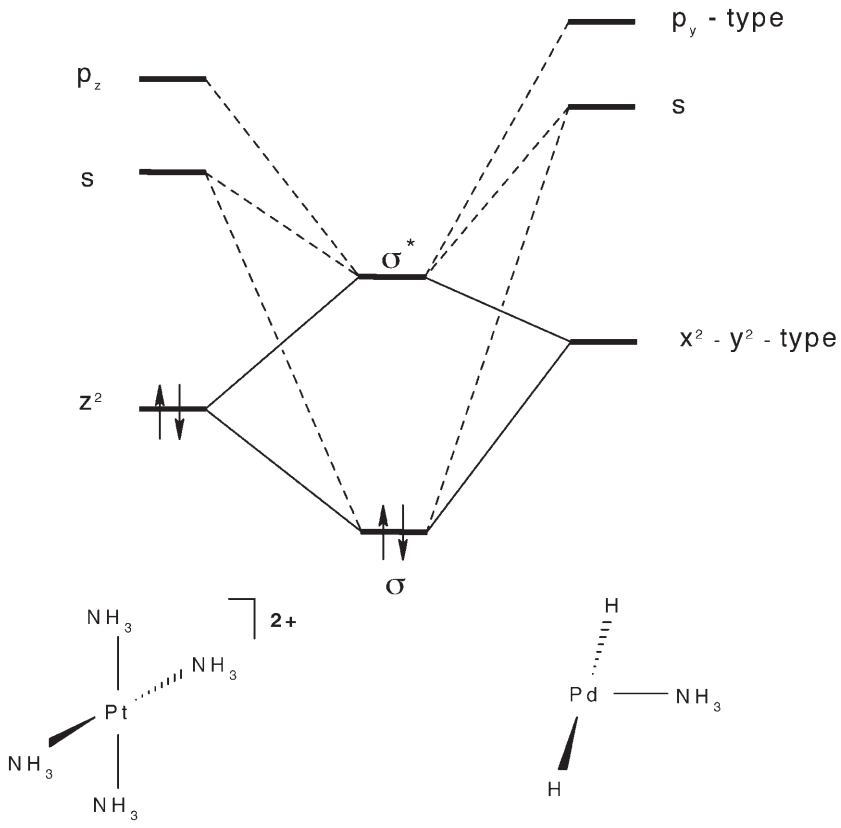


Fig. 11. *Molecular-orbital diagram for the* $[(NH_3)_4Pt^{II}–Pd^{II}(NH_3)H_2]^{2+}$ *model (*4:3 heterobimetallic species*).* σ-Bonding MO is part of 7 (omitted for clarity) +1 filled d orbitals.

By taking into account the isolobal analogy between the FMOs, the proposed bonding pattern can be extended to other heterobimetallic complexes, with or without nucleobases [57], to organometallic complexes without bridging ligands, such as the 4:1 type $[R_4Pt^{II}Ag^{I}X]^{-}$ ion (R = perhalophenyl, X = neutral ligand), where a Pt→Ag dative bond of *ca.* 2.65 Å in length has been reported [59]. For $L_4Pt\text{-}ML_n$ (n = 5, 3, 1) systems, the conditions for n and for the number of electrons in the ML_n fragment to produce a dative bond are summarized in *Fig. 12*.

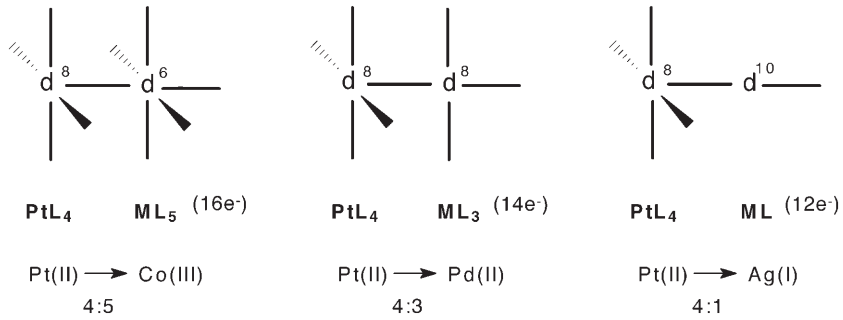


Fig. 12. *Number of electrons and of ligands at the heterometal M, which fulfill the condition for a dative Pt→M bond*

Polynuclear Complexes

Many polynuclear complexes containing pyrimidine ligands are formally derived from the *cis*- or *trans*-bispyrimidinate (or pyrimidine) mononuclear complexes, as shown in *Figs. 4* and *5*. They range from trinuclear to higher nuclearity species, with pyrimidine nucleobases (pym) acting as bridging ligands generally in the anionic form. The most numerous trinuclear species of formula *h-h,cis*-$[A_2Pt^{II}(pym)_2M(pym)_2Pt^{II}A_2]^{m+}$ with (A = neutral ligand and M = Cu^{II}, Mn^{II}, Pd^{II}, Tl^{I}, Ag^{I}, and Pd^{III}) have been recently reviewed [24]. They usually show a linear Pt-M-Pt arrangement where M is coordinated by the exocyclic O (generally O(4)) donors and Pt by the endocyclic N(3) ones (**II**, *Fig. 4*). Since all these cations but two (M = Ag, Tl) possess a crystallographic symmetry center, the MO_4 unit is strictly planar and the two intermetallic distances are equal. The Pt–M distances are relatively long, close to those found in the corresponding *cis*-dinuclear complexes, with the exception of those involving Pd^{III} which are around 2.65 Å. The analogous *trans*-trinuclear complexes (**V**, *Fig. 5*) have not been observed with pyrimidinate, the only example being a complex having 2-pyridonate ligands [60], in which a strong tetrahedral distortion of Cu^{II} allows

the relief of the steric hindrance between ligands around the adjacent metal centers.

Distinct examples of trinuclear (Pd,Pt,Pd) complexes with two 1-methylcytosinate ligands in *h-t* arrangement are shown by the structures **IV** (*Fig. 4*) and **VI** (*Fig. 5*). In the former, *cis*-$[(NH_3)_2Pt(1\text{-}MeCyH^-,N(3),N(4),O(2))_2\{Pd(en)\}_2]^{4+}$ [32], the Pt is coordinated by N(3), while the Pd ions have a mixed N(4),O(2) donor set, with the model nucleobases acting as tridentate ligand. In the second, *trans*-$[(NH_3)_2Pt\{(1\text{-}MeCyH^-,N(3),N(4))Pd(dien)\}_2]^{4+}$, obtained from the reaction of *trans*-$[(NH_3)_2Pt(1\text{-}MeCyH_2,N(4))_2]^{2+}$ with a heterometal complex carrying a bulky ligand (*i.e.*, diethylenetriamine), the cytosines act as singly bridging base coordinating Pt (through N(4)) and Pd (through N(3)) [35].

A number of polynuclear Pt,Ag complexes have been reported with uracilates acting as tridentate ligand [13]. Both *cis* and *trans* configurations of the anions are found in polynuclear species with (Ag,Pt_2,Ag), (Pt_2,Ag), (Pt_2,Ag,Pt_2) and (Ag,Pt,Ag) collinear units (*Figs. 4* and *5*). A metal arrangement, similar to the latter (**IV**, *Fig. 5*), is also found with 1,5-Me_2CyH^- as bridging tridentate ligand [31].

Recently, trinuclear diamagnetic species of formula $[(XCu^{II}(1\text{-}MeCyH^-,N(4),N(3))_2Pt^{II}(1\text{-}MeCyH^-,N(3),N(4))_2Cu^{II}X]^{m+}$ ($m = 2$, X = H_2O and $m = 0$, X = Cl), have been prepared, starting from *h-t-h-t*-$[Pt(1\text{-}MeCyH_2)_4](NO_3)_2$ (*Fig. 8, b*), and structurally characterized [48]. The values of the Pt–Cu distances (*ca.* 2.51 Å) and the diamagnetism of the complexes suggest a significant bonding interaction due to the electron charge delocalized over the Cu-Pt-Cu fragment.

All the above described polynuclear species are characterized by an open chain arrangement, with approximately collinear metal centers, interacting to a different extent. Very recently, attempts to obtain cyclic polynuclear metal complexes containing pyrimidinate ligands have been successful. The dinuclear *cis*-$[\{(Me_3P)_2Pt^{II}(1\text{-}MeCyH^-,N(3))_2\}_2]^{2+}$ species in DMSO solution at 80 °C converts quantitatively into the trinuclear *cis*-$[\{(Me_3P)_2Pt^{II}(1\text{-}MeCyH^-,N(3),N(4))\}_3]^{3+}$. The Pt ions, 5.3 Å apart, lie at the corners of an approximately equilateral triangle, bridged on each side by the three cytosinates, through N(3) and N(4) (*Fig. 13*). The ligand rings, all on the same side of the Pt triangle plane, are inclined at about 60° to the latter, so that the cation may be regarded as basin-shaped [36].

Spontaneous self-assembly of cyclic metal complexes, ('molecular squares' or 'molecular boxes') represents an area of great current interest [61]. They are built up by placing suitable organic ligands at the corners and metal units along the edges of a square, in such a way that the metal centers and the ligands are essentially coplanar. In the reverse situation, where the metals provide the right angular components and the ligands the sides, the

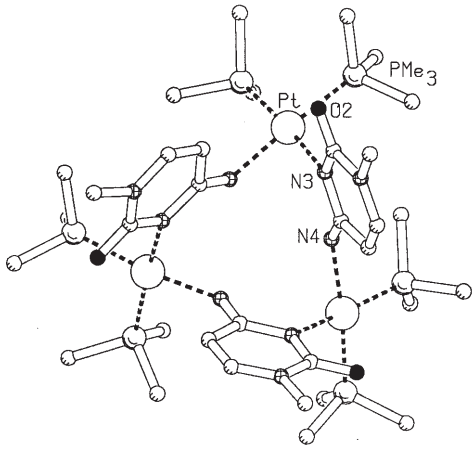


Fig. 13. *Molecular structure of the trinuclear cation* cis-*[{$(Me_3P)_2Pt^{II}$(1-MeCyH$^-$,N(3), N(4))}$_3$]$^{3+}$*

ligand planes are perpendicular to the metal square plane. In this case the more appropriate term should be molecular boxes, taking into account the overall three-dimensional structure. Six-membered heterocycles, such as pyrimidines, where the metal donor vectors make angles of 120°, provide molecular hexagons with linear metal units. However, use of different metal moieties may lead to molecular boxes. In fact, a cyclic tetranuclear uracilate complex [(en)Pt^{II}(UrH^-,*N(1)*,*N(3)*)]$_4$(NO_3)$_4$, which formed spontaneously from an aqueous solution of *cis*-[(en)Pt^{II}(UrH^-,*N(1)*)(H_2O)] (NO_3), has been structurally characterized [24][49]. The structure shows that the (en)Pt^{II} entities (Pt···Pt distances of 5.86 Å) are at the corner of the box, and are bridged through N(1) and N(3), by UrH^- in its rare tautomeric form **VIII** or **X** (*Fig. 2*), with ligand planes approximately perpendicular to the Pt_4 square. In the solid state the cation adopts the 1,3 alternate conformation of the four UrH^- rings, with an approximately C_2 symmetry, so that the O(2) and O(4) atoms of adjacent rings are next to each other, connected by a strong H-bond (*Fig. 14*).

There is indirect evidence that in solution (see below) the 1,3-alternate conformer is in equilibrium with a cone conformer of idealized C_4 symmetry (*Fig. 15, a*). The tetramer of *Fig. 14* can be considered to be a metal analogue of a calix[4]arene [24], and this analogy includes also the propensity of this compound to coordinate metal ions. In fact, after further deprotonation of UrH^- to the dianion Ur^{2-}, the complex binds additional divalent cations to yield octanuclear species, [{(en)Pt^{II}M(Ur^{2-},*N(1)*,*N(3)*,*O(2)*, *O(4)*)}$_4$]$^{8+}$ with M = *cis*-(NH_3)$_2$$Pt^{II}$, (en)$Pt^{II}$, ($H_2O$)$_3$$Ni^{II}$, (en)$Pd^{II}$, and Cu^{II}. The nitrate salts of these cations were isolated and the structures of the first

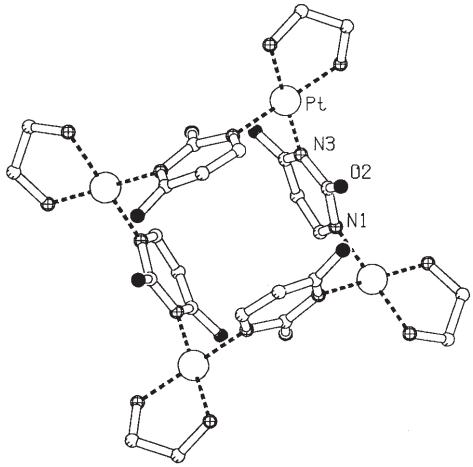


Fig. 14. *Molecular structure of the [{Pt^{II}(en)(UrH^{-},N(1),N(3))}$_4$]$^{4+}$ cation with 1,3 alternate arrangement of the bases*

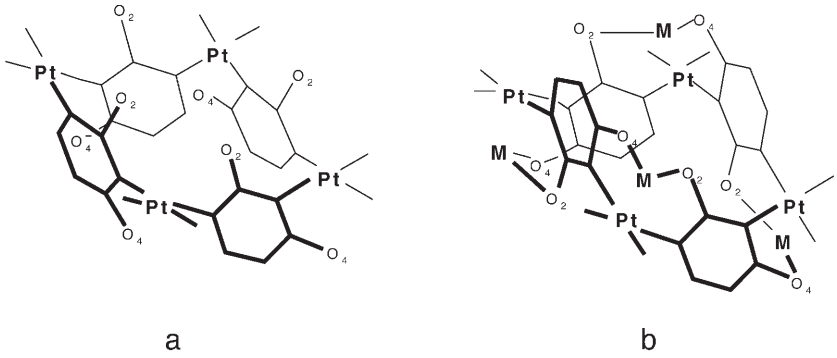


Fig. 15. *Schematic representation of cone conformer of [{Pt^{II}(en)(UrH^{-},N(1),N(3))}$_4$]$^{4+}$* (a) *and of the octanuclear cation [{(en)Pt^{II}M(Ur^{2-},N(1),N(3),O(2),O(4))}$_4$]$^{8+}$* (b). Ethylenediamine ligands at Pt^{II} are omitted for sake of clarity.

three derivatives were found to be similar [21]. This structure may be derived from that of the 1,3 alternate conformer of *Fig. 14*, in which the protons between the atoms O(2) and O(4) of adjacent uracilates are substituted by the M units (*Fig. 15,b*).

The nitrate salt of the analogous [{(en)Pt^{II}Ag(UrH^{-},*N(1),N(3),O(2),O(4)*)}$_4$]$^{8+}$ cation exhibits a different structure, which can be formally derived from that of the pinched-cone conformer of *Fig. 15,a*, by coordination of silver ions to the uracilate oxygens. In all the octanuclear complexes, two metal units, Pt(en) and M, represent each corner of the boxes. These results demonstrate that, irrespective of the 120° angle between the Pt-N(1)

and Pt-N(3) vectors, molecular boxes are formed as long as the metal units have *cis*-configuration.

For metal units in *trans*-configuration, the formation of cyclic polynuclear species is favored by a *h-h* arrangement of the two nucleobases which suppresses formation of open chain oligomers. Thus, the octanuclear species, $[\{(MeNH_2)_2Pt(1\text{-}MeCy^-H^-)_2Hg_3(OH)(NO_3)\}_2]^{4+}$, has been obtained by electrophilic attack, with excess of $Hg(NO_3)_2$, at the C(5) position of the two nucleobases in the dinuclear $[(MeNH_2)_2Pt(1\text{-}MeCyH^-,N(3),N(4))_2Hg]^{2+}$ parent cation (**II** of *Fig. 5*) [21]. The basic structure of the centrosymmetric cation can be described as a compressed hexagon of alternate sides of 7.0 and 5.5 Å (*Fig. 16*). Four Hg^{II} and two Pt^{II} cations are at the edges and four nucleobases and two OH^- groups represent the corners. Two additional Hg^{II} ions bridge pairs of deprotonated N(4) donors of $1\text{-}MeCy^-H^-$ along the Pt···Pt direction. The cation may be considered as being made up by two units of **II** (*Fig. 5*) linked by two Hg–OH–Hg bridges.

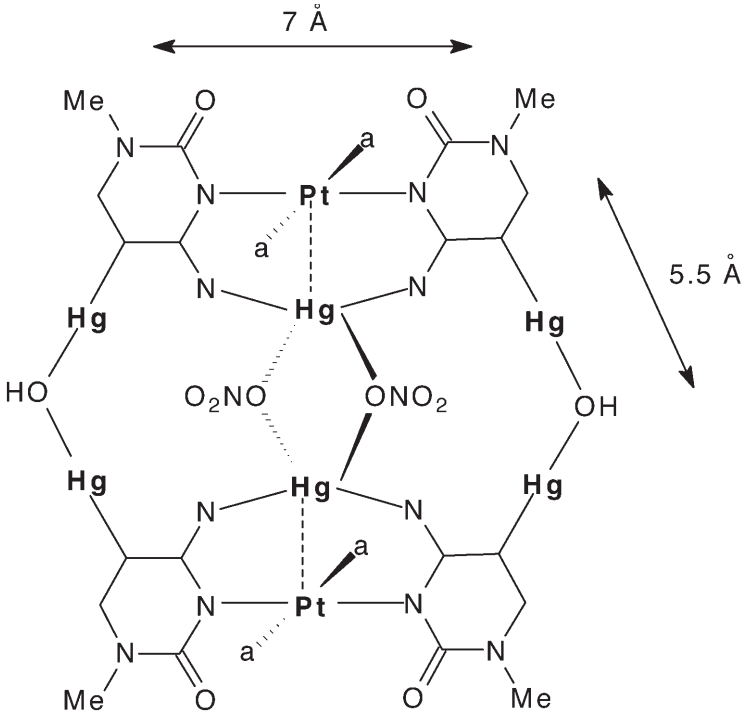


Fig. 16. *Scheme of the cation* $[\{Pt^{II}(MeNH_2)_2(1\text{-}MeCy^-H^-,N(3),N(4),C(5))_2Hg^{II}_3(OH)(NO_3)\}_2]^{4+}$ *with the dimensions of the hexagon*

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Diplatinum(III) Complexes: Chemical Species More Widely Spread Than Suspected

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Oxidation of Pt^{II} to Pt^{III} takes place spontaneously (by atmospheric oxygen, solvent, or ligand molecules) when the *face-to-face* association of two Pt^{II} units by covalently bridging ligands leads to a short intermetallic distance (< 2.7 Å). When the bridging ligands allow a longer intermetallic distance, stronger oxidants (such as halogens) are required. In chains of metal atoms only some platinum atoms can be oxidized to Pt^{III} resulting in a non-integer average oxidation state (such as in 'platinum blue'). Pt^{III} dimers can also form in the oxidation of Pt^{II} monomers (the platinum unit undergoing two-electron removal by an oxidant can interact *face-to-face* with a second platinum unit contributing its d_{z^2} electrons). Thus, the formation of Pt^{III} dimers appears to be a rather common, although overlooked, feature of platinum chemistry. The formation of mixed-valence one-dimensional materials, the occurrence of two-electron redox reactions between Pt_2^{II} and Pt_2^{III} which are fundamentally different from those between Pt^{II} and Pt^{IV}, the photo-induced formation of highly reactive metal-centered radical species, and, in addition, the biological relevance of some related materials (such as 'platinum blue') represent only some of the interesting aspects of Pt^{III} chemistry.

Introduction

Much of the Pt_2^{III} chemistry came about as a result of the serendipitous discovery of the first 'platinum pyrimidine blue', formed from *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and polyuracil left incubating for several days at pH 7 and 37 °C. This material proved to have high antitumor activity as well as low renal toxicity [1–4]. However, interest in Pt^{III} chemistry [5–10] also arises from its possible occurrence in mixed-valence one-dimensional materials [11][12] and from the participation of Pt^{III} in Pt^{II}/Pt^{IV} redox processes [13], as well as in the photocatalytic activation of C-M and C-X bonds [14].

Monomeric Pt^{III} has one unpaired electron, and only a few complexes of this type have so far been reported [15–20]. Well-documented examples are $[PtR_4]^-$ ($R = C_6Cl_5$ and C_6F_5) [15][16], $[PtCl_3(creatininate)]^-$ [17], [Pt(di-

Cisplatin. Edited by Bernhard Lippert

phenylglyoximate)$_2$]$^+$ [18], [Pt(3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine)]$^{3+}$ [19], and [Pt(1,4,7-trithiacyclononane)$_2$]$^{3+}$ [20]. In all cases, the ability of the ligands (often macrocycles) to shield the metal center appears to be crucial for stabilizing such monomeric compounds. Monomeric Pt^{III} species generated by γ-radiation [21] or existing as short-lived reaction intermediates [10] have also been reported.

In contrast to the mononuclear species, the list of binuclear Pt^{III} compounds containing a metal-metal bond is much larger and steadily increasing [22]. In most cases, the two metal centers are bridged by either two or four ligands of suitable bite, such as amidates [23–30] including pyrimidine nucleobases [31–37] and thio analogs [38][39], acetates [40–43] and thioacetates [44], sulfates [45], phosphates [46], and diphosphites [8][47][48]. The bridging ligands usually contain a OXO (X = C, S, P), NCO, NCS, SCS, or PXP (X = O, C) three-atom chain resulting in an overall five-membered ring including the Pt-Pt interaction. There are, however, also a few examples of Pt^{III} dimers unsupported by any bridging ligand [49–53].

At an early stage of the investigation it became evident that the *face-to-face* association of the Pt^{II} precursor in a dimer, with only one axial position per platinum atom open to attack by the oxidant, was the prerequisite allowing a straightforward formation of Pt^{III} dimers. The presence of covalently bridging ligands was therefore required to stabilize the Pt^{II} precursor (for which a direct intermetallic interaction could only provide a weak contribution), rather than to support the covalent Pt-Pt bond in the Pt^{III} dimer.

More recent evidence has been gained that shows that the formation of dimeric Pt^{III} species may be a relatively common, although overlooked, feature of platinum chemistry and, also in the case of monomeric Pt^{II} species, might play an important role in redox reactions. A mechanistic interpretation of their formation will be proposed.

In contrast with the common structure presented by polynuclear Pt^{III} complexes in which the Pt-Pt axis is perpendicular to a four-ligand equatorial plane, there are also a few reports of formal Pt^{III} species for which all bonds involving the platinum center are in a plane [48][54]. These compounds, having a structure essentially different from that of any other polymeric Pt^{III} species, will not be described in detail in this chapter.

Four-Bridge Dimers

Pioneering Work in the Field

An acetamidate-bridged Pt^{III} complex was proposed before any Pt^{III} dimer had been discovered [55][56]. The yellow compound, obtained from

$K_2[PtCl_4]$ and acetamide in water/ethanol and purified by boiling in concentrated HCl, was originally thought to be a novel Pt^{II} monomer with a monodentate acetamide and a chelating acetamidate $[Pt\{CH_3C(O)NH_2\text{-}N\}\{CH_3C(O)NH\text{-}N,O\}X]$ (X = Cl in the initial compound but similar compounds were obtained by substitution of Cl by NO_2, NO_3, Br, and I). However, because of some anomalous properties (such as exceptional stability towards strong oxidizing agents, lability of ligand X, and strength of platinum-acetamide bond), the same complexes were later formulated as $[Pt_2\{CH_3C(O)NH\text{-}N,O\}_4X_2]$ [48]. X-Ray photoelectron spectroscopy (XPS) studies showed that the platinum atoms were equivalent and in the +3 oxidation state [48][54]. *Raman* studies were also performed to substantiate the given formulation [57]. Finally, a single-crystal X-ray structure analysis for the $[Pt_2\{CH_3C(O)NH\text{-}N,O\}_4I_2]$ complex has been performed (*Fig. 1*). The lantern-type structure has been confirmed and the Pt-Pt distance has been found to be exceptionally short (2.480(2) Å) [58].

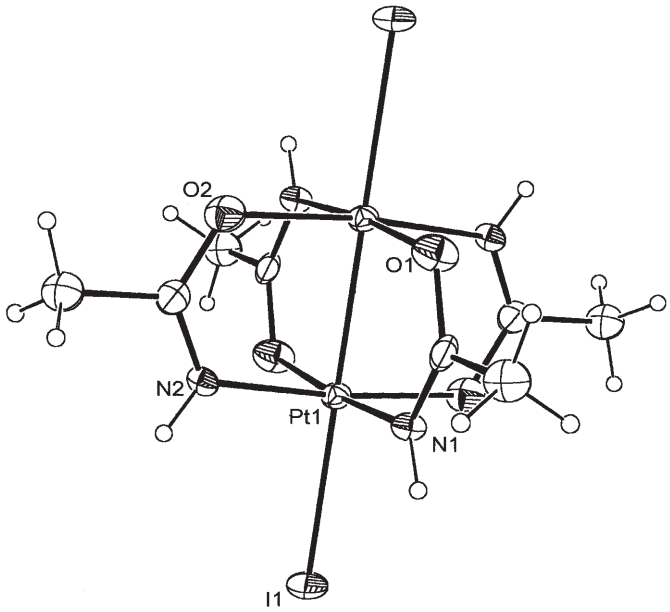


Fig. 1. *Structure of* $[Pt_2\{CH_3C(O)NH\}_4I_2]$ (based on data from [58])

Bridging Ligands with Very Short Bite: Facile Oxidation to Pt^{III}

Among the bridging ligands with the shortest bite distance are the oxygen-donor anions sulfate, hydrogen phosphate and acetate. The sulfate

$[Pt_2(SO_4)_4(H_2O)_2]^{2-}$ and hydrogen phosphate $[Pt_2(HPO_4)_4(H_2O)_2]^{2-}$ compounds have been prepared by heating the Pt^{II} nitro complexes, $K_2[Pt(NO_2)_4]$ [59] or $[Pt(NH_3)_2(NO_2)_2]$ [60], in the corresponding concentrated acid (*Scheme 1*).

Scheme 1

$$[Pt^{II}(NO_2)_4]^{2-} \xrightarrow[\Delta]{H_2SO_4} [Pt^{III}{}_2(SO_4)_4(H_2O)_2]^{2-}$$

$$[Pt^{II}(NH_3)_2(NO_2)_2] \xrightarrow[\Delta]{H_3PO_4} (NH_4)_2[Pt^{III}{}_2(HPO_4)_4(H_2O)_2]$$

The axial ligands can be substituted by halide ions [46][61], pyridine [62][63], guanine and various amines [64], thioethers and thiolates [46]. The Pt-Pt distance is in the range of 2.466–2.471 Å for sulfate (*Fig. 2*) [61][65] and 2.487–2.534 Å for phosphate-bridging ligands. The twist angle is less than 1.3°, except in the case of $[Pt_2(H_2PO_4)(HPO_4)_3(Py)_2]^-$ which has a twist angle of 13.3°.

More recently, also the tetraacetate diplatinum complex $[Pt_2^{III}(CH_3CO_2)_4(H_2O)_2]^{2+}$ was prepared by refluxing a solution of $K_2[Pt(NO_2)_4]$ in a 2:1 mixture of glacial acetic acid and 1M perchloric acid

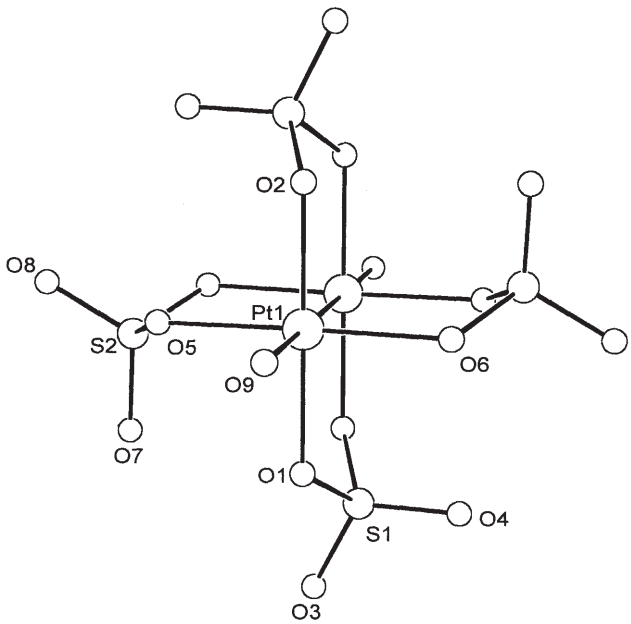
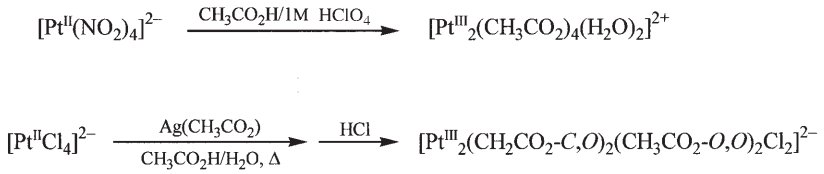


Fig. 2. *Structure of* $[Pt_2(SO_4)_4(H_2O)_2]^{2-}$ (based on data from [61])

in air [43] (*Scheme 2*). The complex is unstable in aqueous solution and this explains why previous attempts to obtain evidence for the tetraacetate structure in solution failed. The tetraacetate complex has the shortest Pt-Pt distance so far detected (2.390 Å).

Scheme 2



The extremely short bite distance of the acetate ligand forces the platinum atoms to deviate from the PtO_4 plane towards the axial ligands; the structure is highly strained and this may be the reason for the observed instability in solution. Greater stability is found in the analogous complexes with one or two *C,O*-bonded $CH_2CO_2^{2-}$ ligands, $[Pt_2^{III}(CH_2CO_2\text{-}C,O)(CH_3CO_2\text{-}O,O)_3Cl_2]^-$ [66] and $[Pt_2^{III}(CH_2CO_2\text{-}C,O)_2(CH_3CO_2\text{-}O,O)_2Cl_2]^{2-}$ [67]. They were prepared by reaction of $[Pt^{II}Cl_4]^{2-}$ and $Ag(CH_3CO_2)$ in aqueous acetic acid at high temperature followed by addition of HCl (*Scheme 2*). It is observed that as the number of $CH_2CO_2^{2-}$ ligands increases, the Pt-Pt distance becomes longer and the strain smaller (2.43(2) and 2.46(2) Å for one and two $CH_2CO_2^{2-}$-bridging ligands, respectively).

The fact that only Pt^{III} dimers have been isolated for quadruply-bridged sulfate, phosphate, acetate, and acetamidate complexes indicates that the short bite distance of these ligands destabilizes the Pt_2^{II} state, which requires a much longer Pt-Pt distance.

Bridging Ligands with Long Bite: Isolation of Both Pt^{II} and Pt^{III} Dimers

SCS-Bridging Ligands. Ligands of greater bite stabilize the Pt_2^{II} state since it requires a longer Pt-Pt distance. As a consequence, quadruply-bridged diplatinum complexes with dithiocarboxylates, RCS_2^-, bridging ligands are known for both the Pt^{II} and the Pt^{III} states. The Pt^{II}-dithiocarboxylate complex $[Pt_2(CH_3CS_2)_4]$ was prepared by reaction of $[PtCl_4]^{2-}$ with dithioacetic acid in hot toluene [68] (Scheme 3). The Pt^{II} dimer was reacted

Scheme 3

with halogens (X_2) to give the corresponding Pt^{III} dimer, $[Pt_2(CH_3CS_2)_4X_2]$ [44]. The Pt^{II}-Pt^{III} mixed-valence complex $[Pt_2(CH_3CS_2)_4I]$ was also prepared either by oxidation of the Pt^{II} dimer with a half-equivalent amount of iodine, or by reaction of a 1:1 mixture of the Pt^{II} and Pt^{III} species. For the mixed-valence complex, the crystal structure consists of infinite chains of equidistant iodo and diplatinum units.

The bite distance for the bridging ligands, which ranges from 2.25 to 2.55 Å for carboxylate and amidate, sulfate, phosphate, and *C,O*-bonded $CH_2CO_2^{2-}$, increases to 3.00–3.10 Å for dithiocarboxylates. The Pt-Pt distance is significantly shorter than the ligand bite distance even in the Pt^{II} dimer, where no Pt-Pt interaction is formally expected. The Pt^{II}-Pt^{II} distance, shorter than the ligand bite, is recognized as a manifestation of the d^8-d^8 metal-metal bonding interaction which arises from valence-shell d-p mixing in σ-type orbitals [6][7][69]. A short intramolecular Pt-Pt distance induces a twist of the two platinum coordination planes – as defined by the four sulfur atoms – which can be as large as 27° [69].

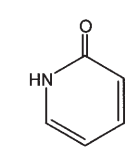
NCS-Bridging Ligands. NCS-bridging ligands are intermediate between SCS and NCO and OCO ligands. Different types of NCS-bridging ligands are known for quadruply-bridged complexes, namely pyridine-2-thiolate (pyt) and its 4-methyl derivative (4-Me-pyt) [39], pyrimidine-2-thionate (pymt) [38][70][71], and 2-thiouracilate (turac) [38] (*Scheme 4*).

The Pt^{II} dimers *cis*-$[Pt_2(L)_4]$ (L = pyt or 4-Me-pyt) were obtained by refluxing a mixture of *cis*-$[PtCl_2(NH_3)_2]$ and LH in dioxane (*Scheme 5*). The Pt^{II} dimer was then reacted with $CHCl_3$, $CHBr_3$, or CH_3I to give the Pt^{III} complex *cis*-$[Pt_2(L)_4X_2]$ (X = Cl, Br, I) [5][39]. The Pt^{III} complexes $[Pt_2(L)_4X_2]$ (L = pymt or turac) were obtained directly on reaction of $[PtX_4]^{2-}$ (X = Cl, Br, I) with the bridging ligand (L) in alcohol and heating [38][70]. The Pt-Pt distance in *cis*-$[Pt_2^{II}(4\text{-Me-pyt})_4]$ and *cis*-$[Pt_2^{III}(pyt)_4Cl_2]$ are 2.680(2) and 2.532(1) Å, respectively. The former is the shortest Pt-Pt distance in structurally characterized Pt^{II} dimers and is very similar to the ligand bite (2.70–2.75 Å).

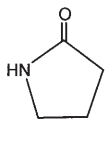
When ligands with bite distances longer than *ca.* 2.7 Å are reacted with Pt^{II} salts, Pt^{II} complexes are obtained. In contrast, with ligands of bite distances less than 2.7 Å, Pt^{III} dimers are obtained. Conversion of $[Pt_2^{II}(pyt)_4]$ into $[Pt_2^{III}(pyt)_4Cl_2]$ by chlorine abstraction from chloroform is viewed as an intermediate case, and the bite distance of *ca.* 2.7 Å is then taken as the cross-over between preference for Pt_2^{III} and preference for Pt_2^{II} states [72]. The Pt_2^{III} complexes with ligands having bite distances longer than 2.7 Å are prepared by oxidation of the corresponding Pt_2^{II} compounds.

Scheme 4

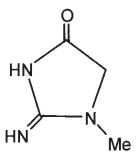
For linear ligand molecules, the chemical formula will be used. For cyclic ligand molecules, for which the chemical formula can be rather cumbersome, an abbreviation of its current name will be used. The indication of a substituent will precede the ligand name. In the case in which the ligand is not present in the usual deprotonated form, a 'H' will follow the ligand name



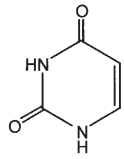
α-pyridone or 2-hydroxypyridine 'hp'



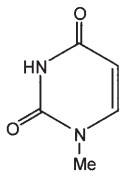
α-pyrrolidone 'pyrl'



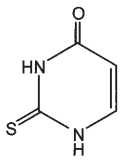
creatinine 'creat'



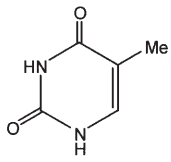
uracil 'urac'



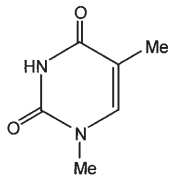
1-Me-uracil '1-Me-urac'



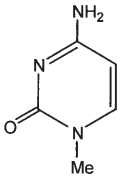
2-thiouracil 'turac'



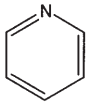
thymine 'thym'



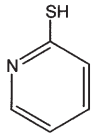
1-Me-thymine '1-Me-thym'



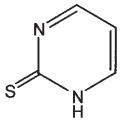
1-Me-cytosine '1-Me-cyt'



pyridine 'py'

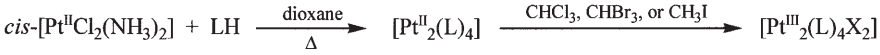


pyridine-2-thiol 'pyt'

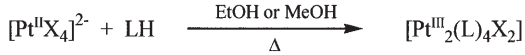


pyrimidine-2-thione 'pymt'

Scheme 5



L = pyt or 4-Me-pyt

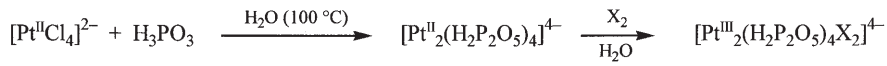


L = pymt or turac

POP- and PCP-Bridging Ligands. The pyrophosphite complexes are the most studied among lantern-type diplatinum complexes [6][8][73]. The Pt^{II} complex $[Pt_2(H_2P_2O_5^{-}P,P)_4]^{4-}$ is easily prepared by reaction of K_2PtCl_4

with H_3PO_3 in aqueous solution [74–76] (*Scheme 6*). Oxidative addition of halogens (X_2) to the Pt^{II} complex gives the Pt^{III} dimers with X ligands at both axial positions [77][78].

Scheme 6



From the 1:1 mixture of the Pt^{II} and Pt^{III} dimers, or by partial oxidation of the Pt^{II}-dimer [12][78], the mixed-valence $Pt^{2.5+}$ dimers, $[Pt_2(H_2P_2O_5)_4X]^{4-}$, have also been prepared. The species possess infinite chain structure with X equidistant from contiguous dimeric units [78–81]. In solution, the mixed-valence species disproportionate to Pt_2^{II} and Pt_2^{III} dimers. The Pt-Pt distance of the Pt_2^{II} dimer is the longest among lantern-type platinum dimers: 2.92–2.95 Å for pyrophosphite ligand, and 2.98 Å for the related methylenebisphosphite ligand (the latter differs from pyrophosphite in having a methylene group in place of an O-atom bridging the two phosphorus atoms) [74][78][82–84]. On the other hand, the intermetallic distances of the Pt^{III} dimers fall in the range 2.67–2.78 Å. The pyrophosphite ligand is very flexible and adjusts the P-O-P angle in order to fit the required Pt-Pt distance [47]. It is interesting to note that unbridged Pt_2^{III} compounds have Pt-Pt bond distances (2.69–2.75 Å) very similar to those of complexes with four pyrophosphite bridges (see below).

Two-Bridge Dimers

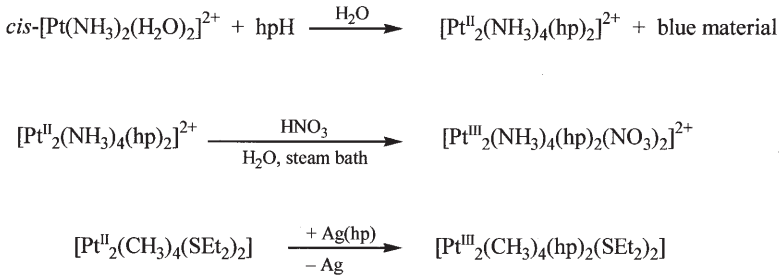
As discussed in the previous section Pt_2^{III} compounds quadruply bridged by amidate ligands are rare [85][86]. This is due to the fact that the short bite distance of these ligands leads to a highly strained structure similar to that observed for the tetraacetate complex. In contrast, amide type ligands such as 2-hydroxypyridine (or α-pyridone) [87][88], α-pyrrolidone [89][90], pyrimidines [91], and other amides [92–94] have provided a great number of dinuclear and oligonuclear platinum complexes of the two-bridge type.

The two-bridge Pt^{III} dimers are invariably characterized by a tilting of the two platinum coordination planes of *ca.* 25° and a frequent twist about the platinum-platinum vector averaging a torsion angle of *ca.* 25°. Responsible for these distortions are the steric interactions between the nonbridging equatorial ligands, in most cases NH_3 or CH_3.

The tetraamine Pt_2^{III} compounds are generally prepared by reaction of PtA_2X_2 species (A = monodentate or half-a-bidentate amine ligand, X = an-

ionic ligand such as halide or nitrate ions) with the required amide-type ligand, followed by oxidation of the Pt^{II} complexes (or related mixed-valence oligomers) with nitric acid [24][95] (*Scheme 7*).

Scheme 7



The tetramethyl Pt_2^{III} complexes, on the other hand, are prepared by reaction of $[Pt_2(CH_3)_4(SEt_2)_2]$ with a salt (Ag^I, Hg^{II}, or Tl^{III}) of the required ligand. The latter reaction involves not only ligand coordination to platinum, but also oxidation of Pt^{II} to Pt^{III} and reduction of Ag^I, Hg^{II}, or Tl^{III} [26][96][97].

α-Pyridone-Bridging Ligands

The work on dinuclear complexes with two bridging α-pyridone ligands, has been carried out mainly by two research groups, *i.e.*, those led by *Cotton* and *Lippard*.

The work from *Cotton* and coworkers [26][96][97] extends their study of metal-metal interactions.

The work by *Lippard* and coworkers [2][24][25][88][95][98–100] derives its chief motivation from the understanding of the interaction between the anticancer drug *cis*-$[PtCl_2(NH_3)_2]$ and pyrimidine nucleobases. Unfortunately, the reaction of *cis*-$[PtCl_2(NH_3)_2]$ with molecules such as uracil or thymine leads to non-crystalline dark blue materials ('platinum blues') which are difficult to characterize. The use of a ligand with similar but more restricted number of donor sites, such as α-pyridone (hp), allowed isolation and full characterization of relevant platinum complexes. Related work has used 1-methyluracil (1-Me-urac) and 1-methylthymine (1-Me-thym) in which one of the pyrimidine nitrogens has been blocked [101].

Reaction of *cis*-$[PtCl_2(NH_3)_2]$ (pretreated with silver nitrate) with α-pyridone (pH = 1 with HNO_3) affords yellow crystals containing the dinuclear Pt_2^{II} cation $[Pt_2(NH_3)_4(hp)_2]^{2+}$, together with dark blue crystals (absorp-

tion band centered at 14.705 cm^{-1}) of a compound containing the tetranuclear unit $[Pt_2(NH_3)_4(hp)_2]_2^{5+}$ and having the platinum in a noninteger oxidation state of +2.25 [2]. Oxidation of the dinuclear Pt^{II} complex with nitric acid (3–5M) affords the corresponding Pt^{III} species $[Pt_2(NH_3)_4(hp)_2(NO_3)_2]^{2+}$ [24][95].

All complexes (Pt^{II}, Pt^{III}, and Pt with noninteger oxidation states) feature Pt_2 units bridged by two α-pyridone ligands in a *cis* arrangement on each metal. There are two possible isomers for this arrangement: a *head-to-head* (*HH*) isomer where the two hp ligands are attached *via* oxygen to one Pt atom and *via* nitrogen to the second Pt atom, or a *head-to-tail* (*HT*) isomer in which both metals are bound to one oxygen and one nitrogen from the two bridging hp ligands. Steric factors suggest that, if only one axial ligand has to be attached to the platinum dimer, the *HH* isomer should be preferred. If, instead, two axial ligands must be attached (for pyridone with small substituents in the 6-position), the HT isomer should be preferred.

HH Isomers of Pt_2^{II} complexes containing two ammines, or a 1,2-diaminoethane chelate, can also dimerize. This represents, to some extent, an alternative to the taking up of axial ligands. Hydrogen bonding between the exocyclic oxygen at the pyridone ligands of one dimer and the amine ligands of the second dimer must contribute to the stability of the dimer of dimers. The interdimer Pt-Pt contact distance can be significantly less than 3.3 Å (*Fig. 3*).

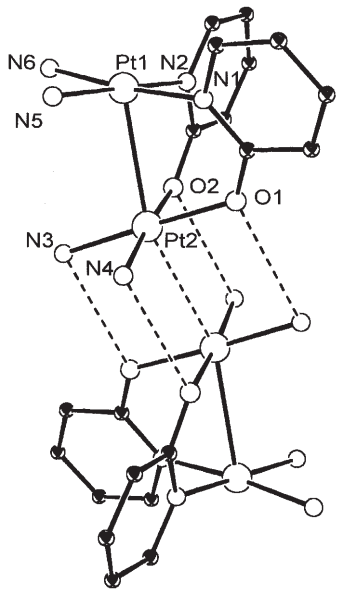


Fig. 3. *Structure of the Pt^{II} cation $[Pt_2(NH_3)_4(hp)_2]_2^{4+}$* (based on data from [99])

It is apparent how removal of one electron from the four aligned Pt^{II} atoms (*Fig. 3*) leads directly to the $[Pt_2(NH_3)_4(hp)_2]_2^{5+}$ 'platinum blue'.

In Pt_2^{II} complexes, there is no net metal-metal bond. In Pt_2^{III} complexes, instead, there is a net single bond. The metal-metal bond distance is, in fact, much shorter for the Pt_2^{III} complexes than for the corresponding Pt_2^{II} species (2.56 Å for $[Pt_2(NH_3)_4(hp)_2XX]^{2+}$ and 2.90 Å for $[Pt_2(NH_3)_4(hp)_2]^{2+}$). Thus removal of two electrons from a σ^* orbital reduces the metal-metal contact distance by > 0.3 Å. For the 'platinum-blue' 'dimer-of-dimers' $[Pt_2(NH_3)_4(hp)_2]_2^{5+}$, containing the platinum in an average oxidation state of +2.25, the intradimer Pt-Pt bond length is 2.775 Å, predictably between the lengths found in the +3 and +2 oxidation state complexes. A comparison between the interdimer Pt-Pt distance in the Pt^{II} complex (3.13 Å) and in the blue complex with fractional oxidation state of +2.25 (2.88 Å) indicates that, in the latter case, there is also partial bonding interaction between dimers. In agreement with the latter conclusion is the assignment of the absorption band responsible for the blue color of $[Pt_2(NH_3)_4(hp)_2]_2^{5+}$ to a transition involving the two central Pt-atoms of the tetranuclear cation, specifically a transition between Pt-Pt σ-bonding and σ^*-antibonding orbitals [102].

^{195}Pt-NMR Studies of both Pt_2^{II} [100] and Pt_2^{III} [25] compounds have shown that the chemical-shift range for the Pt_2^{II} species is between –1308 and –2495 ppm, while for the Pt_2^{III} complexes the range is between –1141 and +541 ppm. The $^1J_{Pt\text{-}Pt}$ coupling constant is unresolved in the Pt_2^{II} complexes, but is around 6850 Hz for the Pt_2^{III} species. Electrochemical studies carried out with the *HT* Pt^{II} dimer $[Pt_2(NH_3)_4(hp)_2]^{2+}$, the *HT* Pt^{III} dimer $[Pt_2(NH_3)_4(hp)_2(NO_3)_2]^{2+}$, and the *HH* Pt^{III} dimer $[Pt_2(NH_3)_4(hp)_2(H_2O)(NO_3)]^{3+}$ have shown that these species undergo a quasi-reversible two-electron redox process interconverting Pt_2^{II} and Pt_2^{III} species.

Several tetramethyl diplatinum(III) complexes with two bridging α-pyridone ligands have been prepared by reaction of $[Pt_2(CH_3)_4(SEt_2)_2]$ with the silver salt of the required X-hp ligand [26][96][97]. The reaction involves X-hp coordination to platinum and oxidation of Pt^{II} to Pt^{III} by silver ion. The SEt_2 ligands are found in axial position and can be displaced by other ligands such as pyridine. The bis-pyridine complex has the α-pyridone ligand in the *HT* arrangement, removal of one pyridine causes rearrangement to the *HH* isomer [97] (*Fig. 4*).

α-Pyrrolidone-Bridging Ligands

Matsumoto et al. [103][104] used α-pyrrolidone as bridging ligand and obtained a tan colored compound having analogous structure to the 'platinum blue' of *Lippard*, except that the average oxidation state was +2.50

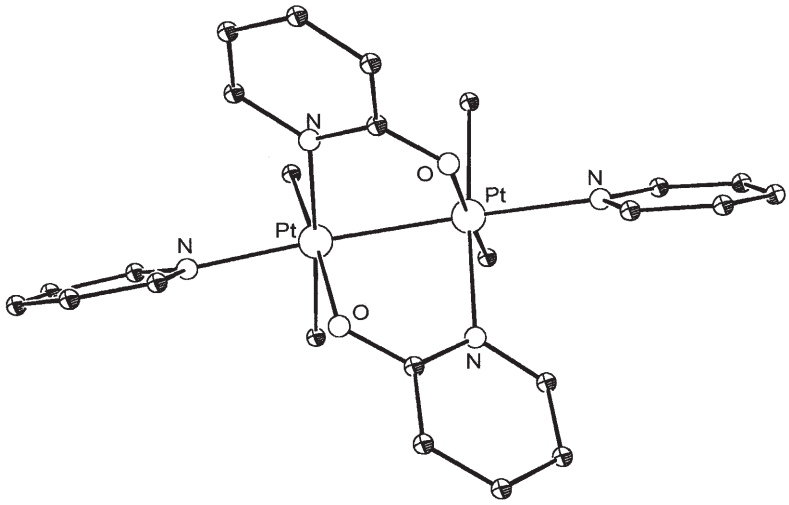


Fig. 4. *Structure of the* head-to-tail *complex [$Pt_2Me_4(hp)_2(py)_2$]* (based on data from [96])

rather than +2.25, $[Pt_4(NH_3)_8(pyrl)_4]^{6+}$. The related Pt^{II} complex $[Pt_2(NH_3)_4(pyrl)_2]^{2+}$ is binuclear in solution but tetranuclear in the solid state, with a structure similar to that of the previous tan cation with oxidation state of +2.5 for platinum [89]. The difference in the oxidation state affects markedly the Pt-Pt distances. The values for the terminal and internal Pt-Pt distances were, respectively, 3.033 Å and 3.186 Å in the compound with oxidation state +2, and 2.70 Å and 2.71 Å in the compound with oxidation state +2.5. In the latter case, there are six electrons for three intermetallic bonds.

A green α-pyrrolidone compound made of a mixture of $[Pt_4(NH_3)_8(pyrl)_4]^{5+}$ and $[Pt_4(NH_3)_8(pyrl)_4]^{6+}$ cations with average oxidation states of 2.25 and 2.5, respectively, was also isolated and characterized by X-ray crystallography [90].

From a solution of the tetranuclear mixed-valence platinum complexes reported above, and in the presence of pyrazine a tetranuclear Pt^{III} complex of formula $[(NO_3)(NH_3)_2Pt^{III}(pyrl)_2Pt^{III}(NH_3)(\mu\text{–}NH_2)]_2^{4+}$ was obtained [28]. It was composed of two α-pyrrolidonate-bridged Pt^{III} dimers which, in turn, were bridged by two NH_2 ligands. In the Pt^{III} species, the intradimer Pt-Pt distance drops to 2.608 Å.

Pyrimidine-Bridging Ligands

An interesting route to platinum 1-methyluracil blue was reported by *Lippert* and *Neugebauer* [105]. They reacted the 1-methyluracil-bridged *head-to-head* compound $[Pt_2(NH_3)_4(1\text{-Me-urac})_2]^{2+}$, with silver nitrate to

give the heterometallic $[Ag\{Pt_2(NH_3)_4(1\text{-Me-urac})_2\}_2]^{5+}$, which consists of two Pt_2^{II} units bridged by an Ag^I ion. This latter compound left standing in solution affords a microcrystalline compound which is identified as $[Pt_4(NH_3)_8(1\text{-Me-urac})_4]^{5+}$ and is believed to be the 1-methyluracil analog of the α-pyridone blue.

The *HH* and *HT* Pt^{III}-Pt^{III} complexes of formula $[Pt_2(NH_3)_4L_2XY]^{n+}$ (L = 1-Me-uracil, 1-Me-thymine and 1-Et-thymine, and 1-Me-cytosine; X,Y = axial ligands such as NO_2^-, Cl^-, H_2O or no ligand at all) have been prepared by oxidation of the corresponding Pt^{II}-dimer with nitric acid, eventually followed by metathesis of the axial ligands [22][23][31–33][36] [106][107]. In general $[Pt^{III}]_2$ complexes contain two hexacoordinated Pt-atoms (including the intermediate bond), but in the case of a strong *trans*-influence from an axial ligand (*e.g.*, X = NO_2 [33] or a *C*-bonded nucleobase [36]), asymmetric structures with six- and five-coordinated metal centers may result. An effect of the bulk of the amine ligands (*e.g.*, CH_3NH_2 in place of NH_3) upon the coordination number of the metal atom has also been observed [22] (*Fig. 5*).

The mutual orientation of the bridging ligands also influences the reactivity of the Pt^{III} dimers. The *HT* dinuclear complexes are generally more robust than the corresponding *HH* isomers as far as ligand exchange and redox chemistry are concerned.

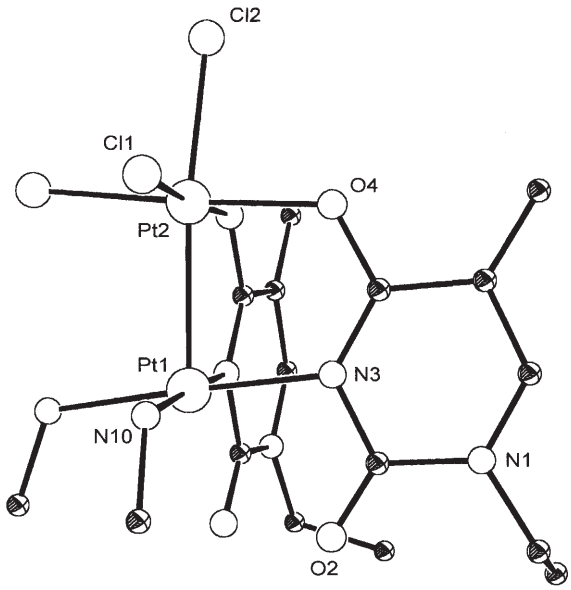


Fig. 5. *Structure of the formal Pt^{III} dimer $[(CH_3NH_2)_2Pt(1\text{-Et-thym})_2PtCl_3]^+$ containing one six- and one five-coordinate metal center* (based on data provided by the authors of [22])

In this context the greater stability (no change after several days in a pH range 3–9 in contrast with other $[Pt^{III}]_2$ species which readily reduce when the pH is not very low) of the Pt^{III}-dimeric species $[Pt_2^{III}(NH_3)_2$(1-Me-cyt-*N(3),N(4)*$)_2$(aminoacid-*N,O*$)_2]^{2+}$ should also be mentioned. Initial *O*-binding of the amino acid *via* H_2O substitution at the Pt_2^{II} precursor $[Pt_2^{II}(NH_3)_2$(1-Me-cyt$)_2(OH_2)_2]^{2+}$ facilitates subsequent *N*-binding *via* the axial position, thereby forcing the Pt^{II} to adopt a higher coordination number and oxidation state. On the other hand, the chelating amino acid makes the $[Pt^{III}]_2$ compound much more inert towards axial substitution reactions, thus preventing its reduction by water oxidation [37].

Carboxylate and Acyclic Amidate-Bridging Ligands

A number of *cis*-biscarboxylate diplatinum complexes such as $[Pt_2(CH_3)_4(CH_3CO_2)_2(py)_2]$ [96] and $[Pt_2(CH_3)_4(CF_3CO_2)_2$(4-me-py$)_2]$ [42] are known. In these complexes the strain is released by a much longer Pt-Pt distance, 2.557(1) Å [42] as compared to the 2.390(1) Å of $[Pt_2(CH_3CO_2)_4(H_2O)_2]^{2+}$ [43].

The 'platinum blue' compound $[Pt_4(NH_3)_8\{(CH_3)_3CC(O)NH\}_4]^{5+}$ was prepared from *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ and pivalamide (the reactants were heated in water at 70 °C for 1 h, then $NaNO_3$ was added and slow crystallization at 5 °C was performed). The 'platinum blue' was oxidized by $Na_2S_2O_8$ (50 °C for 5 min) to the Pt^{III} dimer $[Pt_2^{III}(NH_3)_4\{(CH_3)_3$-$CC(O)NH\}_2(H_2O)_2]^{4+}$ [108]. The Pt^{III} dimer in strongly acidic aqueous solution reacted with acetone to give the acetonyl complex $[Pt_2^{III}(NH_3)_4$-$\{(CH_3)_3CC(O)NH\}_2\{CH_2C(O)CH_3\}(NO_3)]^{2+}$ [30]. The involvement of these species in the catalytic oxidation of olefins adds interest to their chemistry (*cf.* chapter by *Matsumoto*).

Unbridged Dimers

Pt^{II}-Dimer Formation by Weak Face-to-Face Interaction

In several crystal structures it has been observed that two Pt^{II} units are joined *face-to-face* by four H-bonds. This was the case for *cis*-$[PtCl_2\{N,N'$-$(CHMePh)_2en\}]$, with four hydrogen bonds between NH groups of one unit and Cl-atoms of the other unit and a Pt-Pt separation of 3.344 Å [109]. Analogous interactions have been observed in strictly related complexes such as *cis*-$[PtCl_2\{CH_3C(OCH(CH_3)_2)NH\}_2]$ [3.327 Å] [110] and *cis*-$[PtCl_2\{C_6H_5C(\overline{NCH_2CH_2})NH\}_2]$ [3.443 Å] [111].

In one case, with *cis*-$[PtCl_2\{(CH_3)_3CC(OH)NH\}_2]$, the Pt-Pt separation was particularly short [3.165 Å] and geometrical features suggested that there was a net intermetallic attractive interaction (*Fig. 6*) [112]. A pure H-bond interaction between the two subunits would tend to place the dimer into an eclipsed conformation, this, however, was not the case. The X-Pt-Pt-X dihedral angles were close to 45° and the N-Cl distances [3.38 Å] were 0.22 Å longer than the Pt-Pt distance. It was concluded that the force driving the closer approach of the platinum atoms could only stem from a direct intermetallic interaction which can take place by mixing the filled d_{z^2}–d_{z^2} orbitals with the higher lying p_z–p_z orbitals [113]. Further support for the existence of a Pt-Pt attractive interaction came from the observation of a weak interaction of two extra amides along the axial direction [Pt-O distances of 3.229 Å]. This caused a considerable lengthening of the intermetallic distance (*ca.* 0.23 Å) and conversion from the staggered to the eclipsed conformation [112].

The bonding nature of the M···M contact between square-planar d^8-ML_4 molecules and, in particular, of the model dimer *cis*-$[PtCl_2\{HC(OH)NH\}_2]_2$ has also been the object of a theoretical investigation. It was predicted that the dimer was stable towards dissociation into two monomers, with bonding energies in excess of 3 kcal/mol [114]. In the d^8-ML_4-dimer each met-

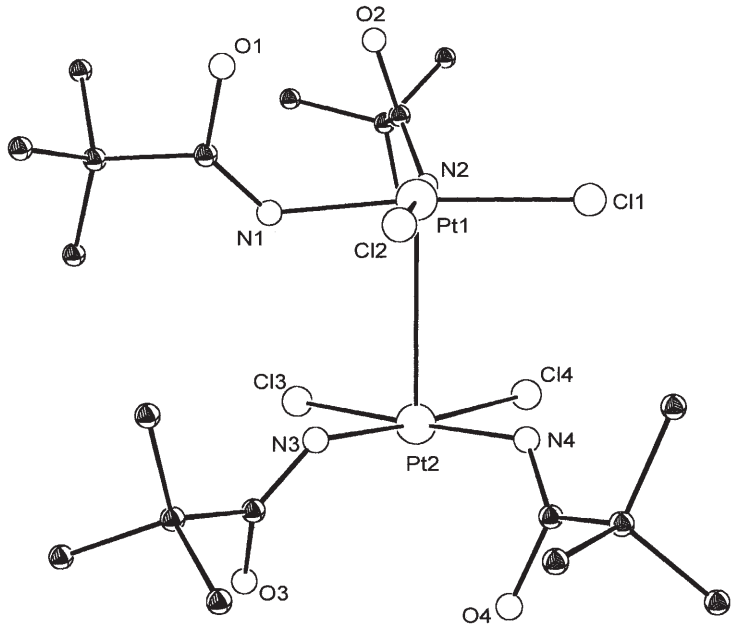


Fig. 6. *Structure of* $[PtCl_2\{(CH_3)_3CC(OH)NH\}_2]_2$ (based on data provided by the authors of [112])

al center contributes two d_{z^2} electrons to bonding along the *z*-direction, hence there is an excess of electron density along this direction (four electrons for only one M···M contact). Strengthening of the M···M interaction implies the formation of two-electron bonds in the *z*-direction. For instance, addition of a *Lewis* acid ($X^{(A)}$) to a metal atom in an axial position allows the formation of two bonds (M-M and M-X) with the four d_{z^2} electrons. Alternatively, one can add two radicals ($X^{(\cdot)}$) or a *Lewis* acid and a *Lewis* base ($X^{(A)}$ and $X^{(B)}$, respectively) to the axial positions, thus forming three bonds with a total of six electrons. On the other hand, the addition of one or two *Lewis* bases to the metal atoms results in an excess of *z* electron density and less stable systems [114]. The latter situation is envisaged in the $[Pt^{II}Cl_2\{(CH_3)_3CC(OH)NH\text{-}N\}_2\{(CH_3)_3CC(O)NH_2\text{-}O\}]_2$ dimer where two extra amide ligands interact along the axial sites [112].

Pt^{III} Dimers Formed by Oxidation of Formally Monomeric Pt^{II} Species

Oxidation of yellow Pt^{II} species, $PtCl_2L_2$ [L = amide type ligands such as $(CH_3)_3CC(OH)NH$ and $CH_3C(OCH_3)NH$] with excess Cl_2 leads instantaneously to an intermediate giving rise to a red solution that slowly returns to yellow. The final products are the Pt^{IV} species, $PtCl_4L_2$, and the intermediate red species that can be trapped by performing the reaction at low temperature (< 0 °C) in the dark, is the dimeric species $[PtCl_3L_2]_2$ (yield > 50%) [49][52].

The molecular structures of three of such complexes, *cis*-$[PtCl_3\{(CH_3)_3CC(OH)NH\}_2]_2$ [49] (*Fig. 7*) and *cis*- and *trans*-$[PtCl_3\{CH_3C(OCH_3)NH\}_2]_2$ [52], have shown that the compounds are neutral dimers composed of two planar $PtCl_2L_2$ units perpendicularly connected by a Pt-Pt bond and capped by axially coordinated chloride ligands. Bond distances and angles within the equatorial $PtCl_2N_2$ moiety are in the range of those reported for square-planar Pt^{II} and octahedral Pt^{IV} complexes. However, the axial Pt-Cl bond lengths are *ca.* 0.15 Å longer than the equatorial Pt-Cl bonds. Similar lengthening of the axial Pt-X bonds with respect to Pt-X values found in mononuclear complexes, has been also observed in most of the Pt^{III} dimers with two or four bridging ligands revealing that the metal-metal bond has a strong *trans*-influence. A parallel trend observed in isoelectronic $[Rh^{II}]_2$-complexes [7] further demonstrates the strong *trans*-influence of the metal-metal bond.

The Pt^{III}-Pt^{III} bond lengths for the three compounds mentioned above range from 2.69 to 2.76 Å, and are in good agreement with the values found in $K_4[Pt_2(H_2P_2O_5\text{-}P,P)_4Cl_2]$ and other pyrophosphite four-bridge dimers [77][78][113][115]. They are, however, much longer than those found in

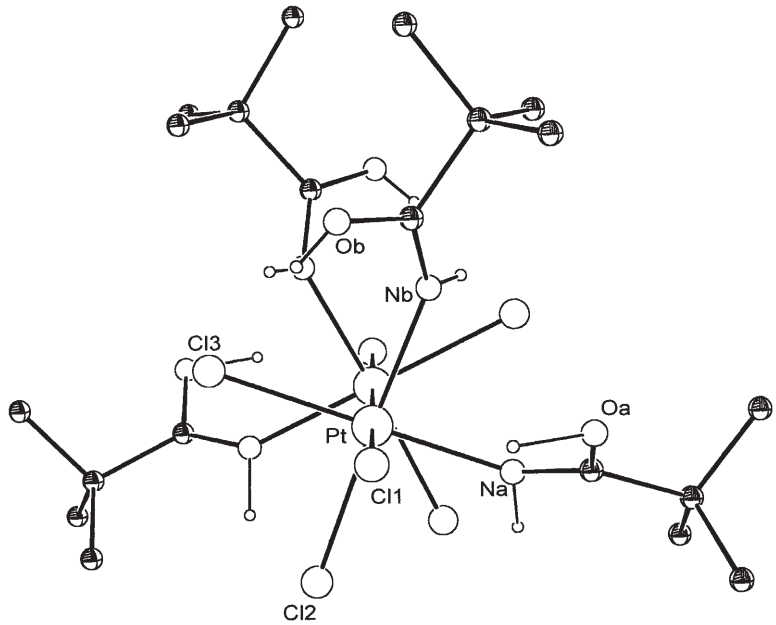


Fig. 7. *Structure of* $[Pt_2Cl_6[(CH_3)_3CC(OH)NH]_4]$ (based on data provided by the authors of [49])

other Pt_2^{III} complexes with either two (> 0.10–0.15 Å) or four bridging ligands (> 0.20–0.30 Å) [23–26][29][32–34][40–42][44–46][61–63][65][70][95–97][105][107][116][117] revealing, once again, the severe steric constraints introduced by covalently bridging ligands. Moreover, the 0.07-Å-difference in Pt^{III}-Pt^{III} bond lengths observed in the strictly related compounds discussed above indicates that the intermetallic distance is sensitive to interfacial steric interactions. In order to reduce interligand steric interactions, the complexes adopt the staggered conformation, and the energy barrier to rotation about the Pt^{III}-Pt^{III} bond goes up to 17 kcal · mol^{-1} [49][52].

Another Pt^{III} dimer unsupported by covalent bridges has been reported and structurally characterized by X-ray crystallography [50]. Each platinum unit carries two 1,2-(cyclooctane)dioximate ligands and is capped by chloride ligands. Structural features are very similar to those found for the previous three species a part from slightly shorter Pt-Cl axial bonds (mean value of 2.40 Å as compared with a mean value of 2.46 Å of the previous compounds) which reveals that also axial ligands can be very sensitive to steric interactions with the equatorial ligands.

A length of 2.70 Å may now be regarded as the standard value for an unhindered Pt^{III}-Pt^{III} single bond *trans* to two chloride ligands. It is also

worth remembering that, for covalently bridging ligands, a bite distance of 2.7 Å was found to be the cross-over between preference for Pt_2^{III} and preference for Pt_2^{II} states [72].

A Mechanistic Interpretation for Dimerization in the Oxidation of Pt^{II} Species

The formation of dimeric Pt^{III} species may be a relatively common, although overlooked, feature of platinum chemistry. Transient red colors in the Pt^{II}/Cl_2 oxidative addition reaction were noted and reported since the early 1950's [118] from time to time [119]. It is most likely that these red colors arise from the temporary formation of Pt_2^{III} species similar to those discussed above (which have been structurally characterized by X-ray crystallography) and others such as the $[PtCl(\beta\text{-diketonate})_2]_2$ species characterized by ^{1}H-NMR spectroscopy [51].

Starting from monomeric Pt^{II} compounds, the dimerization reaction, leading to binuclear Pt^{III} species, must occur during the oxidation process. In a simple model, the oxidation of Pt^{II} to Pt^{IV} requires the removal of two electrons by the oxidant (a *Lewis* acid, $X^{(A)}$), assisted by a ligand (a *Lewis* base, $X^{(B)}$) entering the coordination sphere from the opposite side (*Fig. 8*). A second Pt^{II} unit could act as a base, contributing two d_{z^2} electrons and binding along the axial direction in a *face-to-face* fashion. The resulting dimer still requires another ligand to complete the coordination sphere of the adjoined platinum unit.

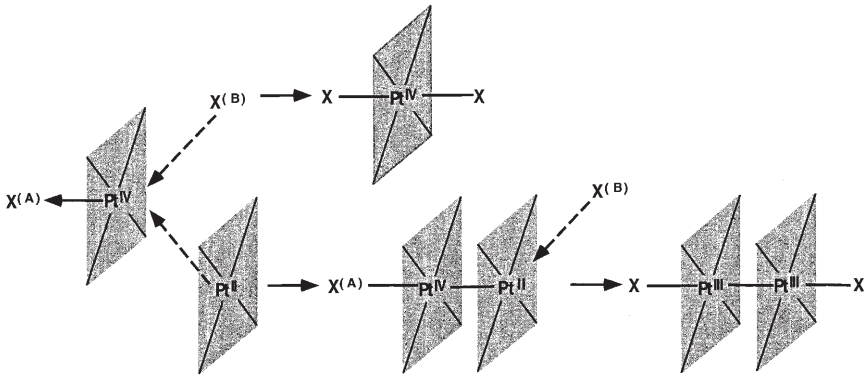


Fig. 8. *Donation of a lone pair of electrons to the transient Pt^{IV} species* (formed by interaction of the Pt^{II} substrate with the oxidant ($X^{(A)}$)) *by either a base* ($X^{(B)}$) *or an extra molecule of Pt^{II} complex leads to the formation of either a Pt^{IV} monomer* (upper line) *or a Pt^{III} dimer* (lower line)

Interfacial hydrogen bonds can play a crucial role in the early stage of the dimerization process, therefore the presence in the Pt^{II} precursors of two ligands capable of donating H-bonds, and of another two ligands capable of accepting H-bonds, could be particularly suitable for promoting the association reaction. Stacking interactions can play a similar role as H-bonds and probably contribute to the dimerization in the case of $[PtCl(\alpha\text{-dioximate})_2]_2$ and $[PtCl(\beta\text{-diketonate})_2]_2$ complexes [50][51].

Support for the hypothesis of Pt^{III}-dimer formation by attack of a second Pt^{II} unit on the sixth coordination position of a Pt^{II} species undergoing two-electron removal by an oxidant, came from the observation that oxidation of mononuclear species by Cl_2, in the presence of an excess of Cl^- ion, leads directly to formation of the Pt^{IV} species with a negligible amount of the Pt^{III} dimer [52].

Pt^{III} dimers unsupported by covalently bridging ligands are also rather inert to further oxidation by excess Cl_2 if kept in the dark (in the presence of light, photoreactions with homolytic cleavage of Pt-X and Pt-Pt bonds can take place [120]). Moreover, the presence of excess Cl_2 inhibits the disproportionation of the Pt^{III} dimer into monomeric Pt^{II} and Pt^{IV} species [49][52]. Therefore, it is conceivable that the disproportionation reaction takes place by reductive elimination of Cl_2 from the Pt_2^{III} species and back formation of the Pt^{II} monomers, rather than by heterolytic intramolecular Pt^{III}-Pt^{III} bond cleavage (half of the Pt^{II} monomeric species is then reoxidized to Pt^{IV} by the released Cl_2). This mechanism would account for the stabilizing effect of the excess Cl_2 (inhibition of the reductive elimination step). The facile reductive elimination of chlorine from the Pt^{III} dimer is also in accord with the rather long Pt^{III}-Cl_{ax} distance.

The weak bonding affinity of Pt^{III} dimers along the axial sites is further confirmed by the recent report of a Pt^{III} dimer without axial ligands $[Pt(o\text{-benzoquinonediiminate})_2]_2^{2+}$. However, in the solid state, a weak interaction along the axial sites with the iminic double bonds of two adjacent units takes place (*Fig. 9*) [53].

Valence Localization in Formal Pt^{III} Dimers

Formal oxidation numbers are essentially a convention and do not necessarily provide information on the structure and reactivity of a given compound. This is particularly true for a platinum dimer of average oxidation state +3 for which there are difficulties in deciding whether to classify the compound as $Pt^{II}Pt^{IV}$ or $Pt^{III}Pt^{III}$. This subject has been dealt with previously [9][121][122] and will be treated only briefly here.

In the case in which only one end of the Pt-Pt axis carries a ligand, while the other end does not have any axial ligand, the Pt-atom carrying the

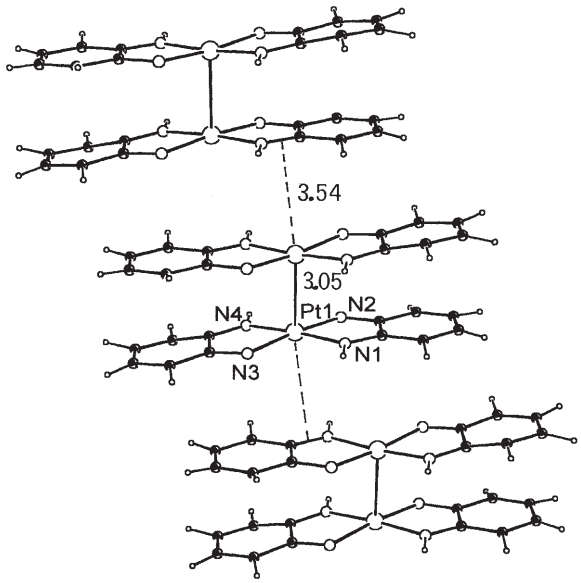


Fig. 9. *Structure of* $[Pt_2$(o-*benzoquinonediiminate*)$_4]^{2+}$ (based on data from [53])

axial ligand is close to Pt^{IV}, whereas the other Pt atom is close to Pt^{II}. This valence localization has been observed by ^{195}Pt NMR in the case of an axial alkyl ligand [108]. In $[Pt_2(NH_3)_4\{(CH_3)_3CC(O)NH\}_2R]^{3+}$ (R = $CH_2\overline{CH(CH_2)_3O}$), one Pt has a chemical shift of –272 ppm (close to values expected for a Pt^{IV}) while the second Pt has a chemical shift of –2253 ppm (close to values expected for a Pt^{II}).

A similar conclusion can be drawn from crystallographic data of a formal Pt^{III} dimer in which only one platinum carries an axial ligand. A clear-cut example is the compound recently reported by *Lippert* and coworkers $[(CH_3NH_2)_2Pt(1\text{-Et-thym})_2PtCl_3](ClO_4) \cdot 4(H_2O)$, in which the Pt-$Cl_{ax}$ bond length (2.31(1) Å) is surprisingly short and compares well with the Pt-Cl_{eq} bond length (2.294(3) Å) (*Fig. 5*) [22].

On the other hand, a perfect Pt^{III} dimer can be recognized in those dimers in which both platinum atoms carry similar axial ligands. We have already pointed out that in symmetrical Pt^{III} species the axial ligands are weakly bound if compared with similar equatorial ligands (*ca.* 0.15 Å longer in the case of Cl^- ligands), revealing that, for Pt^{III} species, the affinity for axial ligands is intermediate between those of Pt^{IV} and Pt^{II} species.

The great polarizability along the Pt-Pt axis appears to be a characteristic feature of Pt^{III} dimers which therefore can span the whole range of intermediate character between Pt^{II} and Pt^{IV}. The olefin oxidation catalyzed by amidate-bridged Pt^{III} dinuclear-species is believed to take place through ol-

efin coordination to a platinum atom having a Pt^{II} character, and nucleophilic attack on the α-carbon of an alkyl bound to a platinum atom having a Pt^{IV} character [108] (*cf.* also chapter by *Matsumoto*).

Perspectives

The unique electron buffer function of the Pt-Pt bond allows Pt^{III} dimers to exhibit various reactivities.

Among quadruply-bridged platinum dimers, the pyrophosphite (POP) and methylenebisphosphite (PCP)-bridged species, which are the most widely studied [6][84][123–125], exhibit a strong red luminescence at 77 K [126]. The substitution of X^- by Y^- in $Pt_2^{III}X_2$ species is markedly accelerated by light, and the conversion to Pt_2^{II} and X_2 is photoinduced [127][128]. The excited-state reactivities of these species could be usefully compared with those of 17-electron monomers and other trapped diradical species.

Two-bridge amidate Pt_2^{III} complexes catalyze the oxidation of olefins in acidic aqueous solution with a mechanism resembling that of the *Wacker* reaction [30][108]. Compounds of this type can also promote the nitration of benzene ring through reductive elimination from a Pt^{III} dimer, containing a nitrite (NO_2^-), to form the nitronium ion (NO_2^+) and the Pt_2^{II} species. The latter can be reoxidized to Pt_2^{III} by nitric acid [25]. Thus a two-electron redox process can take place between Pt_2^{II} and Pt_2^{III} (resembling the two-electron redox reaction between Pt^{II} and Pt^{IV}), but with the obvious advantage that the axial coordination sites in Pt_2^{III} species are much more labile than in octahedral Pt^{IV}.

Finally, the photolytic cleavage of the intermetallic bond in a Pt^{III} species unsupported by covalent bridges, can lead to formation of highly reactive 17-electron metal-centered radicals which can also activate C-M and C-X bonds [14].

It is rather surprising, however, that, although much of the Pt_2^{III} chemistry has come about as result of the serendipitous discovery of the first 'platinum pyrimidine blue' which proved to have high antitumor activity as well as low renal toxicity [1], research on anticancer activity of most of these Pt_2^{III} compounds has not started until very recently [129].

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Inorganic and Organometallic Chemistry of Cisplatin-Derived Diplatinum(III) Complexes

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Cisplatin-derived di- and tetranuclear Pt^{III} complexes, having amidate bridges between the metals have been prepared during the study of the mode of reaction of cisplatin with nucleobases. Their crystal structures, redox properties, the complicated dissociation and association equilibria between the dinuclear and the tetranuclear complexes, and disproportionation reactions in solution are introduced. The reversible redox reactions with O_2 and H_2O are examined by UV-VIS spectroscopy, and these properties are utilized in catalytic oxidation of hydroquinone to quinone. The dinuclear Pt^{III} compounds have also been found to catalyze the oxidation of olefins to aldehydes, ketones, and epoxides in a diluted H_2SO_4-dichloroethane mixed solvent with the addition of O_2. These reactions suggest that the dinuclear Pt^{III} compounds coordinate olefins at their axial positions. Corresponding to this assumption, several dinuclear alkyl Pt^{III} compounds have been synthesized in the reactions with pent-4-en-1-ol and ethylene glycol vinyl ether, and the crystal structures have been solved. These alkyl compounds show that the coordinated olefinic carbon atoms easily are subject to nucleophilic attack of the hydroxy group of the ligand to form dinuclear cycloalkyl-Pt^{III} compounds. What is more significant about the alkyl compounds is that the α-carbon atoms undergo nucleophilic attack with release of dinuclear Pt^{II} compounds.

Syntheses and Structures of Cisplatin-Derived 'Platinum Blues' and the Related Diplatinum(III) Complexes

Historical Background of Amidate-Bridged Pt^{III} Complexes

Since the discovery of the antitumor activity of *cis*-$Pt(NH_3)_2Cl_2$ (cisplatin, *cis*-DDP) by *Rosenberg et al.* [1], the interactions of cisplatin with nucleotides and nucleobases have attracted attention towards gaining an understanding of the mechanism of the antitumor activity of cisplatin at a molecular level. In the course of such studies, dark-blue platinum complexes called 'platinum blues' were obtained when hydrolysis products of cis-

Cisplatin. Edited by Bernhard Lippert

platin were reacted with pyrimidines, or their nucleosides and nucleotides [2]. The dark-blue color was very unusual, since most platinum complexes are colorless to yellow and orange. What is more worthy of attention is that these reaction products themselves possess also antitumor activity towards several tumor lines [2].

In spite of the intensive effort of many chemists to elucidate the identity of these compounds, their formulas and structures long remained unclear as the compounds were always obtained as mixtures, and the isolation of a single compound was difficult. It was only known that the compounds were paramagnetic, and presumably mixed-valent (*cf.* also chapter by *Lippert*).

The First Structure Determination of the 'Platinum Blues'

The first structural evidence for the 'platinum blues' was provided by the single-crystal X-ray structure determination of the 'α-pyridonate blue' of *cis*-$(NH_3)_2Pt^{II}$, $[Pt_2(NH_3)_4(\mu\text{-}\alpha\text{-pyridonato-}N,O)_2]_2(NO_3)_5 \cdot H_2O$ [3][4]. In these studies, *Barton*, *Lippard* and co-workers selected α-pyridone as a simplified model for pyrimidine bases (*Fig. 1*). It was revealed that the complex cation is mixed-valent, formally comprising of three Pt^{II} ions and a Pt^{III} ion. The Pt^{III} ion, having one unpaired electron, is the source of the paramagnetism of this 'platinum blue' compound. The complex is a tetranuclear zigzag-chain made up of two dinuclear platinum units, each doubly bridged with deprotonated amidate ligands (*Fig. 2*, *B-1*). Both the intra- and interdimer Pt-Pt distances (2.7745(4) and 2.8770(5) Å, respectively) revealed that the platinum centers are metal-metal bonded to each other [3]. An additional important feature is that the interdimer interaction is stabilized by four hydrogen bonds formed between the ammine ligands and the oxygen atoms of the amidate ligands. Spin-density measurements by ESR spectroscopy [3], as well as the magnetic-susceptibility measurements [4]

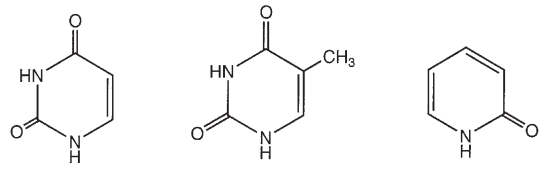


Fig. 1. *Structures of pyrimidine bases uracil and thymine, and the model ligand α-pyridone*

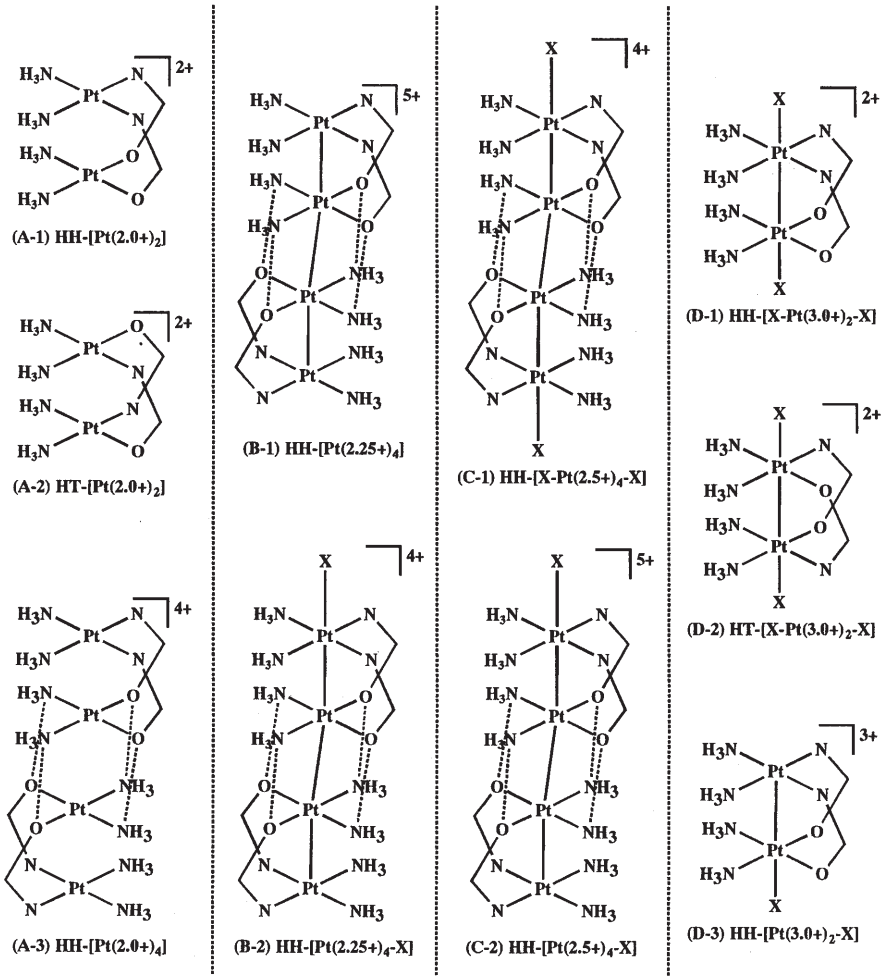


Fig. 2. *Schematic structures of compounds related to 'platinum blues'*. Diplatinum(II) species with *HH* (A-1) and *HT* (A-2) oriented amidate ligands, tetraplatinum(II) species (A-3), tetranuclear $[Pt^{2.25}]_4$ 'blues' without (B-1) and with axial ligand (B-2), tetranuclear $[Pt^{2.5}]_4$ 'tans' (C-1, C-2), and various types of diplatinum(III) species with *HH* (D-1, D-3) and *HT* (D-2) orientation of the bridging ligands. The amidate ligands are expressed with their *N*- and *O*-coordinating atoms only.

indicated the presence of one unpaired electron within the Pt_4 chain (three Pt^{II} (d^8) and one Pt^{III} (d^7)). The unpaired electron of Pt^{III} was further confirmed to be delocalized over the four platinum ions. As a result, the mixed-valence state of this tetranuclear 'platinum blue' is generally expressed as $[Pt^{2.25}]_4$ or $Pt_3^{II}Pt^{III}$.

Syntheses of 'Platinum Blue' and Related Compounds

Following the X-ray study on the 'α-pyridonate blue', various 'platinum blues' and related complexes of exocyclic amidates and imidate ligands have been prepared and structurally analyzed by X-ray diffraction. These include yellow $[Pt_2(NH_3)_4(L)_2]^{2+}$ [5–24], blue $[Pt_4(NH_3)_8(L)_4]^{5+}$ [25–35], dark red $[Pt_4(NH_3)_8(L)_4]^{6+}$ [36][37], and yellow $[Pt_2(NH_3)_4\ (L)_2L'L'']^{n+}$ [38–48] (L = amidate bridging ligand, L′, L″ = neutral or anionic axial ligand). Thus, the 'platinum blue' family is part of four closely related types of species which differ in the average platinum oxidation state, Pt^{II}, $Pt^{2.25}$, $Pt^{2.5}$, and Pt^{III}. Note that the $Pt^{2.75}$ level, corresponding to a $Pt^{II}Pt^{III}_3$ state, has never been isolated to date. In addition to this classification based on the Pt-oxidation state, the structures can be classified based on the orientation of the two bridging amidate ligands within a dimeric unit: *head-to-head* (*HH*) and *head-to-tail* (*HT*) isomers are known of $[Pt^{II}]_2$ and $[Pt^{III}]_2$ species [7][8][39][40] (*Fig. 2*). However, only the *HH* isomers afford a dimer-of-dimers, leading to the tetraplatinum chain structure of the 'platinum blues'. The *HT* isomers do not dimerize to give the tetramer due to the steric bulk of the exocyclic amidate rings at both ends of the unit. The third classification is related to the nuclearity of the complex; two major types exist, namely dimer and tetramer. The last classification is based on whether the terminal platinum atoms accept an axial donor or not. These structure classifications are summarized in *Fig. 2*. In addition to these dimeric and tetrameric structures, two other groups, as illustrated in *Fig. 3*, are known. Compounds E-1 [49] and E-2 [50] are produced as a result of deprotonation at one of the four equatorial ammine ligands of the dinuclear α-pyrrolidinonate Pt^{III} species. Two octanuclear 'platinum blues' (F-1) are known when acyclic amidate (acetamidate and 2-fluoroacetamidate) is employed instead of heterocyclic ones [51][52].

Solution Behavior

Tetranuclear 'platinum blues' usually give several chemical species as described in the previous section when dissolved in aqueous solution. Such complicated behaviors had long been unexplored, but gradually became unveiled as a result of detailed equilibrium and kinetic studies in recent years. The basic reactions can now be classified into four categories: *i*) *HH-HT* isomerization; *ii*) redox disproportionation reactions; *iii*) ligand-substitution reactions, especially at the axial coordination sites of both $[Pt^{III}]_2$ and $[Pt^{2.5}]_4$; *iv*) redox reactions with co-existing species, such as water and O_2. In this chapter, reactions *ii*) – *iv*) are summarized.

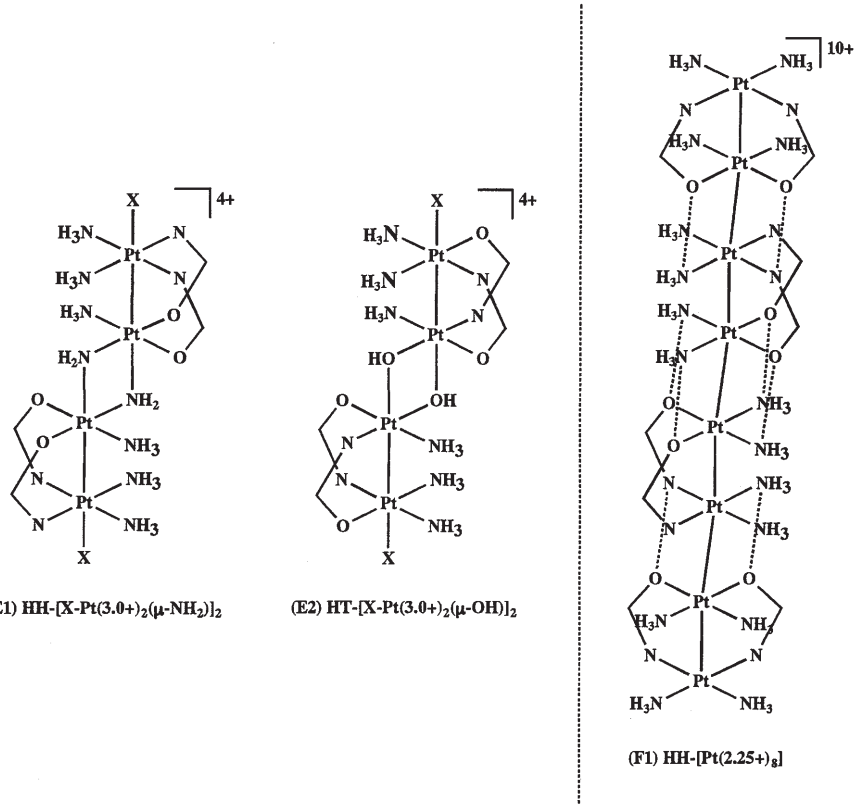


Fig. 3. *Schematic structures of tetraplatinum(III) amidate species with additional* μ-NH_2 (E-1) *and* μ-*OH* (E-2) *bridging, as well as octanuclear* $[Pt^{2.25}]_8$ *compound* (F-1). The amidate ligands are depicted with their *N*- and *O*-coordinating atoms only.

Redox Disproportionation of the Mixed-Valence Compound

Upon dissolving the mixed-valence tetranuclear or octanuclear compounds in water, partial dissociation of the high nuclearity structures to amidate-bridged dinuclear compounds occurs. This can be observed as the decay of the visible absorption bands and growth of new UV bands. The spectral features and the rates of the decay or growth are generally sensitive to pH, counterion, and complex concentration. A visible-absorption decay was first reported for the 'α-pyridonate blue' as mere decomposition [26], but was later suggested to be due to a disproportionation reaction to the corresponding $[Pt^{II}]_2$ and $[Pt^{III}]_2$ dimers [27], without any direct experimental evidence. The first experimental attempt to clarify such phenomena was the spectroscopic study of a disproportionation reaction of the α-pyrrolidonate $[Pt^{2.5}]_4$ compound (*Eqn. 1*) [53][54]. The observed spectral changes are

shown in *Fig. 4*.

$$[Pt^{2.5}]_4 \rightleftharpoons [Pt^{II}]_2 + [Pt^{III}]_2 \quad (1)$$

The reaction rate was found to obey the rate law defined by $k_{obs} = k_1 + k_2/[H^+]$ [54]. The acetamidate-bridged $[Pt^{2.25}]_8$ octamer $[Pt_8(NH_3)_{16}(acetamidato)_8]^{10+}$ was also reported to exhibit similar behavior after dissolution in aqueous media (*Eqn. 2*) [55].

$$[Pt^{2.25}]_8 \xrightarrow{\text{extremely rapid}} \begin{cases} [Pt^{2.5}]_4 \rightleftharpoons [Pt^{II}]_2 + [Pt^{III}]_2 \\ + \\ 2\,[Pt^{II}]_2 \end{cases} \quad (2)$$

It was proposed that $[Pt^{2.25}]_8$ rapidly releases the outer two dimeric units upon dissolution to aqueous media, and that the $[Pt^{2.5}]_4$ species formed undergoes a disproportionation reaction similar to that shown in *Eqn. 1* [55].

Disproportionation of $[Pt^{2.25}]_4$ has also been described; recent studies on the α-pyrrolidonate $[Pt^{2.25}]_4$ revealed that a reaction pathway as shown in *Eqn. 3*, occurs very quickly in solution.

$$2\,[Pt^{2.25}]_4 \xrightarrow{\text{very rapid}} [Pt^{2.5}]_4 + [Pt^{II}]_4 \xrightarrow{\text{very rapid}} [Pt^{2.5}]_4 + 2\,[Pt^{II}]_2 \quad (3)$$

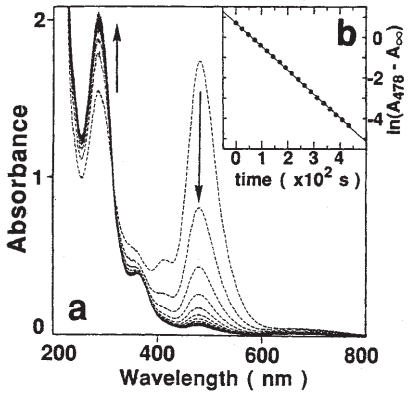


Fig. 4. a) *Spectral change observed for* $[Pt_4(NH_3)_8(\alpha\text{-pyrrolidonato})_4]^{6+}$ *in* 2M H_2SO_4 *at 25 °C in air* (every 2 min), b) *the first-order decay at 478 nm in 0.254*M H_2SO_4 *at 23 °C in air*

Axial Ligand Substitution Reactions of Dinuclear Pt^{III} Compounds

Spectral features in the UV region of the 'platinum blue' solutions are dramatically affected by anions, such as halides, sulfate, and acetate. These also cause bleaching of the visible absorption bands. Recent studies on the α-pyrrolidonate-bridged *HH*-$[Pt^{III}]_2$ species greatly facilitate the understanding of such behavior [54][56][57]. They suggest that the intense UV-absorption bands can be mainly attributed to the $[Pt^{III}]_2$ species, and that the bands are affected by axial ligand substitution. A good example demonstrating the ligand substitution of $[Pt^{III}]_2$ is shown in *Fig. 5* [58]. *Fig. 5,a*, shows that dissolution of HH-O_2NO-$[Pt^{III}]_2$-NO_2 results in a gradual release of the axial NO_2^- (nitro) ligand according to *Eqn. 4*. Since addition of NO_3^- to the solution of $[Pt^{III}]_2$ does not affect the spectral feature, nitrate coordination is expected to be extremely weak. Therefore, it is assumed that the axial nitrate undergoes rapid displacement by other ligands, including water (*Eqn. 4*). The reversibility of this process was also confirmed by adding $NaNO_2$ to the resulting solution (see *Fig. 5,b*).

$$O_2NO\text{-}[Pt^{III}]_2\text{-}NO_2 \xrightarrow{H_2SO_4\ aq.} X\text{-}[Pt^{III}]_2\text{-}NO_2 \underset{}{\overset{+SO_4^{2-}}{\rightleftharpoons}} X\text{-}[Pt^{III}]_2\text{-}OSO_3 + NO_2^- \quad (X = OH_2, OH^-, \text{and } OSO_3^{2-}) \tag{4}$$

Similar spectrophotometric studies were also carried out for the ligand substitution reactions with SO_4^{2-} [54][56] and Cl^- [56][59]. However, the reaction rates were so fast that only the spectra at the respective equilibrated states could be observed. The gradual displacement of NO_2^- discussed above is possible only because most of the NO_2^- exists in its protonated form in the strongly acidic solution. Hydrolysis of axial aqua ligand was also examined spectrophotometrically [56][57]. The stepwise formation constants

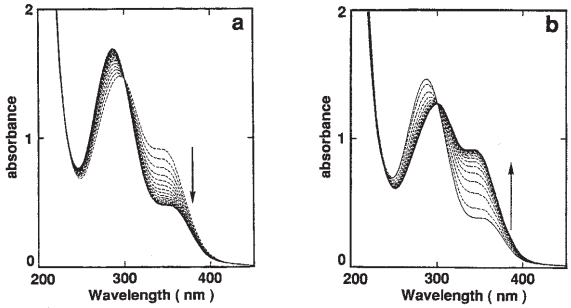


Fig. 5. a) *Spectral changes observed for* HH-*[$Pt_2(NH_3)_4$(α-pyrrolidonato)(NO_2)(NO_3)] (NO_3)$_2$(H_2O) in 0.5*M* H_2SO_4 after dissolution* (0.1 mM, 25 °C in air)*, recorded every 1 min*, b) *a small amount of $NaNO_2$ was added to the resulting solution in* a)

(*Eqn. 5*) and hydrolysis constants (*Eqn. 6*) are: $K_{1,SO_4} = 3.4 \times 10^2$ м$^{-1}$, $K_{2,SO_4} = 2.6 \times 10$ м$^{-1}$ [54][56]; $K_{1,Cl} = 1.7 \times 10^5$ м$^{-1}$, $K_{2,Cl} = 4.0 \times 10^3$ м$^{-1}$ [56][59]; $K_{1,NO_2} = 3.7 \times 10^7$ м$^{-1}$, $K_{2,NO_2} = 7.8 \times 10^4$ м$^{-1}$ [56]; $K_{h1,OH_2} = 9.6 \times 10^{-4}$ м [57]; $K_{h1,SO_4} = 4.6 \times 10^{-4}$ м [57].

$$\begin{aligned} H_2O\text{-}[Pt^{III}]_2\text{-}OH_2 + X &\overset{K_{1,X}}{\rightleftharpoons} H_2O\text{-}[Pt^{III}]_2\text{-}X \\ H_2O\text{-}[Pt^{III}]_2\text{-}X + X &\overset{K_{2,X}}{\rightleftharpoons} X\text{-}[Pt^{III}]_2\text{-}X \end{aligned} \qquad (5)$$

$$\begin{aligned} X\text{-}[Pt^{III}]_2\text{-}OH_2 &\overset{K_{h1,X}}{\rightleftharpoons} X\text{-}[Pt^{III}]_2\text{-}OH + H^+ \\ H_2O\text{-}[Pt^{III}]_2\text{-}OH &\overset{K_{h2,OH2}}{\rightleftharpoons} HO\text{-}[Pt^{III}]_2\text{-}OH + H^+ \end{aligned} \qquad (6)$$

As for the hydrolysis of the axial aqua ligand, pK_a values were determined for the 1-methyluracilate-bridged *HT*-O_2N*O*-$[Pt^{III}]_2$-*O*H_2 by means of potentiometric titration ($K_{h1,OH_2} = 3.2 \times 10^{-4}$ м, $K_{h2,OH_2} = 2.0 \times 10^{-7}$ м) [60]. In addition, it was also suggested that the first ligation in *Eqn. 5* selectively occurs at one of the two chemically nonequivalent Pt atoms of *HH*-$[Pt^{III}]_2$, as a clear isosbestic point was observed when the first ligation was dominating [54][56][59].

Redox Reactions with Molecular Oxygen and Water

One of the most important redox reactions of this family of compounds is the oxidation of $[Pt^{II}]_2$ by O_2. This is the main cause of the appearance of blue, purple, or dark-red colors of the mixed-valence species. Although no detailed examination has been performed, kinetics of the O_2 oxidation of $[Pt_2^{II}(NH_3)_4(\alpha\text{-pyrrolidonato})_2]^{2+}$ to $[Pt_2^{III}(NH_3)_4(\alpha\text{-pyrrolidonato})_2(H_2O)_2]^{4+}$ (*Eqn. 7*) was spectrophotometrically examined [57]. The study showed that the reaction proceeds over several days at room temperature. The first-order rate constants in acidic media were in the range of $k_{obs} = 4.2 \times 10^{-5}$ s^{-1} (pH = 0.23) – 1.13×10^{-5} s^{-1} (pH = 2.1) (at 25 °C, in air, $I = 1.5$м) [57].

$$2\,[Pt^{II}]_2 + O_2 + 4\,H^+ \longrightarrow 2\,[Pt^{III}]_2 + 2\,H_2O \qquad (7)$$

On the other hand, some O_2-evolving reactions (*Eqns. 8* and *9*), corresponding to the reverse reaction of *Eqn. 7*, were reported for the α-pyrrolidonate family [61][62]. In these studies, dissolution of the $[Pt^{III}]_2$ or $[Pt^{2.5}]_4$

compounds resulted in O_2 production from water. As expected from *Eqns. 8* and *9*, addition of NaOH to an aqueous solution of $[Pt^{2.5}]_4$ affords the $[Pt^{II}]_2$ dimer [63][64].

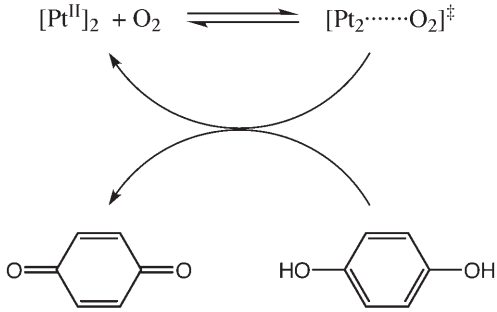
$$2\,[Pt^{2.5}]_4 + 2\,H_2O \longrightarrow 4\,[Pt^{II}]_2 + O_2 + 4\,H^+ \quad (8)$$

$$2\,[Pt^{III}]_2 + 2\,H_2O \longrightarrow 2\,[Pt^{II}]_2 + O_2 + 4\,H^+ \quad (9)$$

Catalysis of Amidate-Bridged Platinum Complexes

Catalytic Oxidation of Hydroquinone to Quinone via *O_2-Activation*

The O_2 oxidation of hydroquinone to quinone, which is very slow in the absence of a catalyst, was found to be accelerated by the addition of the α-pyrrolidonate-bridged $[Pt^{2.5}]_4$ [57]. The detailed kinetic investigation revealed that the $[Pt^{II}]_2$-species, formed according to *Eqn. 1*, plays a major role as the catalyst. The reaction rate of quinone formation is higher than that of O_2 oxidation of $[Pt^{II}]_2$ to $[Pt^{III}]_2$, and was linear with respect to the hydroquinone concentration. Therefore, it was suggested that the quinone formation proceeds *via* an intermediate formed between the $[Pt^{II}]_2$ species and molecular oxygen (*e.g.*, a peroxo species). The schematic mechanism is illustrated in *Eqn. 10*.

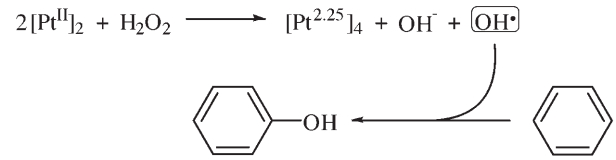


(10)

Oxidation of Benzene to Phenol by H_2O_2

The α-pyrrolidonate $[Pt^{2.25}]_4$ was also found to catalyze the oxidation of benzene to phenol by hydrogen peroxide [65]. As confirmed by HPLC, ESR, and UV-VIS absorption spectroscopy, the main reaction pathway is

shown in *Eqn. 11*.



(11)

As shown above, addition of H_2O_2 to a solution of the complex caused prompt color change to blue with formation of the hydroxyl radical, which was detected with a spin-trapping method. The reaction was therefore found to be a *'Fenton'*-like reaction.

Reactions of Dinuclear Pt^{III} Complexes with Olefins

Catalytic Ketonation and Epoxidation of Olefins

Since tetranuclear 'platinum blues' are oxidized by O_2 to dinuclear Pt^{III} complexes and are reversively reduced to the 'platinum blues' and further to the dinuclear Pt^{II} complexes, an attempt was made to use these complexes as catalysts for olefin oxidation to ketones and epoxides. The catalysts used were α-pyrrolidonato-bridged 'Pt tan' $[Pt_4(NH_3)_8(C_4H_6NO)_4](NO_3)_6 \cdot 2\,H_2O$ (**1**), pivalamidato-bridged 'Pt blue' $[Pt_4(NH_3)_8(C_5H_{10}NO)_4](NO_3)_5$ (**2**), α-pyrrolidonato 'Pt tan' $[Pt_4(NH_3)_8(C_4H_6NO)_4](C_{12}H_{25}SO_3)_6$ (**3**), pivalamidato 'Pt blue' $[Pt_4(NH_3)_8(C_4H_6NO)_4](C_{12}H_{25}SO_3)_5$ (**4**), 3-methyl-2-pyrrolidonato 'Pt tan' $[Pt_4(NH_3)_8(C_5H_8NO)_4](NO_3)_6 \cdot 3H_2O$ (**5**), and 5-methyl-2-pyrrolidonato 'Pt blue' $[Pt_4(NH_3)_8(C_5H_8NO)_4](ClO_3)_5$ (**6**). Since most of the platinum complexes are insoluble in dichloroethane, the reaction was carried out in a H_2O/CH_2ClCH_2Cl biphasic solution. In a typical experiment, 10 μmol of the platinum complex and a fivefold (**2**, **4**, **6**), or a sixfold (**1**, **3**, **5**) equivalent amount of phase-transfer agent, $C_{12}H_{25}SO_3Na$, were dissolved in a mixture of 1 ml of 0.05M H_2SO_4 and 1 ml of CH_2ClCH_2Cl containing 400 equiv. of olefin. The solution was stirred vigorously in a closed O_2-filled *Teflon* vial (5 ml), which was placed inside an O_2-filled glass bottle with a screw stopper to prevent leakage of air from the *Teflon* vial. The reaction was carried out at 50 °C for 5 days, and aliquot of the solution was analyzed by gas chromatography.

The results of the olefin oxidation catalyzed by **1** to **6** are summarized in *Tables 1–3*. *Table 1* shows that linear terminal olefins are selectively oxidized to 2-ketones, whereas cyclic olefins (cyclohexene and norbornene) are selectively oxidized to epoxides. Cyclopentene shows an exceptional behavior; it is oxidized exclusively to cyclopentanone without any produc-

tion of epoxypentane. This exception is tentatively attributed to the strain intrinsic to the planar cyclopentene ring, compared with other larger cyclic nonplanar olefins in *Table 1*. The linear inner olefin, oct-2-ene, is oxidized to both octan-2-one and octan-3-one, 2-methylbut-2-ene is oxidized to 3-methylbutan-2-one, while the ethyl vinyl ether is oxidized to acetaldehyde

Table 1. *Olefin Oxidation Catalyzed by 'Platinum Blue' Compounds*

Substrate	Product	Turnover number	
		Catalyst	
		1	2
Hex-1-ene	Hexan-2-one	11.9	3.8
	1,2-Epoxyhexane	8.6	8.8
Hept-1-ene	Heptan-2-one[b])	13.3	4.5
Oct-1-ene	Octan-2-one[b])	15.8	12.4
Dec-1-ene	Decan-2-one	10.9	4.1
Oct-1-ene	Octan-2-one	1.7	1.6
	Octan-3-one	2.2	1.6
Cyclohexene	Epoxycyclohexane	22.8	15.0
	Cyclohexanone[c])	1.9	1.4
Cyclopentene	Cyclopentanone	2.0	2.5
Norbornene	Epoxynorbornane	7.2	5.2
	Norbornanone[d])	2.3	0.6
2-Methylbut-2-ene	3-Methylbutan-2-one		
Ethyl vinyl ether	Acetaldehyde		
	Ethanol		
1-Chloroprop-1-ene		No reaction	
1-Bromoprop-1-ene		No reaction	
α-Methylstyrene		No reaction	
β-Methylstyrene		No reaction	
Allylbenzene		No reaction	

[a]) Turnover number = [product]/[complex]. Minor products (less than 1%) are [b]) 1,2-epoxide, [c]) cyclopentanecarbaldehyde, and [d]) norborneol.

Table 2. *Effect of Platinum Oxidation State in the Catalytic Oxidation of Cyclohexene*

Catalyst	Turnover number	
	Product	
	Epoxycyclohexane	Cyclohexanone
$[Pt_2^{III}(NH_3)_4(C_4H_6NO)_2(H_2O)_2](NO_3)_4$ [a])	24.7	1.4
$[Pt_2^{II}Pt_2^{III}(NH_3)_8(C_4H_6NO)_4](NO_3)_6$ (1)	22.8	1.9
$[Pt_2^{II}(NH_3)_4(C_4H_6NO)_2](NO_3)_2$ [b])	0.5	1.8

[a]) The complex was prepared *in situ* by electrochemical oxidation at 0.60 V *vs*. SCE [8].
[b]) The complex was prepared *in situ* by electrochemical reduction at 0.35 V *vs*. SCE [8].

and ethyl alcohol. These products were identified by NMR, but could not be quantitatively determined because of the existence of small overlapping peaks in the GC chart. Those olefins having bulky (α-methylstyrene, β-methylstyrene, allylbenzene) or electon-withdrawing substituents (1-bromoprop-1-ene, 1-chloroprop-1-ene, fumaronitrile, acrylonitrile, and methylacrylate) are not oxidized.

Since it is known that the tetranuclear mixed-valent 'platinum blue' and 'tan' complexes such as **1** and **2** undergo disproportionation and reduction by water as *Eqns. 1–3* and *7–9* show [54][66], all the species appearing in *Eqns. 1–3* and *7–9* are present in the solution. However, only one or several of the four species in the solution may in fact be active during catalytic olefin oxidation. To clarify this point, the effects of the Pt oxidation state in the platinum complexes were compared. The results are summarized in *Table 2*. It clearly shows that the dinuclear Pt^{III} complex is most effective, and is likely to be a true catalyst. Compound **1** also exhibits high activity, whereas the dinuclear Pt^{II} complex is ineffective. All other factors expected to affect the catalytic efficiency, including the presence of O_2, the surfactant and the choice of solvent, have been examined and the results are summarized

Table 3. *Effects of Surfactant, Solvent, and Atmosphere on the Catalytic Oxidation of Cyclohexene*

Run	Catalyst	Surfactant [a]) $C_{12}H_{25}SO_3Na$	Solvent	Atmosphere	Turnover number	
					Product	
					Epoxycyclohexane	Cyclohexanone
1	None	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	0	0.4
2	**1**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	22.8	1.9
3	**1**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	air	18.6	1.2
4	**1**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	N_2	4.7	0.8
5	**1**	–	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	0	0.8
6	**1**	+	CH_2ClCH_2Cl	O_2	0	0
7	**1**	+	0.05M H_2SO_4	O_2	0	0.3
8	**1**	+	H_2O/CH_2ClCH_2Cl	O_2	2.3	0.1
9	**1**	+	CF_3SO_3H [b])/CH_2ClCH_2Cl	O_2	0	0
10	**3**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	20.3	1.5
11	**3**	–	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	17.3	1.2
12	**2**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	15.0	1.4
13	**4**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	14.5	1.0
14	**4**	–	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	12.2	0.7
15	**5**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	21.7	1.3
16	**6**	+	0.05M H_2SO_4/CH_2ClCH_2Cl	O_2	4.2	0.3

[a]) +: added; –: not added. [b]) Two drops of CF_3SO_3H added to CH_2ClCH_2Cl.

in *Table 3*. It is clear from the runs *2*, *3*, and *4* that O_2 is required for the oxidation reaction. Addition of surfactant, or the presence of surfactant as the counterion, is necessary (runs *2*, *5*, *10*, *11*, *13*, and *14*), and the reaction must be carried out in a biphasic solution, *i.e.*, a mixture of CH_2ClCH_2Cl and 0.05M H_2SO_4. Neither 0.05M H_2SO_4 nor CH_2ClCH_2Cl alone gave appreciable products, not even with addition of surfactant (runs *6* and *7*). It is also clear that water is essential for the reaction (runs *6* and *9*). Acid is also necessary for the reaction; the oxidation does not proceed in a biphasic solution of H_2O/CH_2ClCH_2Cl (run *8*). The effect of various acids was also examined, and the results showed that $HClO_4$ was as effective as H_2SO_4, while other coordinating acids, such as HNO_3 and HCl, were much less effective. The effect of a substituent on the α-pyrrolidone ring is compared in runs *2*, *15*, and *16*, and it is evident that a substituent near the amidate group of the α-pyrrolidonate ring suppresses the reaction (run *16*).

Although the olefins seem to be oxidized by O_2 from the experiments described above, GC/MS analysis of the oxidation products generated in the presence of $^{18}O_2$ showed that all of the oxygen atoms in the products are from H_2O and not from O_2. When the oxidation reactions were carried out in both $^{16}O_2$ and $^{18}O_2$, and the products were analyzed by GC/MS, all of the oxygen atoms in the products, including ketones and epoxides, were ^{16}O, irrespective of whether the reaction had been carried out in $^{16}O_2$ or $^{18}O_2$. The reactions were also carried out in $^{16}O_2$ with either $H_2{}^{18}O$ or $H_2{}^{16}O$. The GC/MS analysis of these products revealed that water oxygen is exclusively introduced into the oxidation products. The reactions were also carried out under $^{16}O_2$ in $D_2{}^{16}O$, and it was confirmed that deuterium is absent in the products. From these observations, it appears that the mechanism of the catalytic oxidation of olefins to ketones and epoxides is similar to the *Wacker* reaction [68][69], as shown in *Fig. 6* for ketones and epoxides. There exist, however, several differences between this reaction and the *Wacker* process: *i*) The epoxide is not produced as a main product in the *Wacker* process and *ii*) inner olefins are not oxidized in the *Wacker* process, whereas they are oxidized in our reaction. For linear olefins, 1,2-shift of the coordinated Pt takes place, giving selectively ketones or aldehydes, while for sterically more restrained cyclic olefins, a 1,2-shift does not take place and epoxides are formed as major products. Similar ketone *vs*. epoxide selectivity based on the ease of the 1,2-shift, has been proposed for olefin oxidation catalyzed by [Pt(diphoe)(CF_3)(OH)] [70] (diphoe = *cis*-1,2-bis(diphenylphosphino)ethane), where peroxide is the oxidizing agent.

During the reaction, most of the platinum complex is transferred to the organic phase as is observed from the dark blue or tan color of the tetranuclear complexes in the organic phase. After the catalytic reaction ceases, both phases are pale yellow. The tan color and the catalyst can be recovered

at this stage by adding sodium persulfate to the solution. This indicates that the Pt^{III} catalyst is gradually reduced to the dinuclear Pt^{II} complex during the catalytic reaction, and therefore the reaction finally stops. Acid and O_2 are necessary in the catalysis to reoxidize the dinuclear Pt^{II} complex to the dinuclear Pt^{III} complex [57][66]. The oxidation reaction is, however, not fast enough, and the reduced dinuclear Pt^{II} species gradually accumulates in the solution. Addition of $Na_2S_2O_8$ at the beginning of the reaction in order to increase the lifetime of the catalyst, however, decreases the turnover number.

Synthesis of Dinuclear Alkyl-Pt^{III} Complexes from Olefins and Its Implication on the Olefin Oxidation

According to the reaction mechanism in *Fig. 6*, olefins coordinate axially to the dinuclear Pt^{III} complexes. Whether olefins actually coordinate to Pt^{III} is the subject of further research. Pt^{II} is known to coordinate various olefins, whereas Pt^{IV} does not coordinate any of them. Therefore, an attempt was made to isolate the olefin π-complex of the Pt^{III} dimer in order to prove the proposed mechanisms in *Fig. 6*. While no olefin π-complex was obtained despite our intensive efforts, pent-4-en-1-ol and ethylene glycol vinyl ether

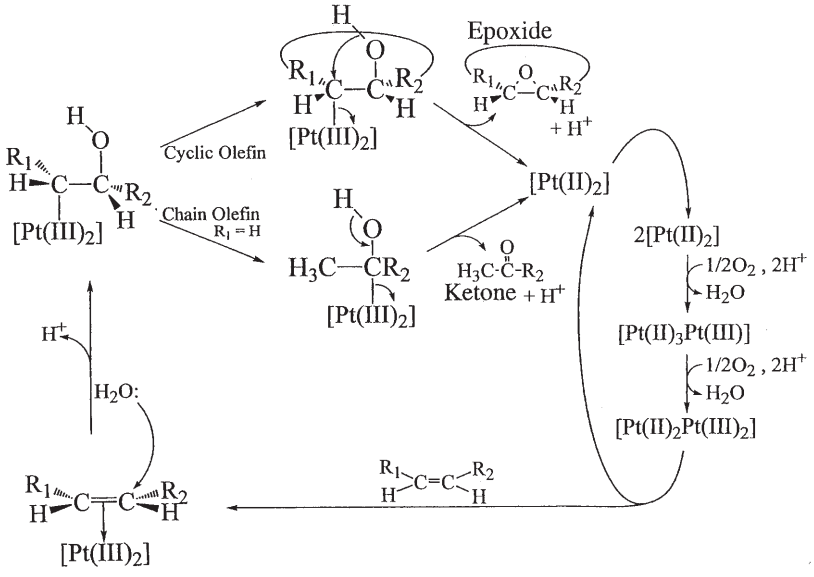


Fig. 6. *Proposed reaction mechanism for olefin ketonation and epoxidation catalyzed by 'platinum blue'*

were found to give alkyl complexes. Although *Fig. 6* shows that tetranuclear 'platinum blue' complexes catalyze the reactions, the actual species responsible for the reaction are dinuclear Pt^{III} complexes produced from the disproportionation of the tetranuclear complexes [71]. The alkyl complexes formed in the present study are the diplatinum(III) 2-methyltetrahydrofurfuryl complex, $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(CH_2\overline{CH(CH_2)_3O})](NO_3)_3 \cdot H_2O$ (**7**), and the oxyethyl complex, $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(CH_2CHO)](NO_3)_3 \cdot H_2O$ (**8**), which were obtained from the reaction of $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(H_2O)_2]^{4+}$ with pent-4-en-1-ol and ethylene glycol vinyl ether, respectively. The reactions support π-coordination of the olefins to the Pt^{III}-axial position in the first step of the reaction, although π-coordination is very unstable and the π-complex cannot be isolated. The reactions are also very important in the sense that they give a general route for synthesizing dinuclear Pt^{III}-alkyl complexes [72].

The crystal structures of **7** and **8** are shown in *Figs. 7* and *8*, respectively. The reactions of pent-4-en-1-ol and ethylene glycol vinyl ether with the amidate-bridged dinuclear Pt^{III} complex are shown in *Eqns. 12* and *13*, re-

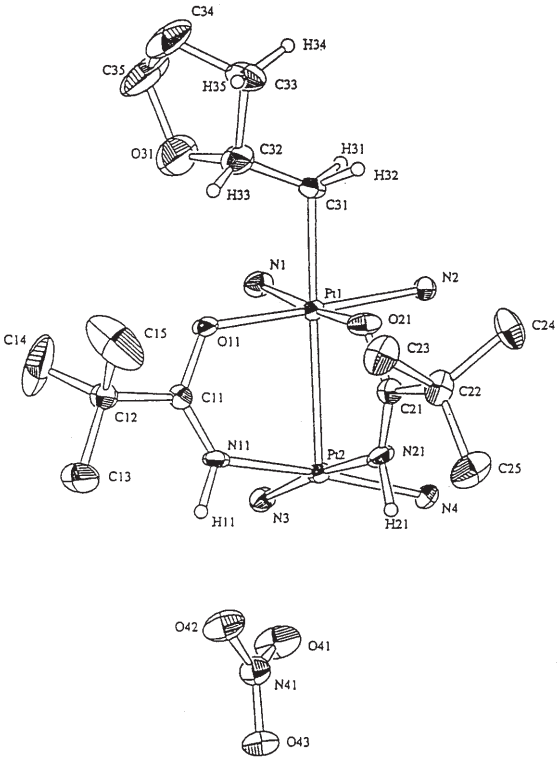


Fig. 7. *ORTEP Drawing of* $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(CH_2\overline{CH(CH_2)_3O})](NO_3)_3 \cdot H_2O$ (**7**)

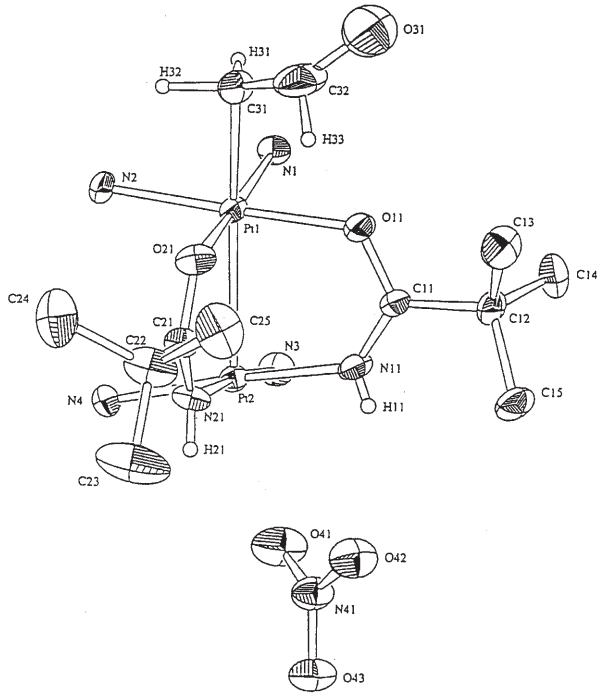
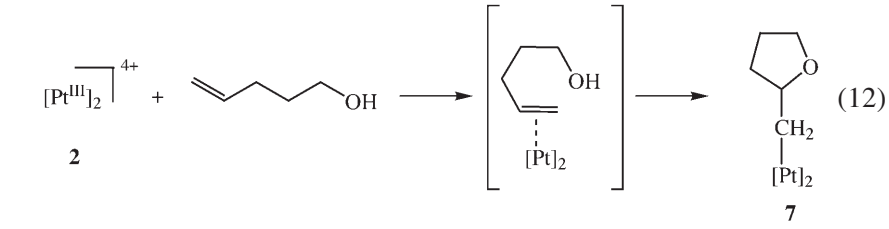
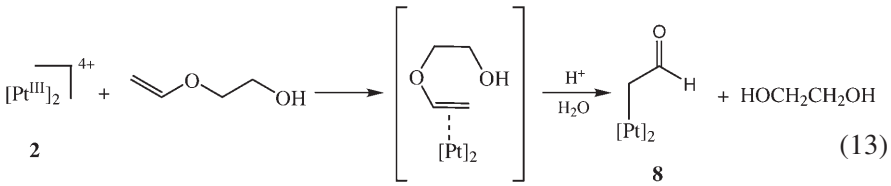


Fig. 8. *ORTEP Drawing of* $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(CH_2CHO)](NO_3)\cdot H_2O$ (**8**)

spectively. Both **7** and **8** are not very stable in air and must be kept in a desiccator.



(12)



(13)

Both the Pt-Pt distances (2.7687(8) Å in **7** and 2.7106(7) Å in **8**) are significantly longer than those in previously reported amidate-bridged dinuclear Pt^{III} complexes with non-alkyl axial ligands such as a halide, H_2O, NO_2^-, or NO_3^- (2.165(10) to 2.644(1) Å) [5–9]. The long Pt-Pt distances are brought about by the strong *trans*-influence of the alkyl ligand, and this strong *trans*-influence extends further to the other axial end. Therefore, the Pt-O (axial nitrate) distances of **7** (2.92(1) Å) and **8** (2.7498(8) Å) are longer than the usual nitrate-coordination distances to Pt^{III} (2.71(1) to 2.36(3) Å) [5–9][38–44], in fact too long to be still considered a bond. Such remote *trans*-influence *via* a Pt-Pt bond would be caused by a strong dipole-inducing effect of the alkyl ligand, and a dipole along the Pt-Pt axis, R-$Pt^{\delta+}$-$Pt^{\delta-}$-L, is induced. Judging from the fact that one end of the Pt-Pt axis is bonded to an alkyl, while the other end does not have any axial ligand, the Pt-atom bonded to alkyl (R) has an oxidation state close to Pt^{IV}, whereas the other Pt-atom has an oxidation state close to Pt^{II}. This valence localization is also observed in the ^{195}Pt-NMR spectra [72].

The bond distance of the alkyl group in **8** is shown in *Fig. 9*, together with the related C=C, C–C, C–O, and C=O distances. The C–C distance in **8** is between typical C–C and C=C distances, and the C–O distance of **8** is

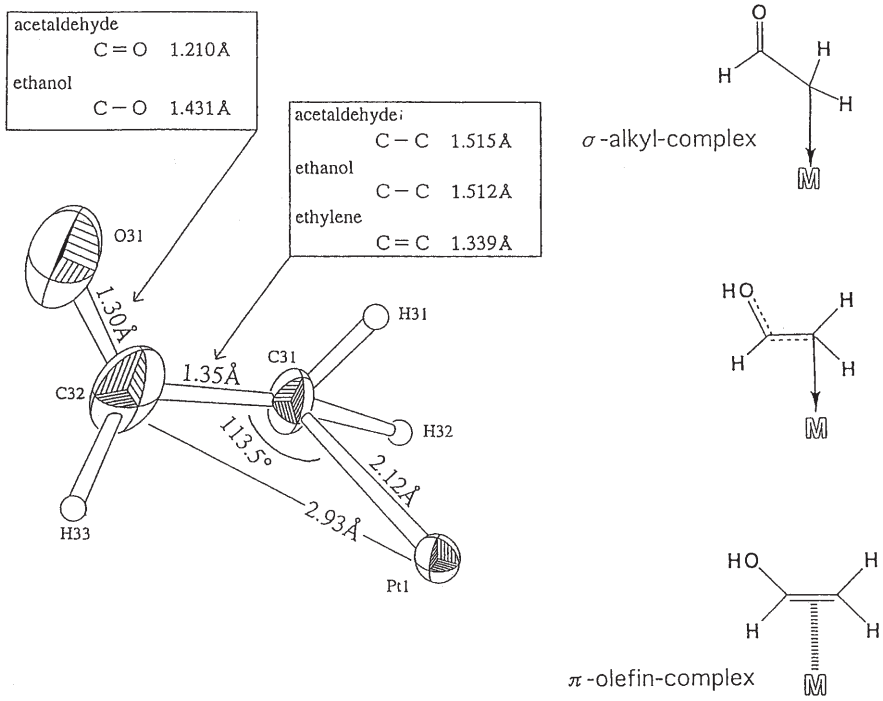
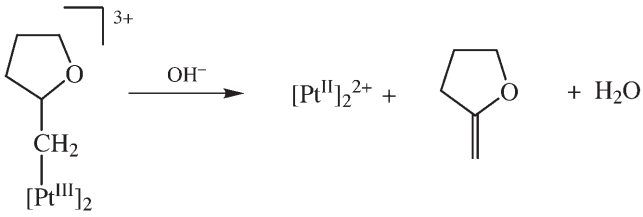


Fig. 9. *Bond distances and angles of the β-oxyethyl group in* **8**

also between the typical C–O and C=O distances. This fact means that the actual electronic state of the alkyl group is in between the σ- and π-complexes as shown in *Fig. 9*. The Pt–C–C angle of 113.5° in **8** is close to that of the σ-complex. Although complexes **7** and **8** do not directly prove the existence of olefin π-complexes, the discussed reactions strongly support the mechanism given in *Fig. 6*, suggesting that an alkyl complex is produced by nucleophilic attack of H_2O on the olefinic carbon of the π-complex.

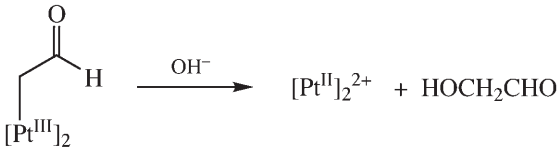
Complex **7** is stable in acidic to weakly basic aqueous solution. However, on addition of 0.1M NaOH, nucleophilic attack at the β-carbon takes place as shown in *Eqn. 14*. In this reaction, it may also be considered that initially α-hydroxylation takes place, which eventually leads to dehydration to give the product.



(14)

7

Complex **8** is unstable at room temperature even as a solid and is easily hydrolyzed in neutral water to produce glycol aldehyde (*Eqn. 15*). In 0.1M $HClO_4$, **8** produces glycol aldehyde and acetic acid in a *ca*. 9:1 ratio.

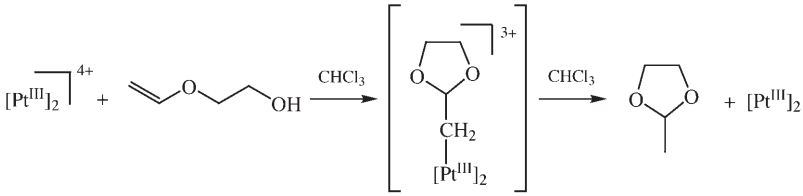


(15)

8

The absence of attack on the α-carbon of **7** by OH^-, in contrast to the reaction in *Eqn. 15*, would be due to the electron-donation by the β-carbon of **7**. Addition of ethylene glycol vinyl ether to the *p*-toluenesulfonate salt of $[Pt_2(NH_3)_4((CH_3)_3CCONH)_2(H_2O)_2]^{4+}$ in $CDCl_3$ catalytically yields 2-methyl-1,3-dioxolane (*Eqn. 16*). The reaction proceeds almost instantane-

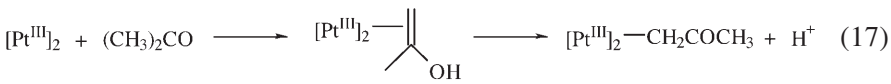
(16)



ously, and 50 equiv. of the substrate was completely converted to 2-methyl-l,3-dioxolane, as confirmed by ^{1}H-NMR. After the reaction, the dinuclear Pt^{III} complex without the alkyl ligand, which is still catalytically active, is left in solution.

A similar reaction was not observed for pent-4-en-1-ol. It should be noted that a dinuclear $[Pt^{III}]_2$ species is released (*Eqn. 16*), in contrast with the release of olefin and a Pt^{II} dimer complex in aqueous solution by reductive elimination (*Eqn. 14*). The dependence of reactivity on the alkyl residue and the solvent could be caused by a difference in electron density at the α-carbon atom, and in the dipole vector along the Pt-Pt bond in solvents of different polarity. In an aprotic organic solvent, the electron distribution along the Pt-Pt bond would be less polar, *i.e.*, close to $R\text{-}Pt^{III}\text{-}Pt^{III}$, whereas in aqueous solution, it would be more polar, being close to $R\text{-}Pt^{IV}\text{-}Pt^{II}$, as indicated in the X-ray structure of **7**. Recent work of ours [73], and of *Labinger*, *Bercaw,* and coworkers [74][75] on C-H activation of alkanes by a mixture of Pt^{II} and Pt^{IV} shows that $R\text{-}Pt^{IV}$ undergoes nucleophilic attack by Nu: to release NuR and Pt^{II}; no such behavior is observed for $R\text{-}Pt^{II}$. It is therefore reasonable that $R\text{-}Pt^{IV}\text{-}Pt^{II}$ undergoes nucleophilic attack by OH^- to release ROH or olefin and a Pt^{II} dinuclear complex in aqueous solution. Although it is essential to compare the reactivity of an identical compound in organic and aqueous solutions, **7** and **8** cannot be dissolved in organic solvents, and exhibit complicated decomposition if forced to dissolve with the aid of a surfactant.

Similar to the above reactions, we found that acetone easily reacts with diplatinum(III) complexes as follows (*Eqn. 17*):



(17)

In this reaction, the enol form of acetone reacts with the diplatinum(III) complex, and therefore the reaction mechanism seems to be the same as that of the olefin reactions [74].

Synthesis of **7** and **8** supports the olefin oxidation mechanisms in *Fig. 6*. These mechanisms have several important and noteworthy points about Pt^{III} chemistry: *i*) Olefins coordinate to Pt^{III} at the axial position, which is contrasted to the π-coordination of olefins perpendicular to the square planar coordination plane of Pt^{II}. Olefin coordination to Pt^{III} should also be contrasted to the behavior of Pt^{IV} species which do not bind olefins. *ii*) Pt^{III} is strongly electron-withdrawing, and the coordinated olefin undergoes nucleophilic attack. *iii*) The alkyl α-carbon on the Pt^{III} undergoes nucleophilic attack in aqueous solution, whereas in aprotic solvent, aldehyde (and possibly also ketone in other cases) is produced by reductive elimination.

The Pt^{III}-Pt^{III} bond in the alkyl complexes exhibits a unique character in that the Pt-atom acts both as Pt^{II} and Pt^{IV} or as an intermediate through electron localization and delocalization along the Pt-Pt axis: *i*) Coordination of olefin is a Pt^{II} characteristic, since no olefin-Pt^{IV} complex is known. *ii*) Nucleophilic attack on the coordinated alkyl α-carbon atoms takes place, which is a Pt^{IV} characteristic, and does not occur at α-carbon atoms of alkyl-Pt^{II} complexes [75–77].

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Part 6. New Developments

Structure-Activity Relationships Within Di- and Trinuclear Platinum Phase-I Clinical Anticancer Agents

Nicholas Farrell, Yun Qu, Ulrich Bierbach, Mariella Valsecchi, and *Ernesto Menta*

The Development of Orally Active Platinum Drugs

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Methods for Screening the Potential Antitumor Activity of Platinum Compounds in Combinatorial Libraries

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Jiří Kozelka

Cisplatin. Edited by Bernhard Lippert

Structure-Activity Relationships within Di- and Trinuclear Platinum Phase-I Clinical Anticancer Agents

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During the last ten years we have investigated a number of novel dinuclear and trinuclear platinum compounds as potential antitumor drugs. This undertaking was driven by the hypothesis that alteration of the antitumor activity of platinum-containing drugs will be achieved by modification of the mode of DNA-binding in comparison to cisplatin. We and our collaborators have extensively studied these new classes of compounds in order to understand the patterns of DNA modification induced by various structural motifs and further related these patterns to cytotoxicity and antitumor activity. The most promising of these compounds have undergone extensive therapeutic tests against human tumors growing as xenografts. This has been successful to the point that a novel trinuclear cationic compound, 1,0,1/*t*,*t*,*t*, or BBR3464, is now undergoing Phase-I clinical trials required to advance to full medical use. This agent is the first genuinely new platinum drug not based on the 'classical' cisplatin structure to enter clinical trials. Notable features are the potency, the ten-fold lower maximum tolerated dose in comparison to cisplatin, and the broad spectrum of tumors sensitive to this agent. The 4+ charge, the bifunctional DNA-binding where the binding sites are separated by large distances and the consequences of such DNA-binding suggest that with this advance the paradigm of cisplatin-based antitumor agents is altered. This contribution outlines early structure-activity relationships we have found within this broad new class of anticancer agents.

Introduction

Dinuclear and trinuclear platinum complexes represent a new class of anticancer agents, distinct in DNA-binding and profile of antitumor activity from their mononuclear counterparts. The dinuclear motif was first reported in 1988 and consisted of two *cis*-$PtCl_2(NH_3)$ units linked by a flex-

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ible diamine chain [1]. Thus, the ability to prepare two antitumor active moieties in the same molecule was shown (*Fig.1*). The use of cisplatin as synthon was further demonstrated by extension to trinuclear systems (*Fig.1*) [2]. Systematic chemical studies since then have made it clear that dinuclear and trinuclear compounds represent a diverse class of structures (*Fig. 1*) which may be differentiated amongst themselves with respect to important parameters of biological activity. The first representative of this class, currently denominated BBR3464, has now advanced to Phase-I clinical trials. Its structure (*Fig. 1*) is best described as two *trans*-$[PtCl(NH_3)_2]^+$ units linked by a non-covalent tetra-amine $[Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2]^{2+}$ unit. This agent is the first genuinely new platinum drug not based on the 'classical' cisplatin structure to enter clinical trials. The profile of antitumor activity (*Table 1*) has recently been detailed [3]. Notable features are the potency, the ten-fold lower maximum tolerated dose in comparison to cisplatin, and

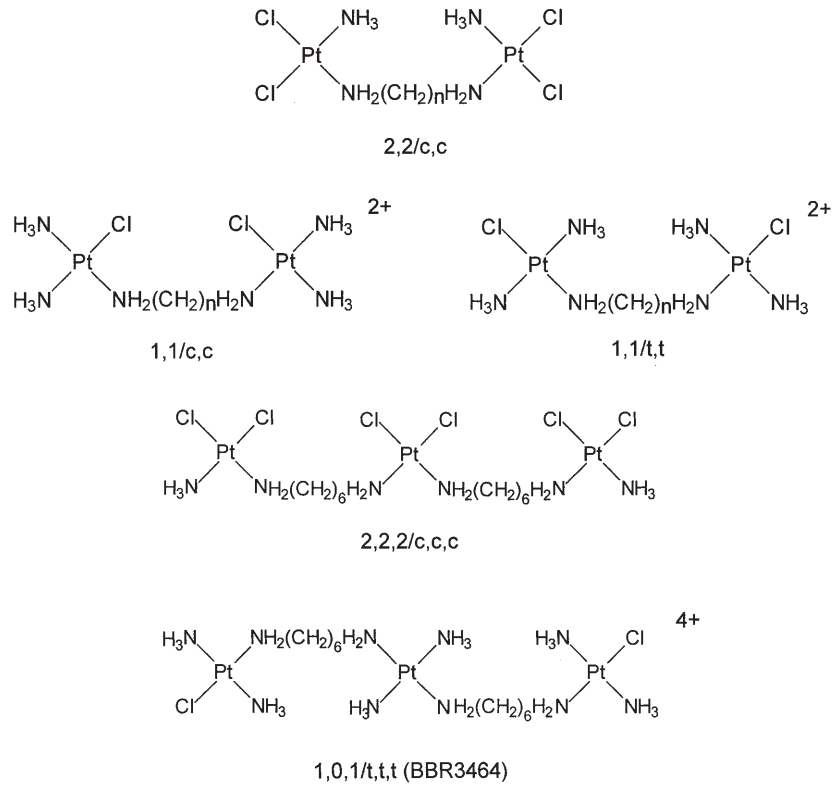


Fig. 1. *Structures of dinuclear and trinuclear compounds containing the cisplatin synthon* (2,2/*c,c* and 2,2,2/*c,c,c*) *and the principal bifunctional DNA-binding agents studied.* Abbreviations refer to the number of chloride leaving groups on each platinum and their geometry relative to the diamine bridge [22].

the broad spectrum of tumors sensitive to this agent. Within 18 human tumor xenografts studied, BBR3464 also maintains high antitumor activity in a subset of 6 tumors classified as mutant p53 [4]. The 4+ charge, the bifunctional DNA-binding where the binding sites are separated by large distances, and the consequences of such DNA-binding suggest that with this advance the paradigm of cisplatin-based antitumor agents is altered.

Dinuclear and Trinuclear Bifunctional DNA-Binding Compounds

The choice of BBR3464 as a clinical candidate, and the recognition of its exceptional antitumor activity, arose from systematic studies on the broad class of dinuclear and trinuclear platinum compounds. It is therefore useful at this point to review the structure-activity relationships that have been found within this new class of antitumor agents. The structures in *Fig. 1* represent a formal array of potential DNA-binding modes from bifunctional to hexafunctional, with parallel increasing level of complexity. This review summarizes di- and trinuclear compounds capable only of *bifunctional* DNA-binding. In this manner, chemical and biological comparisons with the classical mononuclear *cis*- and *trans*-$PtCl_2(NH_3)_2$ can be highlighted wherever possible. The two limiting modes of DNA-binding (*Fig. 2*) are (Pt,Pt) interstrand and (Pt,Pt) intrastrand crosslinks. The frequency and

Table 1. *Comparison at Maximum Tolerated Dose of BBR 3464* (0.2–0.4 mg/kg) *and Cisplatin* (3–6 mg/kg) *after* i.v. *Repeated Treatment on Human Solid Tumors*

Clinical parameter[a])	BBR 3464	Cisplatin
Resistance, TWI < 50%	0	9 (4 NSCLC, 2 ovarian, 2 gastric, 1 prostatic)
Relative resistance (Responsive) TWI 50–70%	3 (1 NSCLC, 1 gastric, 1 prostatic)	7 (2 SCLC, 2 NSCLC, 2 ovarian, 1 bladder)
Sensitivity, TWI > 70%	15 (3 SCLC, 5 NSCLC, 5 ovarian, 1 gastric,1 bladder)	2 (1 ovarian, 1 SCLC)

[a]) TWI% is Tumor Weight Inhibition compared to controls. SCLC: small-cell lung cancer. NSCLC: non-small-cell lung cancer. The clinical parameter refers to the fact that clinical resistance, relative resistance, and sensitivity are most likely to be seen at these TWI levels; a drug achieving a TWI% of < 50% is unlikely to have significant clinical efficacy in that tumor type. Thus, of 18 tumors tested, 15 appear sensitive to BBR3464 while 3 are responsive. In the same panel of 18 tumors, only two are truly sensitive to cisplatin. The sensitivity of tumors difficult to treat in the clinic, such as gastric and bladder, is greater to BBR3464 than to cisplatin. Note that the maximum tolerated dose of BBR 3464 is significantly less than that of cisplatin, approximately thirtyfold on a molar basis. Most direct cisplatin structural analogs need significantly higher doses to achieve similar potency to the parent drug.

structure of these adducts will be affected by geometry and nature of the coordination sphere as well as chain length and steric effects within the linker group. Cross-links between opposite strands have been found where the guanines are either located on neighboring base pairs (1,2-interstrand) or are separated by one or two base pairs (1,3- and 1,4-interstrand, respectively) [5].

In this program, the biological testing has been performed with *Boehringer Mannheim Italia*, now *Roche-Boehringer Mannheim*. The goals of any platinum drug development must be to obtain a wider spectrum of anticancer activity and to retain activity in cisplatin-resistant tumors. The general approach has been to *i*) obtain cytotoxicity and *in vivo* data in murine L1210 and human ovarian A2780 models both sensitive and resistant to cisplatin; *ii*) extend most promising compounds to testing in other human solid-tumor xenografts. This approach has the advantage of obtaining appropriate pharmacological data on effective and toxic doses on the murine L1210 animals and on the A2780 human ovarian animals, where there is considerable historical precedent for cisplatin in both cases. *Table 2* shows how the cytotoxicity in murine leukemia, sensitive and resistant to cisplatin, varies with the nature of the dinuclear and trinuclear compound. Remarkably, L1210 continues to be a good prognosticator for activity in the human tumor systems. Values for cytotoxicity vary with time of incubation. *Table 2* is presented to show trends across the various structures for compounds assayed under identical conditions.

Structure-Activity Relationships for Dinuclear Platinum Compounds

Geometric Isomerism

The classical structure-activity relationship for mononuclear platinum compounds is that only the *cis*-geometry is antitumor active. Most recently, this statement has been found to be strictly true only for the pair of $PtCl_2(NH_3)_2$ isomers [6]. A fascinating general difference in biological activity is seen for the geometric isomers in dinuclear chemistry. The biological activity is of interest because the 1,1/*c*,*c* compound is, if anything, more potent than its *trans* counterpart, but does not overcome resistance to cisplatin (*Table 2*). Thus, unlike the mononuclear case, where one isomer is antitumor-active and the other not, both dinuclear isomers are antitumor-active but are differentiated by their activity in cisplatin-resistant cells. This unusual feature is a powerful one for studying the structural factors determining activity in cisplatin-resistant cell lines. Resistance in L1210 has been attributed to a combination of diminished uptake (or more rapid efflux) of

the drug and enhanced DNA repair of the Pt-DNA adducts formed. Cellular pharmacology studies confirmed that the cellular accumulation of both dinuclear isomers was diminished in L1210/DDP (the resistant cell line) but the level of DNA-binding was greater for the 1,1/*t*,*t* adducts. This result implied that the structures of the DNA adducts formed by the two isomers are

Table 2. *Structure-Activity Relationships in Dinuclear and Trinuclear Platinum Compounds Reflecting Effects of Linker, Chain Length, and Coordination Sphere on Cytotoxicity in Murine Leukemia Cells Sensitive (L1210/0) and Resistant (L1210/DDP) to Cisplatin* [a])

Compound	L1210/0	L1210/DDP	RF
cis-DDP	0.43	12	27.9
$PtCl_2$(en)	1.25	> 20	> 16
Flexible Diamine $NH_2(CH_2)_nNH_2$ Linkers [b])			
1,1/*c*,*c*/NH_3 (*n* = 4)	0.25	2.5	10
1,1/*c*,*c*/NH_3 (*n* = 6)	0.24	2.6	10.8
1,1/*c*,*c* (en) (*n* = 6)	1.2	16.8	14
1,1/*t*,*t*/NH_3 (*n* = 2)	4.7	13	2.8
1,1/*t*,*t*/NH_3 (*n* = 3)	5.6	15	2.8
1,1/*t*,*t*/NH_3 (*n* = 4)	4.7	1.4	0.3
1,1/*t*,*t*/NH_3 (*n* = 5)	3.5	2.8	0.8
1,1/*t*,*t*/NH_3 (*n* = 6)	3.03	2.4	0.7
1,1/*t*,*t*/py (*n* = 4)	> 15	> 15	–
Sterically Rigid Linkers [c])			
cis-1,4-dach	17.6	30	1.8
trans-1,4-dach	9	31	3.4
TETMET	13	13	1
thiourea (*n* = 2)	4.15	39.6	9.5
thiourea (*n* = 6)	6.93	56.63	8.2
Hydrogen-Bonding Linkers			
Spermidine [d])	0.41	0.02	0.05
Spermine	0.60	0.14	0.23
1,0,1/*t*,*t*,*t* (BBR 3464)	0.19	0.12	0.63

[a]) Data obtained according to [24]. Data may vary slightly from previously published numbers due to effect of averaging all repetitions and slightly different exposure times. Nevertheless, the purpose of the table is to show trends, which are consistent across the range of compounds studied. ID_{50} (μM) after 72-h continuous exposure. Resistance factor defined as ID_{50} (L1210/0) / ID_{50} (L1210/DDP).

[b]) Abbreviation refers to nature of inert group (NH_3, py *etc*.) and chain length of diamine backbone. Thus 1,1/*t*,*t*/NH_3 (*n* = 2) is specifically $[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_2NH_2]^{2+}$. Counter-ions (usually nitrate) omitted for clarity.

[c]) See structures in *Figs. 4* and *5*.

[d]) From [23].

different and that the tolerability with respect to DNA repair varied with structure.

DNA-Binding of Dinuclear Bifunctional Compounds. The DNA-binding profiles of $[\{cis\text{- or } trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_n NH_2]^{2+}$ (n = 4, 6) differ in important respects. Both dinuclear compounds bind to DNA more rapidly than cisplatin but steric effects result in diminished binding to Calf Thymus DNA for the 1,1/*c*,*c* isomer relative to the 1,1/*t*,*t* isomer. The binding of dinuclear platinum complexes to poly(dG-dC)·poly(dG-dC) (representing possible interstrand crosslinks) is enhanced relative to polydG··polydC (representing intrastrand crosslinks). Both isomers induce the B → Z transition in poly(dG-dC)·poly(dG-dC) [7]. This transition is irreversible and is dependent on the presence of interstrand crosslinks [8]. This feature is again in contrast to that observed for cisplatin. DNA-DNA interstrand cross-linking is very efficient for both agents but sequencing studies indicated that only the 1,1/*t*,*t* derivative is capable of forming a (Pt,Pt) intrastrand cross-link (*Fig. 2*) to the adjacent guanines of a d(GpG) sequence [5]. Thus, the 1,1/*c*,*c* isomer is a 'pure' interstrand crosslinking agent. This was an important result because it was the first demonstration that DNA adducts

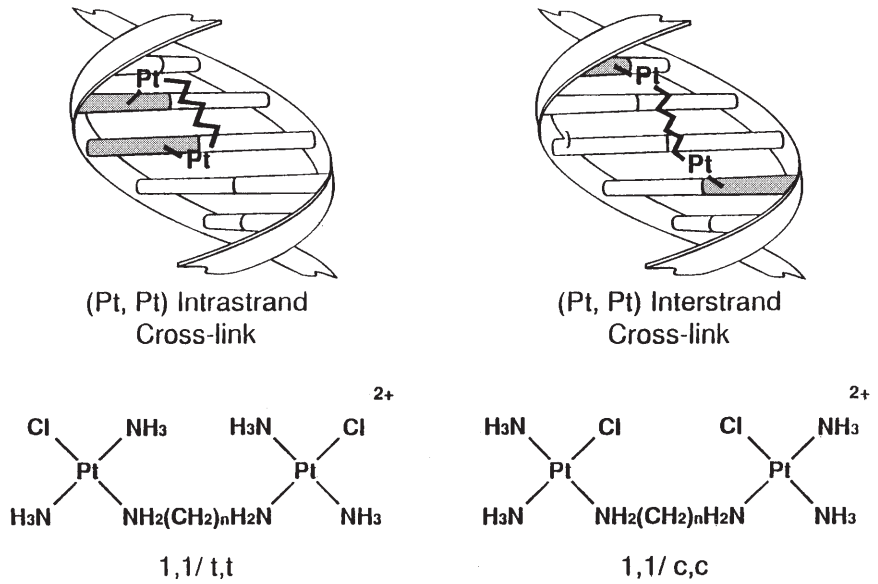


Fig. 2. *Schematic limiting binding modes for a di- or trinuclear bifunctional DNA-binding compound*. The 1,1/*t*,*t* geometry forms both types of adduct; the 1,1/*c*,*c* (n = 4, 6) forms only interstrand cross-links. Long-range intrastrand cross-links can, however, occur for a species such as BBR 3464.

structurally unique from those formed by cisplatin could lead to cytotoxicity and antitumor activity.

Hydrolysis of Dinuclear Platinum Compounds. The rate of binding to DNA of the 1,1/*c*,*c* and 1,1/*t*,*t* geometric isomers is different. The generally accepted understanding of Pt-DNA-binding is that hydrolysis of Pt-Cl bonds to the aqua Pt-H_2O species must first occur, followed by rapid displacement of the labile aqua ligand. For cisplatin, the mechanism of action is generally accepted to involve activation *via* hydrolysis inside cells where the Cl^- concentration is much lower (*ca*. 4 mM) than outside cells (104 mM). The nature of the ligands and their geometry affect hydrolysis of Pt-Cl bonds. It is possible that chloride hydrolysis *trans* to a primary amine (as in 1,1/*t*,*t* compounds) is significantly different from that of Cl *trans* to NH_3. The high affinity of dinuclear compounds for DNA, with a rapid electrostatic interaction because of charge effects coupled with a slow hydrolysis, could imply that DNA-binding does not require activation by hydrolysis, a clear difference to the cisplatin case and with important implications for further drug development. The rigorous kinetic analysis of hydrolysis of dinuclear compounds is complicated somewhat by the fact that the coordination spheres act independently:

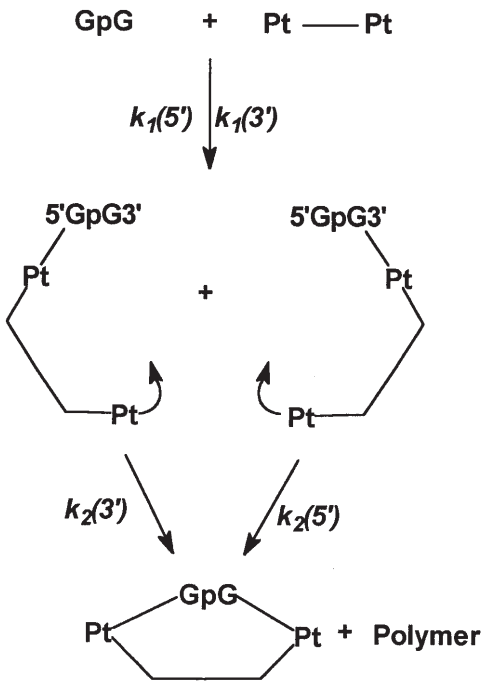
$$\begin{aligned}&[\{PtCl(NH_3)_2\}_2\mu\text{-}H_2N(CH_2)_nNH_2\{PtCl(NH_3)_2\}]^{2+} \rightarrow\\ &[\{Pt(H_2O)(NH_3)_2\}_2\mu\text{-}H_2N(CH_2)_nNH_2\{PtCl(NH_3)_2\}]^{3+} \rightarrow\\ &[\{Pt(H_2O)(NH_3)_2\}_2\mu\text{-}H_2N(CH_2)_nNH_2\{Pt(H_2O)(NH_3)_2\}]^{4+}\end{aligned} \qquad (1)$$

Nevertheless, an estimate of initial rates of aquation may be obtained by standard potentiometric measures such as Cl^- release followed by ion-specific electrodes and conductivity measurements. For all dinuclear compounds we have studied, a rapid loss of Cl^- within the first hours is always observed, followed by a levelling off as the released chloride inhibits further hydrolysis. A rapid increase in conductivity is also observed during the first 30 min, but after *ca.* 120 min the conductivity is invariant. Linear regression analysis shows that the rate of hydrolysis is 1,1/*t*,*t* ($n = 4$) > 1,1/*t*,*t* ($n = 6$) > 1,1/*c*,*c* ($n = 4$) > 1,1/*c*,*c* ($n = 6$) with pseudo-first-order rate constants in the range of 10^{-5} s^{-1}. Early hydrolysis studies on the series $[PtCl_n(NH_3)_{4-n}]^{(2-n)+}$ gave hydrolysis rates (for the first chloride) varying from 0.62 to 9.8×10^{-5} s^{-1} [9]. Thus, no major differences are expected in aquation properties of Pt-Cl bonds *trans* to a primary amine relative to NH_3 – if anything, hydrolysis is slightly faster in the former case. This behavior is in contrast to the recent results confirming slow hydrolysis of $[PtCl(dien)]^+$ (dien = diethylenetriamine) [10]. The observed differences in rates of DNA-binding of the geometric isomers then, especially on template double-strand-

ed DNA, must be due to steric or orientation effects as the cationic molecule approaches the negatively charged backbone of the polymer.

Model Studies of DNA-Binding. Model studies with mononucleotide 5′-GMP and small oligonucleotides have been very useful in understanding structural features of Pt-DNA-binding. HPLC studies using GGCC and GCGC tetranucleotides, at room temperature and therefore existing as single-stranded species, confirmed the preference for the alternating purine-pyrimidine sequence as measured by the rate of disappearance of the free tetranucleotide. The 1,1/*t*,*t* (n = 4) derivative reacted faster than its 1,1/*c*,*c* analog but the presence of multiple product peaks precluded extensive analysis of these systems. Further, HPLC studies and estimates of DNA-binding by atomic absorption cannot readily distinguish the contribution of individual binding steps to the overall rate. ^{1}H-NMR spectroscopy has been very useful in this regard and in model studies with dinucleotides (as a model for the Pt,Pt intrastrand crosslink) individual binding steps have been identified in favorable cases [11]:

Scheme



From ^{1}H-NMR studies, initial rapid binding to 5′-GMP with short $t_{1/2}$ values of 3.1 h (1,1/*c*,*c* ($n = 4$)); 3.6 h (1,1/*c*,*c* ($n = 6$)); 2.4 h (1,1/*t*,*t* ($n = 4$)) and 1.9 h (1,1/*t*,*t* ($n = 6$)) confirm the differences between the two isomers. For the dinucleotide r(GpG) at 37 °C , binding was also slower for 1,1/*c*,*c* compared to 1,1/*t*,*t* confirming the observation with double stranded DNA [12]. However, the second binding step (adduct closure, $k_2(3')$ or $k_2(5')$ above) was actually *faster* for 1,1/*c*,*c* (*Scheme*). The Cl-Cl distances have been calculated from molecular modelling as 16.40 Å and 13.05 Å for the 1,1/*t*,*t*, and 1,1/*c*,*c* (n= 6) isomers, respectively. The shorter distance of the *cis*-isomer, and thus reduced flexibility, presumably will aid in the monofunctional/bifunctional closing step. ^{1}H-NMR spectroscopy showed that, in the absence of a complementary strand, 1,1/*c*,*c* ($n = 4, 6$) does form the 1,2-intrastrand adduct with r(GpG), d(GpG), and d(TGGT). The ^{1}H-NMR spectra of the 1,1/*c*,*c* adducts at 37 °C show two H(8) signals, one of which is broad and becomes sharper on increasing the temperature, indicating restricted rotation around the Pt-N(7) bond.

This steric hindrance may explain the inability of 1,1/*c*,*c* complexes to form 1,2- intrastrand adducts with sterically more demanding double-stranded DNA [7]. More recent studies have shown that, whereas 1,1/*t*,*t* compounds form predominantly long-range cross-links between guanines on opposite strands [13], there is greater structural diversity for 1,1/*c*,*c* compounds. *Maxam-Gilbert* sequencing shows short-range crosslinks and even interstrand crosslinks between a guanine and a cytosine on the *same* base pair, similar to that observed for *trans*-$PtCl_2(NH_3)_2$ [14]. In relation to biological activity, a plausible explanation for the differences in the geometric isomers is, therefore, that, whereas interstrand crosslinks contribute to cytotoxicity in L1210/0 (sensitive to cisplatin), the sterically hindered adducts formed by 1,1/*c*,*c* are more easily repaired in L1210/DDP, resulting in a reduced ability to circumvent cisplatin resistance.

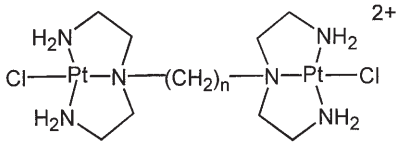
Ethylenediamine-Based Compounds. To further examine steric effects within the 1,1/*c*,*c* geometry the ethylenediamine-based compound was synthesized:

$$2\,[PtCl_2(en)] + H_2N(CH_2)_nNH_2 \rightarrow [\{PtCl(en)\}_2H_2N(CH_2)_nNH_2]^{2+}\ (n = 4, 6) \qquad (2)$$

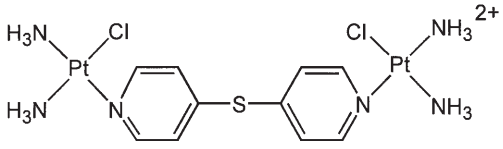
The properties are similar to the NH_3 analog with similar cytotoxicity. Interestingly enough, the hydrolysis products of the en derivative still show NH signals of the linker $H_2N(CH_2)_nNH_2$ in the ^{1}H-NMR spectrum, even after 16 h in D_2O, indicating that these must be protected from solvent due to the presence of the ethylenediamine rings. Reaction with 5′-GMP gives one major product $[\{Pt(5'\text{-GMP})(en)\}_2H_2N(CH_2)_nNH_2]$ with an apparent half-

life for reaction of *ca.* 4 h, not significantly different from that of the NH_3 compound. In D_2O, the diamine NH protons are not observed in the 5′-GMP adduct. The reasons for this are still not clear, but NH-O hydrogen bonding from the amine ligands to phosphate oxygen of the mononucleotide ligand may assist in catalyzing H-D exchange.

Miscellaneous Dinuclear Bifunctional DNA-Binding Agents. A number of similar compounds have been prepared by other groups, extending the structural diversity of the dinuclear class (*Fig. 3*) [15–17]. Extensive antitumor data has only been reported for the Pt(dien)-linked compounds (*Fig. 3,a*), which were disappointingly inactive [15]. The absence of any major cytotoxicity of the Pt^{II}(dien)-linked compounds is somewhat surprising considering their close similarity to the systems developed in our laboratories. It is possible that the tertiary amine involved in Pt binding is unstable in so-



(a)



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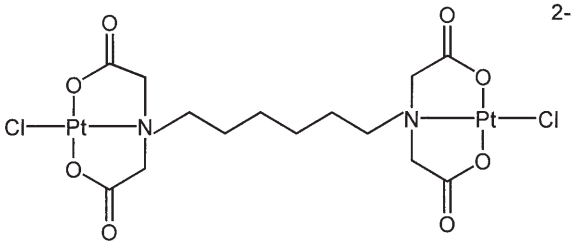

(c)

Fig. 3. *Miscellaneous bifunctional DNA-binding dinuclear compounds reported for their potential antitumor activity*

lution (even as part of a chelate), and rapid breakdown may occur in media and/or serum.

Alternative Non-Amine Linkers

The flexibility of the alkanediamine linkers in dinuclear platinating agents produces a broad array of adducts on random sequence DNA. The multiplicity of adducts formed complicates the detection and structural characterization of a specific lesion on double-stranded DNA. In order to control the sequence specificity of the DNA-targeting drugs more efficiently, alternative linkers exhibiting increased conformational rigidity are required. To examine this question, the spectrum of linkers in dinuclear compounds was extended to bifunctional thiourea derivatives. In solution, *N*,*N*′-disubstituted thioureas exist as an equilibrium mixture of (*E*)- and (*Z*)-conformers due to the pronounced double-bond character of the C(sp^2)-N bonds. Mono- and dinuclear Pt^{II} complexes have been described that derive from the cisplatin analogues $PtCl_2$(en) and $PtCl_2$(dach) (dach = racemic *trans*-cyclohexane-1,2-diamine) [18][19]. Substitution of one chloro ligand in these species by *N*,*N*,*N*′,*N*′-tetramethylthiourea (tmtu) gave $[PtCl(en)(tmtu)]^+$ and $[PtCl(dach)(tmtu)]^+$ [18]. The nucleotide-binding and biological properties of these cationic complexes are strongly suggestive of classical monofunctional chloro-am(m)ine complexes. In model reactions that mimic DNA-binding, chloride is readily displaced by nucleobase nitrogen whereas sulfur-bound thiourea acts as a typical nonleaving group [18]:

$$[PtCl(diamine)(tmtu)]^+ + 5'\text{-GMP} \rightarrow [Pt(5'\text{-GMP-N(7)})(diamine)(tmtu)] + Cl^- \quad (3)$$

$$[Pt(5'\text{-GMP-N(7)})(diamine)(tmtu)] + 5'\text{-GMP} \rightarrow \text{no reaction} \quad (4)$$

^{195}Pt-NMR chemical shifts in the –3000 ppm region, indicative of a $[N_3S]$ mixed-donor environment of platinum, confirm the monofunctional nature of the above Pt-nucleobase adducts. This ultimately prevents the formation of a bifunctional (cytotoxic) lesion on target DNA, in accordance with the lack of *in vitro* cytotoxicity found in L1210 leukemia cells (ID_{50} > 50 μM) [18].

Peralkylated (**I** and **II**) derivatives have been synthesized that act as bridging ligands in analogous dicationic dinuclear complexes (*Fig. 4*) The effective chain lengths (population of rotamers) and flexibility (rotational barriers, nature of thiourea bridging unit) in these species depend on the degree of alkylation and the nature of the substituents on nitrogen. Reactions

employing the bifunctional thiourea derivatives (**I** and **II**, *Fig. 4*) gave the dinuclear complexes $[\{PtCl(en)\}_2(\mu\text{-}\mathbf{I}\text{-}S,S')]^{2+}$ and $[\{PtCl(en)\}_2(\mu\text{-}\mathbf{II}\text{-}S,S')]^{2+}$, respectively, which were isolated as their nitrate salts [19]. ^{195}Pt-

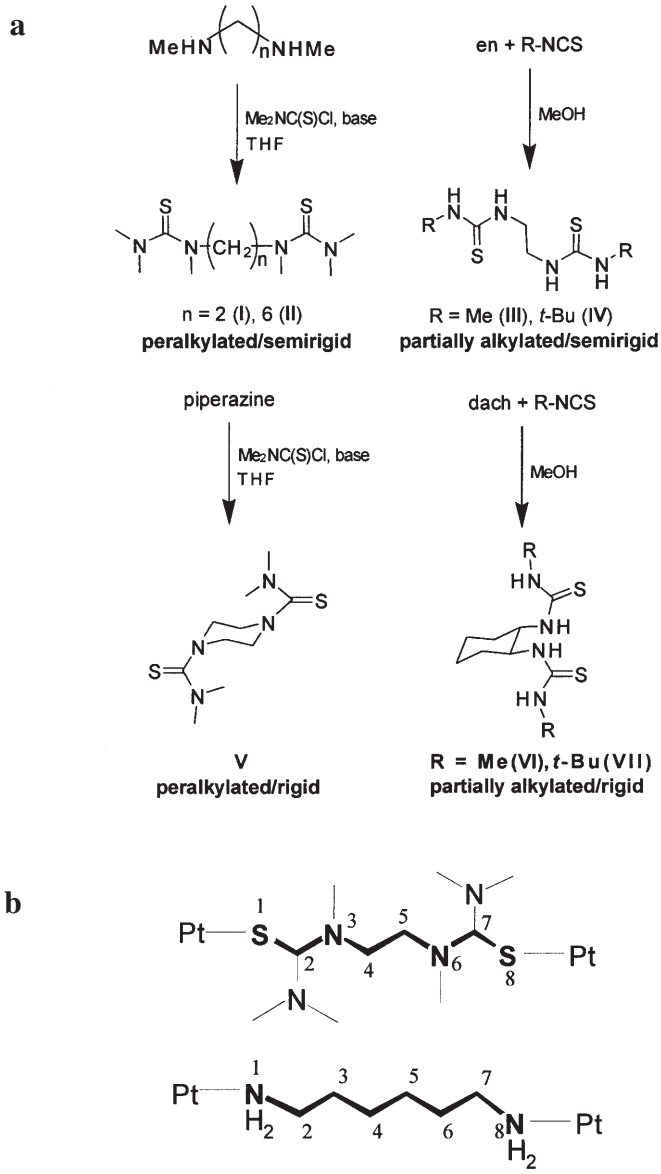

Fig. 4. a) *Peralkylated semi-rigid thiourea bridging groups used in dinuclear chemistry* (**I**, **II**) *and possible approaches to rigid groups* (**III**–**VII**). b) *Distance similarities between a thiourea and diamine bridging group.*

NMR chemical shifts around the –2900 ppm region confirm the mixed-donor [PtN_2ClS] coordination and S-thiourea coordination in both cases. The bifunctional thiourea derivatives **I** and **II** adopt highly elongated conformations in the solid state where the sulfur atoms and the N-$(CH_2)_n$ (n = 2, 6) linkers are (*Z*)-oriented (*i.e.*, S and bis- and hexamethylene linker are *cis*-oriented with respect to the CN bonds).

A drawback in the development of thiourea-bridged dinuclear platinum complexes is the fact that these decompose in solution, giving $PtCl_2$(en) and [Pt(en){bis(thiourea)-*S*,*S*′}]$^{2+}$ (intramolecular 'disproportionation') [20]. For the **II**-based complex this reaction is slow ($t_{1/2}$ = 12 h at 310 K). Formation of a macrochelate containing an intact dinuclear platinum entity has been observed in the model reaction between [{PtCl(en)}$_2$(μ-**II**-*S*,*S*′)]$^{2+}$ and r(GpG), thus simulating a 1,2-intrastrand cross-link on DNA [20]. However, the compound is likely to produce its cytotoxicity through its decomposition product [$PtCl_2$(en)], in accordance with the observed inefficacy of the **II**-based derivative in cisplatin-resistant cells (*Table 2*). To reduce decomposition caused by platinum-bis(thiourea) chelation, new derivatives containing a rigid aliphatic spacer, unfavorable for chelate formation on a single Pt center, are required (*Fig. 4*, **III**–**VII**). For such species, an activity spectrum similar to that of the complexes of the 1,1/*t*,*t* series may result, due to enhanced drug stability and formation of structurally similar long-range cross-links on DNA.

Structure-Activity Relationships in the 1,1/*t,t* Series

Chain Length and Steric Effects. Early Structure-Activity Relationships

Since the 1,1/*t*,*t* series based on alkanediamine bridging units gave consistently better antitumor activity in cisplatin-resistant cells, development concentrated on this series. Shorter chain lengths (n = 2, 3) gave poor results but reasonable cytotoxicity, and indeed *in vivo* antitumor activity is seen with $n > 4$ (*Table 2*). Note also that resistance factors of < 1, implying enhanced activity in cisplatin-resistant cells is seen at $n > 4$. In general *in vivo* antitumor activity was greater for the straight-chain diamine $n = 6$. Steric effects within the diamine linker were examined by the linkers shown based on butane-1,4-diamine (*Fig. 5*). Molecular modelling showed that use of the sterically hindered *cis*-cyclohexane-1,4-diamine could produce only the (Pt,Pt) intrastrand crosslink whereas the *trans*-cyclohexane-1,4-diamine isomer could give both interstrand and intrastrand cross-links. The compounds were thus prepared, but the cytotoxicity was very disappointing and significantly less than that of the straight-chain butane-1,4-diamine

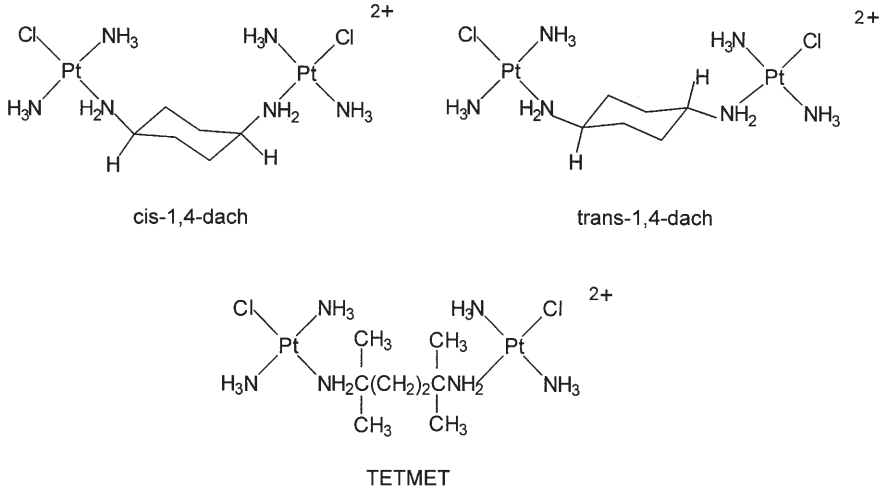

Fig. 5. *Steric hindrance incorporated into bifunctional dinuclear compounds of the 1,1/*t,t *series*

(1,1/*t*,*t*/NH_3 (*n* = 4), *Table 2*). While differences are observed for the two isomers, the activity did not warrant detailed investigation. It is likely that the sterically hindered compounds bind more slowly to DNA, especially in the second bifunctional step – the persistence of monofunctional adducts, as in the mononuclear case, will result in easier excision from DNA.

Effects of Coordination Sphere: Structural Factors Affecting B → Z Conformational Changes

Dinuclear platinum compounds with short chain lengths are poorly cytotoxic. DNA-Binding studies indicated that they can induce the B → Z transition, but they are poor (Pt,Pt) interstrand cross-linking agents. These observations suggested that the ability to form (Pt,Pt) interstrand cross-links and also induce the B → Z transition was a prerequisite for good antitumor activity. A relevant finding is that the presence of planar ligands in the coordination sphere stabilizes the B form of poly(dG-dC)·poly(dG-dC) [5][21]:

$$\textit{trans}\text{-}[PtCl_2(pyr)_2] + H_2N(CH_2)_nNH_2 \rightarrow [\textit{trans}\text{-}\{PtCl(pyr)_2\}_2\, H_2N(CH_2)_nNH_2]^{2+} \quad (5)$$

$$\textit{trans}\text{-}[PtCl_2(NH_3)(quin)] + H_2N(CH_2)_nNH_2 \rightarrow [\textit{trans}\text{-}\{PtCl(NH_3)(quin)\}_2 H_2N(CH_2)_nNH_2]^{2+} \quad (6)$$

Both pyridine (pyr) and quinoline (quin) compounds are efficient cross-linking agents but not potent cytotoxic agents. Interstrand cross-linking is thus not by itself a sufficient requirement for cytotoxicity. These results were also of considerable interest because the mononuclear precursors were found to be similar in cytotoxicity to cisplatin itself, violating the classical structure-activity relationship of platinum compounds. Yet incorporation into the dinuclear structure did not produce active compounds!

Long-Range Cross-Linking Agents Containing Hydrogen-Bonding Capacity in the Linker Chain

BBR3464, A Novel Trinuclear Agent

The results summarized above clearly showed that straight-chain flexible diamines linking two *trans*-$[PtCl(NH_3)_2]^+$ moieties gave the most potent compounds. Models showed that the 1,1/*t*,*t* ($n = 6$) compound could easily span a four-base pair segment of DNA. Simple extension of an aliphatic diamine chain results in lack of aqueous solubility after $n > 8$. To improve water solubility and to further improve DNA affinity for long-range cross-linking, some H-bonding capacity in the backbone is desirable for reaction with the negatively-charged DNA backbone. These features were achieved by preparation of a novel trinuclear compound, which arose from our initial reports on trinuclear systems [2][22]. The central platinum coordination sphere is different from the terminal units (see *Fig. 1*). In general, linear trinuclear platinum compounds are prepared by linking a precursor central molecule, Pt(*c*), to two equivalents of a target terminal platinum coordination sphere, Pt(*t*):

$$2\ \mathrm{Pt}(t) + \mathrm{Pt}(c) \rightarrow \mathrm{Pt}(t)\text{-}\mathrm{Pt}(c)\text{-}\mathrm{Pt}(t) \qquad (7)$$

Specifically for BBR 3464:

$$2\ \textit{trans}\text{-}[PtCl(DMF)(NH_3)_2]^+ + \textit{trans}\text{-}[Pt(NH_3)_2\{H_2N(CH_2)_6NH_3\}_2]^{4+} \rightarrow \textit{trans}\text{-}[\{\textit{trans}\text{-}PtCl(NH_3)_2\}_2\mu\text{-}\{H_2N(CH_2)_6NH_2\}_2Pt(NH_3)_2]^{4+} \qquad (8)$$

(BBR 3464)

The total charges on the compounds reflect the need to neutralize not only the cationic platinum coordination spheres but also the protonated dangling amines of the central platinum precursor.

Initial cytotoxicity data indicated a remarkable activity for this agent, and subsequent exhaustive analysis (*Table 1*) confirmed its promise. As stated, Phase-I trials are currently (1998–1999) being carried out. Note that the

optimal dose of this 4+ compound is at least tenfold lower than that of cisplatin, a remarkable finding considering the previous emphasis on the necessity for neutrality in platinum anticancer agents. The isomers of BBR 3464 such as 1,0,1/*t*,*c*,*t* where the central platinum atom is in the *cis* geometry are also, as might be expected, antitumor active. The hydrolysis chemistry of BBR3464 is similar to that of the dinuclear compounds (*Sect. 2.1.2*) but the extra charge produces a remarkably high DNA affinity, presumably by the contribution of the central unit through electrostatic and H-bonding interactions.

An important point to note is that, in our detailed examination, the *profile* of antitumor activity of a simple dinuclear compound such as 1,1/*t*,*t* (*n* = 6) is generally similar to that of BBR 3464 (activity in cisplatin-resistant cell lines, enhanced activity in solid tumors classified as mutant p53 [3]) – what differs is the potency, producing the remarkable profile in *Table 1*. In the absence of major differences in aqueous chemistry the dramatically enhanced antitumor activity of BBR 3464 may be ascribed to this enhanced DNA affinity.

Polyamine-Linked Dinuclear Compounds

An immediate question relating to the structure-activity relationships within this class of compounds is whether it is necessary to contain a third Pt unit (trinuclear class of compounds) in the molecule to achieve this remarkable antitumor potency. The most closely related compounds to BBR 3464 would contain a diamine backbone with some hydrogen-bonding capacity. This was achieved through synthesis of dinuclear platinum complexes with hydrogen-bonding ligands based on spermine (total charge 4+) and spermidine (total charge 3+) linkers [23]. Platination of the terminal NH_2 group is achieved by selective blocking and deblocking of the secondary nitrogens (*Fig. 6*). Upon complexation, the Boc group may be displaced readily by dilute acid. Designed synthesis of dinuclear platinum complexes with hydrogen-bonding ligands such as spermine (total charge 4+) and spermidine (total charge 3+) linkers mimics the essential biological features of BBR 3464 [23].

Summary

This review summarized our systematic studies on dinuclear platinum compounds leading to identification of a novel clinical agent BBR 3464. The profile of antitumor activity is shared by the general structure with po-

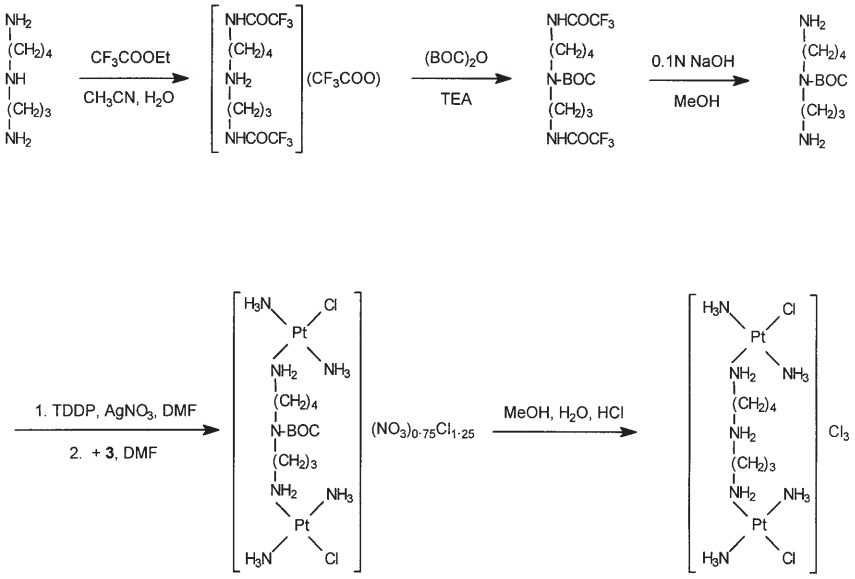

Fig. 6. *Scheme for selective platination of polyamines such as spermidine*

tency being dictated by factors such as charge and chain length. Di- and trinuclear complexes incorporating the principal features of *trans*-Pt monofunctional coordination spheres, separated by a long-chain, flexible linker capable of hydrogen-bonding, and with an overall charge of 3+ or 4+ are now *expected* to reproduce high antitumor activity, activity in p53-mutant tumors, and a predictable DNA-binding profile. This profile is characterized by rapid binding to DNA, long-range inter- and intrastrand cross-links, and the ability to irreversibly induce the left-handed Z conformation in appropriate sequences. Thus, the Pt-DNA adducts are clearly not those found for cisplatin. This work has implications for how we view the future development of platinum-based clinical agents, confirming our hypothesis that new, clinically relevant agents not based on the classic cisplatin structure can be found.

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The Development of Orally Active Platinum Drugs

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A collaborative programme of research was established in the late 1980's between *Johnson Matthey*, the *Institute of Cancer Research* and, until 1994, *Bristol Myers Squibb* to further extend the improvement in quality of life for cancer patients receiving cisplatin-based chemotherapy offered by the less toxic analogue, carboplatin. Its aim was to discover and develop an orally active platinum drug possessing at least comparable antitumor activity to that of cisplatin but a toxicological profile reminiscent of carboplatin. A new class of Pt^{IV} compounds, the mixed ammine/amine dicarboxylates, synthesized specifically to circumvent the poor gastrointestinal absorption of cisplatin and carboplatin possessed the desired properties of relatively low molecular weight, lipophilic, neutral, kinetically inert and acid stable. The resulting lead compound, JM216 (bis(acetato)amminedichloro(cyclohexylamine)platinum (IV)) entered clinical trial at the *Royal Marsden Hospital*, London, in 1992. Preclinically, JM216 was demonstrated to possess oral antitumor activity in mice broadly equivalent to that observed for intravenously administered cisplatin or carboplatin and a toxicological profile similar to that of carboplatin. The metabolism of JM216 is complex with up to six metabolites being formed; the major metabolite in man being *cis*-amminedichloro(cyclohexylamine)platinum(II) (JM118). Although absorption-limited non-linear pharmacokinetics prevented a maximum-tolerated dose being reached in the initial Phase-I bolus administration trial, a second daily administration for 5 days in Phase I showed dose-limiting toxicities in the form of thrombocytopenia and neutropenia. Phase-II trials are currently ongoing in a number of tumor types including prostate, ovarian and lung. More recently, a second complex, AMD473 [*cis*-amminedichloro(2-methylpyridine)platinum(II)] with improved activity over JM216 against acquired cisplatin-resistant tumors, oral activity in mice and less complex metabolism has entered Phase-I clinical trial at the *Royal Marsden* under the auspices of the *UK Cancer Research Campaign* Phase I/II Committee.

Platinum Drug Development – the Pathway to Orally Active Drugs

The parent drug, cisplatin, has proved to be one of the most successful anticancer drugs yet developed and, in particular, has made a significant

Cisplatin. Edited by Bernhard Lippert

contribution to improved response rates in patients presenting with testicular and ovarian cancer. However, over the past 30 years, many hundreds, perhaps thousands of analogues have been synthesised: why? This is a reflection of two major limitations of cisplatin, namely, it is one of the most toxic drugs used in man and second, many tumors exhibit resistance, either *ab initio* (*e.g.*, colon and non-small-cell lung cancers) or it is acquired during therapy (*e.g.*, ovarian cancer, small-cell lung cancer). Cisplatin causes a wide range of side effects including kidney toxicity and neurotoxicity and induces severe nausea and vomiting.

Initially, most analogues concentrated upon reducing the toxicity of platinum-based therapy while retaining the therapeutic benefits, thus making its administration easier and potentially extending opportunities for use in combination with other anticancer agents. The first of this second generation of anticancer drugs to be approved was carboplatin, (*Paraplatin*®) first marketed in 1986 [1]. The dose-limiting toxicity of this compound is haematological, and at the normal maximum tolerated dose there is essentially no kidney toxicity and very little neurotoxicity.

Following on from the success of carboplatin, there have been additional similar 'second-generation' less toxic platinum compounds introduced into Phase-I trial. Generally, these have added little to the properties already provided by carboplatin although at least one (254-S, *Nedaplatin*® in Japan) has now received limited marketing approval. However, to date carboplatin is the only second-generation analogue in widespread use.

The clinical trials of carboplatin established that it shows a high degree of cross-resistance with cisplatin [2]. From a mechanistic DNA-binding point of view, this is not too surprising since the adducts produced on DNA by cisplatin and carboplatin are similar (but differ in their relative rates of formation) [3]. While many tumors initially respond to cisplatin treatment, this therapy is often not completely successful such that the tumor re-grows and is then more resistant to the drugs used initially. *Post* carboplatin, additional compounds which do not show cross-resistance with cisplatin has, therefore, become a major goal in the development of platinum drugs.

Cisplatin and all the second-generation platinum drugs are administered by intravenous infusion. The ability to deliver the drug orally would allow much greater flexibility in dosing and increase the potential for the use of platinum drugs, especially in palliative care. However, the physical properties of cisplatin rule out the possibility of an effective oral formulation. While antitumor activity for cisplatin following oral administration can be demonstrated preclinically in mouse tumor models, the low level of absorption makes this impractical at the clinical level [4]. For example, comparing the antitumor efficacy of cisplatin administered either by the intraperitoneal or the oral route to mice bearing the ADJ/PC6 murine plasmacytoma, the drug

was both less toxic and less effective as an antitumor agent when given orally with no gain in therapeutic index (*Table*). Carboplatin and most of the other second-generation compounds have greater water solubility than cisplatin (desirable for an intravenously administered drug of lower potency than cisplatin) and very low organic/aqueous partition coefficients which would be expected to lead to low absorption. Studies in mice revealed an oral bioavailability of only 11–15% for carboplatin with the major (60–80%) proportion of the dose excreted in the faeces [4]. The poor oral bioavailability properties of carboplatin were also confirmed by a brief clinical study, using carboplatin in solution with lemonade syrup, which revealed poor absorption and severe gastrointestinal effects; bioavailability was only 4–5% [5]. The search for oral activity in platinum drugs thus became an area of chemical development distinct from other programmes. This led to the identification of a new class of Pt^{IV} compounds with suitable properties for oral administration and high antitumor activity (see below). The compound selected for clinical evaluation from this class was JM216 (*Fig. 1*). Subsequently, two other platinum drugs, AMD473 and C(5)-OHP-Cl (*Fig. 1*), have been described to possess oral activity at the preclinical level (see below).

The Discovery of JM216, the First Oral Platinum Drug

A pivotal feature of the preclinical lead discovery process for identifying orally active platinum complexes was a comparison of antitumor activity in mice bearing the ADJ/PC6 subcutaneous murine plasmacytoma by the oral *vs.* intraperitoneal (i.p.) routes of administration. The ADJ/PC6 tumor model had been widely used in earlier platinum drug development pro-

Table. *Intraperitoneal* vs. *Oral Antitumor Activity in Mice Bearing the ADJ/PC6 Plasmacytoma for Selected Platinum Agents*[a])

Compound	i.p.		*TI*	oral		*TI*
	LD_{50} mg/kg	ED_{90} mg/kg		LD_{50} mg/kg	ED_{90} mg/kg	
Cisplatin	11.3	0.6	18.8	140	24	5.8
Carboplatin	180	14.5	12.4	235	99	2.4
JM149	17.4	0.4	44	118	18	7
JM216	30	5.7	5.3	330	5.8	56.9
AMD473	43	3	14.3	560	6.2	90.3

[a]) LD_{50}, 50% lethal dose; ED_{90}, dose required to reduce tumor mass by 90%; *TI*, therapeutic index, LD_{50}/ED_{90}. JM149 = *cis*-[$PtCl_2(OH_2)(NH_3)$(c-$C_6H_{11}NH_2$)]; JM216 = *cis*-[$PtCl_2(OCOCH_3)_2(NH_3)$(c-$C_6H_{11}NH_2$)]; AMD473 = *cis*-[$PtCl_2(NH_3)$(2-picoline)].

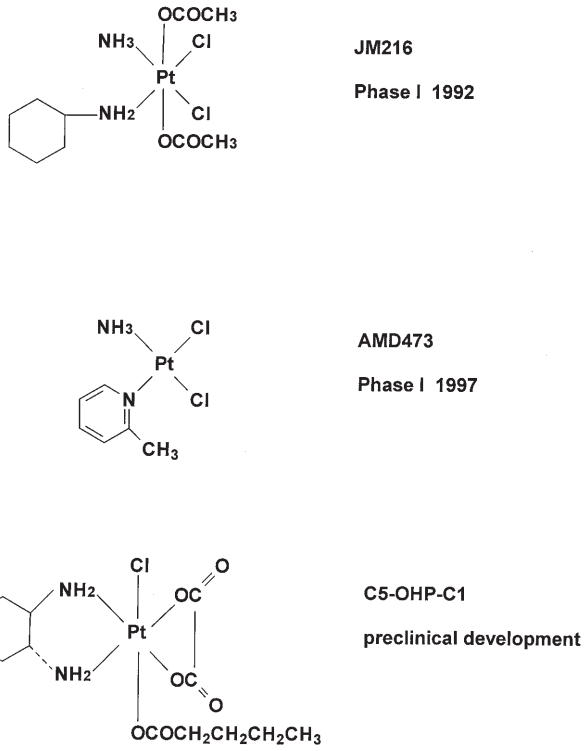

Fig. 1. *Structures of published orally active platinum drugs*

grammes, including in the discovery of carboplatin [6], and is believed to predict well for activity in man. Additional antitumor-efficacy studies were then performed in immune-suppressed mice bearing one of a selected group of subcutaneous human ovarian carcinoma xenografts [7]. These models exhibited a generally good correlation between responsiveness to platinum drugs and corresponding patient-response data and *in vitro* cell-line drug sensitivity [7][8].

Following the initial results with the ADJ/PC6 plasmacytoma model using cisplatin and carboplatin (see above), selected second-generation analogues possessing ammine/amine, so-called 'mixed amine' carrier ligands were evaluated. A typical result is shown in the *Table* for JM149 (*cis*-amminedichloro(cyclohexylamine)dihydroxoplatinum(IV)). As with cisplatin and carboplatin, results clearly demonstrated that, while such compounds are active when given orally, the doses required are significantly higher and that there is some reduction in the therapeutic index (the ratio of a toxic to a therapeutic dose).

Since the amine ligands are an essential feature of the activity of platinum drugs [9], modification of these groups to promote oral absorption is likely to affect the antitumor activity of the complexes. On the other hand, modification of the axial ligands of Pt^{IV} complexes offered the prospect of achieving good absorption without strongly influencing activity, since it is believed that Pt^{IV} complexes are reduced to Pt^{II} compounds (involving the loss of the axial ligands) prior to reaction with DNA [10]. However, Pt^{IV} compounds are inert to substitution under most conditions and thus the synthesis of a suitable range of complexes presented some difficulties. These were overcome by exploiting the inertness of the metal-ligand bonds. Dihydroxoplatinum(IV) complexes are well known, and one such compound CHIP (iproplatin, *cis*-dichlorodihydroxobis(isopropylamine)platinum(IV)) was clinically tested as a potential second-generation drug [11]. The O-atom of the hydroxo ligand retains strong nucleophilic character after bonding to platinum, allowing it to participate in reactions with suitable electrophiles while the inertness of the bond to platinum ensures that the complex remains intact. This reaction allowed the preparation of a class of complexes containing axial carboxylate ligands and greatly increased the scope for synthesising complexes with a wide range of partition coefficients and solubilities.

Reaction of the dihydroxoplatinum(IV) complexes with organic acids was generally unsuccessful in obtaining high yields of the desired product. However, reasonable yields (>50% isolated yield) were obtained for formate complexes. In other cases, conversion of the acid to an active anhydride (*e.g.*, using isobutylchloroformate) or ester (*e.g.*, using *N*-hydroxysuccinimide) generally allowed formation of the desired complex. A reaction with much greater applicability was that of the platinum complex with an acid anhydride as the electrophile. Stirring of the dihydroxo complex in the anhydride as solvent (or using an inert solvent such as hexane for solid anhydrides) at ambient temperature for several hours achieved essentially 100% conversion to the dicarboxylate complex, and this reaction was used to obtain several series of products containing simple alkyl and aryl carboxylate ligands [12]. Carboxylation was also achievable using a variety of other electrophiles such as pyrocarbonates and isocyanates yielding carbonate and carbamate complexes, respectively [12].

It was soon established that, in contrast to the results shown in the *Table* for other ammine/amine Pt^{II} and Pt^{IV} complexes such as JM149, the ammine/aminedicarboxylateplatinum(IV) series generally exhibited no loss of antitumor activity (ED_{90}) by the oral route and substantially lower toxicities resulting in significant improvements in therapeutic indices. A typical result is shown for JM216 itself (bis(acetato)amminedichloro(cyclohexylamine)platinum(IV)) (*Table*) where, in contrast to cisplatin and JM149, the ED_{90} was similar for i.p. *vs.* oral dosing. The therapeutic index (ratio of tox-

ic to antitumor-effective dose) was around tenfold greater for JM216 when given orally compared to intraperitoneally (56.9 *vs.* 5.3). Many dicarboxylates exhibited oral activity in this tumor model; some, such as JM274 (bis(valerato)amminedichloro(cyclohexylamine)platinum(IV)) and JM244 (bis(benzoato)amminedichloro(propylamine)platinum(IV)) exhibited therapeutic indices by the oral route of *ca.* 300 [13][14].

In order to further prioritise a dicarboxylate for selection for phase-I clinical trial, ten compounds shown to be active against the ADJ/PC6 tumor were then also evaluated by oral administration against a panel of 5 human ovarian-carcinoma xenografts [15]. All compounds induced substantial antitumor growth delays (60 days or more) against the cisplatin-sensitive PXN/100 model while only JM244, JM216 and JM221 (bis(butryato)amminedichloro(cyclohexylamine)platinum(IV)) induced growth delays of 10 days or more in the cisplatin refractory SKOV-3 xenograft. Studies with three xenografts of intermediate sensitivity to cisplatin (HX/110, OVCAR-3, PXN/109T/C) showed that JM216, JM225 (bis(acetato)amminedichloro(cyclopentylamine)platinum(IV)), JM269 (bis(acetato)amminedichloro(cycloheptylamine)platinum(IV)) and JM244 were the most active while JM274 was least active.

A final level of selection involved a determination of emesis-inducing properties using the ferret model. Both JM244 and JM221 were shown to be highly emetogenic in the ferret compared to compounds in the acetato series (*e.g.*, JM269, JM216) [14]. Comparative scores (duration of emesis in hours × mean number of episodes following a single oral dose) were 39 for JM244, 85 for JM221, 36 for cisplatin (i.v. administration) but only 4 for JM269, 9 for JM216 and 5 for carboplatin (i.v.). Since emesis in patients was of some concern with orally administered platinum drugs (the 5-HT_3-receptor antagonist antiemetics were not on the market at this time), the more emetogenic JM221 and JM244 were dropped. In contrast, the acetato series was shown to be substantially less emetogenic than cisplatin and comparable to that observed for carboplatin.

Further *head-to-head* oral antitumor studies of the acetato series JM216, JM225 and JM269 *vs.* i.v. cisplatin and carboplatin against four human ovarian carcinoma xenografts were then performed [15][16]. Broadly comparable tumor growth delays to that observed for i.v. cisplatin and carboplatin were obtained, including for JM216 (*Fig. 2*). Further confirmatory antitumor studies were conducted within *Bristol Myers Squibb* with four compounds (JM216, JM225, JM269 and JM251 (bis(formato)amminedichloro(cyclohexylamine)platinum(IV)) using the M5076 murine reticulosarcoma and A2780 human ovarian xenograft [17]. Oral antitumor activity was also observed in these two models, JM216 being the most active of the series against the M5076 sarcoma.

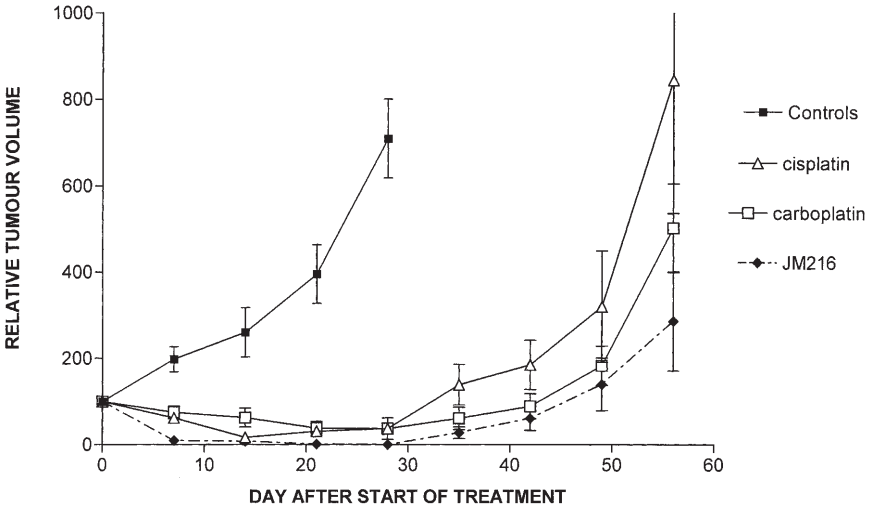

Fig. 2. *Comparative antitumor activity of orally administered JM216, 135 mg/kg q7d×4* (♦), *i.v. administered cisplatin, 3 mg/kg q 7d×4* (△), *and i.v. administered carboplatin, 90 mg/kg q7d×4* (□) *to mice bearing the CH1 human ovarian carcinoma xenograft* (controls, (■))

From the above described antitumor and emesis studies, JM216 was selected on the basis of possessing both good oral antitumor activity against a variety of murine and human ovarian tumor models, a low emesis score in the ferret and favourable physico-chemical properties.

Preclinical Properties of JM216

Chemistry

To confirm the specificity of the synthesis and the analytical procedures for the single isomer JM216, all other isomers of the complex $[PtCl_2(OAc)_2(NH_3)(c\text{-}C_6H_{11}NH_2)]$ were prepared [18]. While there are only *cis*- and *trans*-isomers for square-planar Pt^{II} complexes such as cisplatin, there are six isomers for these octahedral platinum compounds with the two (all-*cis*)-isomers also being optically active. The (all-*trans*)-dihydroxoplatinum(IV) complex may be prepared by oxidation of *trans*-$[PtCl_2(NH_3)(c\text{-}C_6H_{11}NH_2)]$ with aqueous hydrogen peroxide. On treatment with acetic anhydride in the dark, this complex undergoes the simple conversion to the corresponding (all-*trans*)-bis(acetate). However, if the reaction is allowed to continue in light, then an isomerisation takes place to yield the isomer with *cis*-acetate and *cis*-chloride ligands, JM338. Using *cis*-$[PtCl_2(NH_3)(c\text{-}$

$C_6H_{11}NH_2$)] as the starting material, while some intramolecular exchange reduced the yield of the reaction, the desired (all-*cis*)-isomers JM568 and JM2893 could be obtained by non-aqueous oxidation using iodobenzene diacetate. When starting with *trans*-[$PtCl_2(NH_3)$(c-$C_6H_{11}NH_2$)] it was found that the reaction proceeded with rearrangement to give *cis*-amine ligands producing JM394.

JM216 possesses a solubility in water of around 0.3 mg/ml, saline 0.4 mg/ml and octan-1-ol of 0.7 mg/ml (octanol/water partition coefficient of 0.1). A crystal-structure determination for JM216 revealed the expected octahedral coordination around platinum with a number of hydrogen bonds formed within the crystal [19]. The N(1)-Pt-N(2) angle (94.3°) is significantly greater than 90° with corresponding reductions in the N-Pt-Cl angles. Intramolecular interactions occur between both nitrogen atoms and the carbonyl oxygen atoms. The orientation of the cyclohexylamine ligand towards one or other of the acetate ligands may serve as some protection from attack and produce some differentiation in the reactivity of these two groups.

Stability studies with JM216 were conducted in acid, alkali and light. Treatment of JM216 with acids results in protonation of the carboxylate groups and their subsequent substitution. However, the reaction is sufficiently slow (a half-life of several hours in 1M HCl) as to be of little consequence with regard to possible reactions in the stomach prior to absorption. In contrast, JM216 is unstable in alkaline media. While Pt^{IV} complexes are usually relatively inert to substitution reactions, in the presence of strong base, the amine ligands are deprotonated forming amido species. The *trans*-effect of the amido ligand is much greater than that of the amine and so substitution of the ligand in the *trans*-position becomes rapid. As JM216 contains two different amine ligands, the differing equilibria for the ammonia and cyclohexylamine ligands result in differing substitution rates for the two chloride ligands. The cyclohexylamine protons are the more acidic which directs initial substitution *trans* to this ligand [18]. This reaction is of particular importance in the metabolism of JM216 (see below). In common with many coordination complexes, JM216 is unstable to light. In solution, the degradation is complex involving both substitution and reduction. Degradation in the solid state is very much slower, making a solid oral dosage form the most appropriate for complexes of this type.

Since reduction of Pt^{IV} to Pt^{II} is required before reaction with DNA, it is important for JM216 to have a reduction rate which is an effective compromise between maintaining the Pt^{IV} state for uptake and distribution, and reducing to Pt^{II} sufficiently rapidly to achieve reaction with DNA rather than being excreted intact. A suitable model reaction for this reduction is that with ascorbate [13]. For JM216, a half-life for reduction of the order of 50 minutes in 5 mM ascorbate suggests an adequate lifetime for absorption

as Pt^{IV} species, but with the likelihood of reduction occurring in the body to form reactive metabolites (see below).

In Vivo *Antitumor Efficacy*

In addition to the extensive oral antitumor studies described above in mice bearing the ADJ/PC6 plasmacytoma and human ovarian carcinoma xenografts, JM216 has also been evaluated *in vivo* in two murine models of acquired cisplatin resistance. Schedule-dependency effects have also been determined.

Although the demonstration of activity against acquired cisplatin-resistant tumor models was not a prerequisite for selection, JM216 did exhibit evidence of activity against the cisplatin-resistant variant of the ADJ/PC6 [16]. Following oral gavage, a therapeutic index of 2.2 was achieved (ED_{90} of 180 mg/kg). JM216 was not particularly active against the murine L1210 ascitic leukaemia or its cisplatin-resistant subline. Maximum increase in life spans were 41% (i.p. against the parent tumor) and 21% (oral against the resistant tumor).

The schedule dependency of oral JM216 was studied using the ADJ/PC6 and the PXN/109T/C ovarian-carcinoma xenograft and comparisons were made for single-dose (every 21 days) *vs.* once-a-day dosing for 5 consecutive days (every 21 or 28 days) *vs*. once-a-day dosing indefinitely [20]. In contrast to cisplatin and other platinum complexes studied to date, five-times-daily administration of JM216 to mice bearing the ADJ/PC6 tumor improved the tolerance, antitumor potency and therapeutic index (*i.e*., *TI* of 56 single-dose *vs*. > 423 daily × 5 JM216).

The schedule-dependency studies performed in the human ovarian carcinoma xenograft were especially important in helping to guide the clinical evaluation of the drug (see below). These studies clearly showed a gain (2–3-fold in terms of growth delay, $p < 0.01$) in antitumor activity in the daily × 5 arm *vs*. the single-dose or chronic dosing schedules; maximum growth delays, in days, were 30 in the single-dose every 3 weeks arm (100–200 mg/kg), 91 in the daily × 5 every 21 days arm (60 mg/kg/day), 65 in the daily × 5 every 4 weeks arm (60 mg/kg/day) and 16 in the chronic daily dosing arm (9.5 mg/kg/day). Moreover, greater total weekly doses were tolerable on the 5-day split dose schedule *vs.* a single weekly dose (300 *vs.* 200 mg/kg).

More recently, JM216 has been studied *in vivo* against the P388 leukaemia and M5076 sarcoma murine models in combination with orally administered etoposide [21]. When used in combination in non-tumor bearing animals, only *ca*. 25% of each drug's individual maximum-tolerated dose could be safely used. Evidence of a therapeutic synergy was reported in the

leukaemia model but not in the solid M5076 tumor. A phase-I trial of this combination is planned in Canada.

Toxicology, Pharmacology and Metabolism

Toxicology. JM216 showed limited toxicity with the dose-limiting toxicity in rodents being myelosuppression. In mice receiving a single-dose of 200 mg/kg, leucopenia was the prominent effect with a nadir at days 2 to 10 *post* treatment but with recovery by day 14. Mild thrombocytopenia and anaemia were also observed. When administered daily for 5 consecutive days at 55 mg/kg, thrombocytopenia was the most significant effect with nadir reached by day 14 and recovery observed by day 30. Leucopenia and anaemia were mild [22].

Other non-myelosuppressive toxicities were evaluated in rodents, including nephrotoxicity which is a serious concern with cisplatin. Comparative studies with cisplatin, carboplatin and JM216 at the maximum tolerated dose showed that, by a variety of methods, JM216 is devoid of nephrotoxicity [23]. For example, in mice at the maximum tolerated dose, cisplatin caused glycosurea, proteinurea and decreased the glomerular filtration rate after 4 days. These changes were not observed, however, with carboplatin or JM216. In rats, at the maximum tolerated dose, while cisplatin caused a fivefold elevation of plasma creatinine and urea and decreased the creatinine clearance by tenfold, neither JM216 nor carboplatin had any effect.

As described above, emesis studies in the ferret formed part of the selection criteria for developing an oral platinum drug. The duration of the emetic response induced by JM216 was significantly shorter than that induced by cisplatin. In mice emesis is not observed but stomach bloating, which has been described as a good predictor of emesis, was lower with JM216 than that observed following the equivalent dose of cisplatin and comparable to that observed after carboplatin.

Neurotoxicity has been observed both preclinically and clinically with some platinum drugs including cisplatin and tetraplatin (*Ormaplatin*®). For example, in rats after 6 weeks of treatment with cisplatin twice weekly at 2 mg/kg, a significant decrease (17%) in sensory nerve conduction velocity was observed while the motor nerve conduction velocity was unaffected. Tetraplatin at 1 mg/kg twice weekly caused a 14% decrease in sensory nerve conduction velocity. In parallel experiments in the rat, twice weekly treatment with JM216 at 25 mg/kg did not affect the sensory nerve conduction velocity [24].

Histological abnormalities in mice at the maximum tolerated dose were confined to the intestinal tract. This was characterised by villus atrophy,

crypt tip necrosis and reduced frequency of crypt mitosis similar in appearance and severity to that which has been observed with cisplatin and carboplatin. However, there was no sign of mucosal damage as disaccharidase activity (sucrase, threhalase and maltase) were unaffected [22]. Both alkaline phosphatase and alanine aminotransferase were unaffected indicating no liver damage.

Preclinical Pharmacology. The pharmacokinetics of total and free platinum were determined following oral gavage of JM216 as part of the schedule dependency antitumor experiments described above [20]. At doses of 9.5, 40 (day 1), 40 (day 5) and 200 mg/kg, non-linear pharmacokinetics were observed, both in terms of total and ultrafilterable platinum. On comparing doses of 9.5 and 40 mg/kg, the AUCs increased by tenfold and out of proportion to the fourfold increase in dose. Conversely, a further fivefold increase in dose to 200 mg/kg (the maximum tolerated for single-dose administration) was accompanied by only a twofold increase in AUC, consistent with saturable absorption. Similar C_{max} and AUC values for total and ultrafilterable platinum were obtained on day 5 *vs*. day 1 for the 40 mg/kg dose level.

Conversely, in another experiment using oral doses of 20, 50, 100 and 200 mg/kg to Balb C mice, broadly linear pharmacokinetics were observed. The maximum platinum levels in plasma were observed between 30 and 120 min and were delayed with increasing dose. Platinum unbound to proteins was detectable up to 7 h *post* dosing. Elimination of total platinum was biphasic with a terminal half-life of ~30 h. The half-life for ultrafilterable free platinum ranged from 87 to 135 min which is considerably longer than the 10 min reported for cisplatin or 25 min reported for carboplatin [25].

Tissue distribution studies in mice revealed that, 48 h after 200 mg/kg oral administration of JM216, platinum levels were the highest in the liver (6–19 μg Pt/g tissue) and kidney (2.8–12 μg Pt/g tissue). This is 5 times higher than that which has been reported after equivalent doses of cisplatin. All other tissues (spleen, heart, lung) had levels ≤ 3.1 μg Pt/g tissue. In the liver a time course of platinum levels showed that the C_{max} were reached by 2 h *post* administration [22]. Following administration of 200 mg/kg JM216 orally (in oil or in saline) 8% of platinum was eliminated in urine over 72 h and 66% was present in the faeces after 72 h.

Metabolism. JM216 is rapidly biotransformed *in vivo* with no parent drug being detectable in any of the patient samples examined even as early as 15 min *post* administration [26]. Six platinum-containing peaks were observed in patient's plasma ultrafiltrate samples [26]. Initially, metabolic studies were performed in fresh human plasma incubated with JM216 and

^{15}N-JM216 with analysis by HPLC followed by atomic absorption spectrophotometry or LC/MS to evaluate the platinum-containing fractions. This led to the identification of four metabolites: JM118 (*cis*-amminedichloro(cyclohexylamine)platinum(II)), the major metabolite seen in patients, JM383 (bis(acetato)ammine(cyclohexylamine)dihydroxoplatinum(IV)), JM518, JM559, isomers of bis(acetato)amminechloro(cyclohexylamine)hydroxoplatinum(IV) [27]. Selected ion monitoring revealed that these species are also present in patient plasma ultrafiltrate at peak concentration following treatment with JM216 [27] and that JM118 is the main metabolite of JM216 [26]. A further platinum-containing fraction could be detected in patient's plasma ultrafiltrate but not in ultrafiltrate from animals treated with JM216, JM118, JM383 or JM518 [28]. This unidentified metabolite was also not observed in tumor cells exposed to JM216 [29]. An early eluting platinum-containing fraction could be detected in all matrices evaluated (patient artificial plasma incubations, animal plasma, ovarian carcinoma cells). This peak was shown to be more prominent in tumor cells possessing high glutathione levels and decreased if cells were treated with buthionine sulfoximine (which inhibits glutathione synthesis) suggesting that it contains a glutathione adduct [29].

The growth inhibitory activity of the identified metabolites, JM118 and JM518, against a panel of ovarian carcinoma cell lines was of the same order of magnitude as that of the parent compound while the glutathione adduct corresponds to a detoxification product [28]. JM383 was around tenfold less cytotoxic against these cell lines. Furthermore, *in vivo* intraperitoneal administration of the metabolites JM118, JM383 and JM518 to mice bearing the s.c. ADJ/PC6 plasmacytoma, showed that all were active antitumor agents; *e.g.*, JM118-*TI* of 14 (LD_{50} 14 mg/kg, ED_{90} 1 mg/kg) [28].

In conclusion, as expected, the axial ligands were readily lost leading to the main platinum(II) metabolite, JM118. However, surprisingly, ligand-exchange reactions with replacement of a Cl-atom by a hydroxy group was observed up to 4 h *post* treatment with JM216. Conjugation with glutathione appeared to represent the main detoxification pathway.

In Vitro *Antitumor and Cellular Properties*

Cytotoxicity. JM216 was evaluated *in vitro* using the sulforhodamine B (SRB) assay against a panel of human ovarian carcinoma cell lines, established to be representative of the range of clinical responsiveness to platinum-based chemotherapy observed in patients presenting with advanced ovarian cancer (see below) [30]. JM216 showed a similar *in vitro* potency to that of cisplatin itself; mean IC_{50} values across the ovarian cell line pan-

el of 1.7 μM *vs.* 3.5 μM for cisplatin and 26.3 μM for carboplatin [16][31]. In contrast, some of the longer-chain mixed amine Pt^{IV} dicarboxylates (*i.e.*, butyrates and pentanoates of high lipophilicity) with an alicyclic carrier ligand were significantly more cytotoxic than cisplatin (*e.g.*, 100-fold more cytotoxic for JM274) and probably are among the most cytotoxic platinum-containing molecules yet described [31].

Spearman rank analysis of patterns of response across the panel was used to determine whether compounds were acting by similar (correlation coefficient approaching 1) or dissimilar means. Calculated correlation coefficients were: cisplatin/carboplatin 0.93 ($p < 0.01$), cisplatin/JM216 0.86 ($p = 0.01$), carboplatin/JM216 0.96 ($p < 0.01$) and tetraplatin/JM216 0.46 ($p > 0.05$). Thus JM216 *in vitro* appeared to behave similarly to cisplatin and carboplatin but not to the 1,2-diaminocyclohexane compound, tetraplatin. The reactivity of JM216 in comparison to that of cisplatin, carboplatin and tetraplatin was addressed in terms of the effect on cytotoxicity of differing drug exposure times [16]. While cytotoxicity was unchanged in moving from a 24- to 96-h exposure time for cisplatin and tetraplatin, for JM216, the IC_{50} was 1.8-fold lower than that obtained with a 24-h exposure [16].

JM216 was also shown to exert cytotoxic properties against panels of lung [32], cervix carcinoma [33] and murine L1210 leukaemia [34] cell lines. As with the ovarian lines, *Spearman* rank analysis of the pattern of response in the small-cell lung panel showed a high coefficient for the cisplatin/JM216 pair (0.82) [32]. Across a panel of 5 human cervix carcinoma cell lines, the potency of JM216 was similar to that of cisplatin with the exception of the HX/156 cell line which was 13-fold more sensitive to JM216 [33].

Flow cytometric analyses of L1210 leukaemia cells exposed to JM216 revealed that the drug caused a slowdown in the S-phase of the cell cycle followed by a G_2 block [35]. Cell death primarily occurred through apoptosis.

Circumvention of Acquired Cisplatin Resistance. The ability of JM216 to circumvent acquired cisplatin resistance *in vitro* has been addressed in a variety of pairs of tumor-cell lines of predetermined underlying mechanisms of resistance. In common with observations made with the longer-chain dicarboxylates JM221 and JM244 [31], JM216 also circumvented resistance in the transport-deficient 41McisR cell line but showed only partial circumvention against the CH1cisR line where resistance is due to enhanced DNA repair/tolerance to platinum-DNA adducts [20][36]. A similar circumvention of acquired transport-mediated cisplatin resistance was shown for the HX/155 and HX/155*cis*R pair of cervix carcinoma cell lines [33]. Further transport studies using the 41M pair of lines revealed that the mechanism

of JM216 transport across plasma membranes is through passive diffusion, predominantly as a result of its enhanced lipophilicity compared to cisplatin [37]. Circumvention of acquired cisplatin resistance by JM216 was also observed in the murine L1210 [34] and OVCAR-3 human ovarian carcinoma pairs of lines [16]. Partial circumvention of resistance in the CH1, A2780 ovarian, GCT27 testicular, and H69, MOR lung pairs of lines was also observed [16][32].

DNA-Binding Properties. The DNA-binding properties of JM216 have been studied in terms of binding to naked DNA and within human ovarian-carcinoma cell lines [38]. Exposure of plasmid DNA and analysis by agarose-gel electrophoresis showed that, in common with cisplatin, JM216 (or metabolites thereof) was capable of forming platinum-DNA interstrand crosslinks. This was confirmed within cells (CH1 and SKOV-3 ovarian carcinoma) using alkaline filter elution. The nature of platinum-DNA intrastrand crosslinks formed by JM216 was addressed using a competitive enzyme-linked immunosorbent assay (ELISA) and the ICR-4 monoclonal antibody raised against cisplatinated DNA. DNA extracted from CH1 cells exposed to JM216 was recognised by ICR-4 but around twofold less effectively than adducts formed by cisplatin suggesting some differences in adduct recognition for the two drugs.

Acquired Resistance to JM216. Mechanisms of acquired resistance to JM216 were studied in 2 human ovarian-carcinoma cell lines (41M and CH1) which had previously also been made resistant to cisplatin. Notably, in contrast to cisplatin (see above) 41MJM216R showed no deficiency in platinum transport [39]. Instead, resistance to JM216 in the 41MJM216R cell line appeared to be due mainly to elevated glutathione (around 1.7-fold higher) reflected in a similar reduction in total platinum bound to DNA following JM216 exposure. These results suggest that, in contrast to cisplatin, acquired resistance to JM216 may be less likely to occur through reduced drug uptake although this has not yet been addressed *in vivo*. However, as shown in the JM216R cell lines, other resistance mechanisms common to cisplatin such as elevated glutathione (in 41MJM216R) and increased DNA repair (as observed in CH1JM216R) may also apply to JM216.

JM216 in Combination with Radiation. JM216 has been studied *in vitro* in combination with ionizing radiation in RIF1 mouse tumor cells [40]. While no radiosensitization was observed with a 2-h drug exposure (irradiation occurring 15 min prior to the completion of exposure), radiosensitization (1.5 enhancement ratio) was observed with 1-h and 0.5-h exposures. In a second study using 1-h drug exposure and H460 non-small-cell lung can-

cer cells, dose enhancement ratios of 1.39 for 15 μM, 1.2 for 10 μM and 1.57 for 20 μM were obtained [41]. The majority of the sensitisation effect involved a reduction in the shoulder of the radiation survival curve rather than the final slope, suggesting predominant effects on inhibition of DNA repair. These data indicate that JM216 could be used as a clinical alternative to cisplatin for combination with radiotherapy.

JM216 Clinical Trials

Phase-I Single-Dose Study

JM216 entered Phase-I clinical trial in August 1992 as a single oral dose (60–700 mg/m^2) delivered every 21 days using a dry filled gelatin capsule. No hydration or diuresis was used with the treatment. Myelosuppression (leukopenia and thrombocytopenia) was seen but showed marked variability at doses from 420 to 700 mg/m^2. There was no significant neuro-, oto- or nephrotoxicity. Furthermore, in contrast to some initial fears with oral platinums, emesis was mild and easily controllable by prophylactic antiemetics. One of the 37 patients showed a partial response in relapsed ovarian carcinoma following cisplatin treatment. Two other patients showed a significant decrease in tumor markers (CA125) [42]. However, due to limited absorption/dissolution of the drug, no dose-limiting toxicity was observed in this trial. While plasma pharmacokinetics were linear up to dose levels of 120 mg/m^2, at dose levels > 200 mg/m^2, the C_{max} and AUC increased less than proportionally with dose.

Phase-I Daily-for-5-Days Study

Having failed to reach MTD with the single-dose study, the second Phase-I study (from March 1993 to December 1994) used a daily × 5 schedule in 32 patients at doses of 20, 30, 60, 100 and 140 mg/m^2/day [43]. Furthermore, preclinical antitumor studies had shown improved activity using a split-dose schedule (see above). Hard gelatin capsules of 10-, 50-, and 200 mg were employed. Prophylactic antiemetics were used routinely (oral dexamethasone combined with either metoclopramide or ondansetron). Under these conditions, JM216 was well tolerated with good control of emesis. The MTD was 140 mg/m^2 with 2 of 3 patients experiencing grade 4 thrombocytopenia and grade 3 and 4 leucopenia. The hematotoxicity was reversible and non-cumulative with the nadir occurring at days 17 to 21 and recovery by day 28. There was no neurotoxicity, ototoxicity nor renal tox-

icity observed. No objective sign of response was recorded in this trial, perhaps because of the inclusion of 22 patients previously treated with chemotherapy. Although considerable interpatient variability was observed for a given dose, the pharmacokinetic parameters (AUC, C_{max}) in this study proved to be increasing linearly with dose for both total and ultrafiltrable Pt (*e.g.*, $r^2 = 0.78$ for free AUC). The maximum concentrations for free platinum in the plasma were reached around 2 h and the half-life for free platinum varied from 4 to 14 h. There were no differences in the pharmacokinetic parameters observed on day 1 and day 5. A sigmoidal relationship was observed between the plasma ultrafiltrate AUC and the severity of thrombocytopaenia ($r^2 = 0.83$). The doses recommended for phase-II studies were 100 mg/m^2 in patients previously treated with platinum-based chemotherapy and 120 mg/m^2 otherwise.

Phase-I Twice-Daily Study

Nineteen patients received 150 to 350 mg/m^2 JM216 orally twice daily, 12 h apart. Again, the considerable variability in the pharmacokinetic parameters (mainly absorption) in both plasma and plasma ultrafiltrate led to the trial being stopped before a MTD was reached. No response was recorded but 2 patients with mesothelioma had stable disease [44]. Hence, this trial confirmed that the pharmacokinetics of JM216 is nonlinear and highly variable due to saturable absorption and that a daily-for-5-days schedule is optimal.

Phase-II and Other Studies

JM216 (BMS 182751) has been evaluated in small-cell lung cancer [45], non-small-cell lung cancer (NSCLC) [46][47], hormone refractory prostate cancer [48] and ovarian cancer (unpublished), all using the daily × 5 schedule. JM216 exhibited antitumor activity in patients presenting with previously untreated small-cell lung cancer [45]. Responses included 5 partial responses and 5 stable disease, and overall was 5/16 (31%).

A Phase-II trial in non-small-cell lung cancer was conducted under the auspices of the EORTC early clinical studies group [46]. Seventeen patients received JM216 at 120 mg/m^2/day for 5 days repeated every 3 weeks. Toxicity was manageable with < grade 2 myelosuppression, nausea, vomiting, diarrhoea, constipation and asthenia. One patient had a partial response after 3 courses but was progressing again after 4; an additional 5 patients had stable disease (46.2%). The authors concluded that, although JM216 afford-

ed useful palliation in some patients, the drug did not appear to possess significant antitumor activity in this disease. A second randomised phase-II study of oral JM216 (120 mg/m^2/day days 1–5) *vs*. intravenous cisplatin (100 mg/m^2 on day 1) every 21 days, for a maximum of 6 cycles, has begun. In both arms, 14 patients were evaluable for response. Response rates were low (7% for JM216 and 14% for cisplatin) for both drugs, without complete responses in either arm. In terms of toxicities, JM216 exhibited milder non-hematological side-effects but was more myelosuppressive than cisplatin [47].

Patients with hormone refractory prostate cancer (n = 39) received 120 mg/m^2/day × 5 every 28 days with prophylactic oral ondansetron [48]. Of 9 patients with measurable disease, 1 had a partial response and 6 had stable disease. 7 patients (32%) had PSA (prostate specific antigen) reductions of greater than 50% for greater than 28 days; 6 (27%) had PSA reductions of greater than 80%. The drug was generally well tolerated with myelosuppression (grade 3 and 4 neutropenia and thrombocytopenia) reported. Several patients experienced a second neutrophil nadir on days 32–42 *post* therapy. Grade 3 and 4 non-hematological toxicities included transient elevation of AST and bilirubin, diarrhoea, nausea and vomiting. A Phase-III study is planned in hormone refractory prostate cancer against and combined with prednisone.

Following preclinical studies suggesting that JM216 may act as a radiosensitizer (see above) a Phase-I study has begun combining JM216 and radiation in patients with advanced malignancies of the chest [49]. Nineteen patients have received doses of JM216 from 30 to 60 mg/m^2/day daily for 5 days concomitant with 2Gy fractions of standard radiotherapy. Severe toxicities were observed at the 60 mg/m^2/day dose level (grade 3 and 4 neutropenia, grade 3 thrombocytopenia, grade 3 oesophagitis) resulting in a dose reduction to 45 mg/m^2/day. Further patients are being treated at this recommended Phase-II dose; evidence of antitumor activity was observed at all dose levels.

AMD473

Following the selection and development of JM216, the principle objective in the continuing collaborative programme between *Johnson Matthey/AnorMED* and the *CRC Centre for Cancer Therapeutics* was to discover and develop a platinum drug possessing activity against cisplatin-refractory disease. A 'disease-oriented, mechanism-directed' evaluation cascade was established whereby novel molecules were screened against a panel of 8 *in vitro* human ovarian-carcinoma cell lines representative of intrinsic and

acquired cisplatin resistance. Using 3 pairs of cell lines (parent and acquired cisplatin resistant, where the major underlying mechanism of resistance had been determined) agents were sought which circumvented resistance in all pairs of lines. Ultimately, this led to the discovery of AMD473 (formerly JM473; *cis*-amminedichloro(2-methylpyridine)platinum(II)) which was subsequently discovered to possess the additional desirable property of being orally active against preclinical tumor models.

The chemical rationale for the synthesis of AMD473 was based on the desire to generate a platinum compound with reduced susceptibility to inactivation by elevated intracellular thiol concentrations. Many studies, including our own, have shown that thiols, especially glutathione (GSH) represent a significant cause of resistance to cisplatin [50]. Substitution in unhindered platinum complexes such as cisplatin occurs by an associative route and reactions with soft nucleophiles like GSH are much faster than reactions with hard nucleophiles such as nucleotides. Increasing steric bulk at the platinum centre (achieved using the 2-methylpyridine ligand in the case of AMD473) has the effect of shifting the reaction pathway more towards a dissociative mechanism where bond breaking in the starting complex is rate limiting rather than binding of the incoming ligand to platinum.

Preclinical Properties of AMD473

Chemical Reactivity, DNA-Binding. As predicted by the above chemical substitution considerations, AMD473 was shown to be less reactive than cisplatin towards the sulfur-containing molecules thiourea and methionine [51]. Furthermore, AMD473 binding to salmon-sperm DNA was significantly less affected than cisplatin when 5 mM GSH was added [52]. In whole tumor cells, AMD473 exhibited a smaller decrease in growth inhibition effectiveness than cisplatin or the major JM216 Pt^{II} metabolite, JM118, when GSH levels were artificially raised [52]. Finally, by a variety of measures, the DNA-binding properties of AMD473 appeared to differ from those of cisplatin. First, on naked DNA, several adducts unique to AMD473 were observed [51]; second, DNA interstrand crosslinks were formed much more slowly in cells exposed to AMD473 compared to cisplatin (peak formation of 5 h for cisplatin *vs*. 14–24 h for AMD473) [51][52]; third, a polyclonal antibody raised to DNA adducts of AMD473 showed no cross-reactivity with cisplatin-DNA adducts [53].

In Vitro *Effects Against Acquired Cisplatin-Resistant Tumor Cells*. AMD473 was primarily selected for further *in vivo* antitumor studies (and

clinical trial) because of its circumvention of acquired cisplatin resistance in tumor cell lines. Against 3 pairs (parent and cisplatin-resistant) of human ovarian carcinoma cell lines, 41M/41M*cis*R, CH1/CH1cisR and A2780/A2780*cis*R, AMD473 showed generally better circumvention of resistance than carboplatin, tetraplatin, JM216 or JM118 in all 3 pairs [52] (*Fig. 3*). These pairs were selected on the basis of encompassing all of the known major mechanisms of resistance to cisplatin; 41M*cis*R being resistant primarily through reduced drug transport, CH1*cis*R through enhanced DNA repair/tolerance and A2780*cis*R through a combination of decreased transport, enhanced DNA repair/tolerance and elevated GSH levels [16][36][37]. Platinum-transport studies following exposure of the 41M and A2780 pair of lines to AMD473 showed, in contrast to results obtained with cisplatin, equal intracellular drug levels in the parent and acquired resistant lines [52].

In Vivo *Antitumor Properties.* Initial studies using intraperitoneal administration of AMD473 at doses of 35–40 mg/kg showed the drug to confer marked antitumor activity against both murine (ADJ/PC6, *cf. Table*) and human ovarian-carcinoma xenografts [54]. Moreover, activity was observed

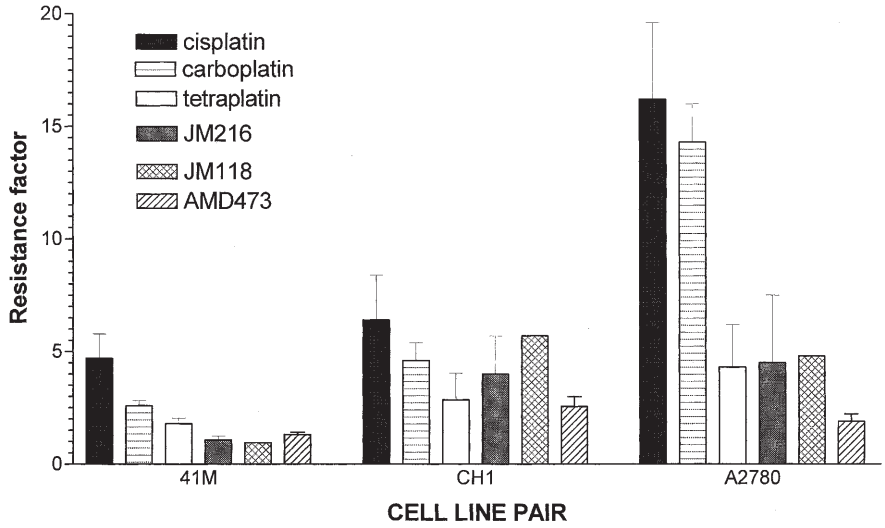

Fig. 3. *Cross-resistance profiles for the 41M/41M*cis*R, CH1/CH1*cis*R and A2780/A2780*cis*R pairs of human ovarian-carcinoma cell lines for cisplatin itself, carboplatin, tetraplatin (1,2-diaminocyclohexane)tetrachloroplatinum(IV)), JM216, JM118* (the major metabolite of JM216) *and AMD473*. Resistance Factor = IC_{50} resistant/parent cell line. Drug exposure was for 96 h, sulforhodamine B growth-inhibition assay, bars = SEM, n = 3–4.

against several tumors possessing acquired resistance to cisplatin and against CH1 xenografts that had regrown following initial treatment with cisplatin [54].

Antitumor activity by the oral route was first realised in the murine ADJ/PC6 plasmacytoma (*Table*) [54] where a therapeutic index of 90 was obtained. Significant antitumor activity following oral dosing was then confirmed, in particular against the acquired cisplatin resistant CH1*cis*R xenograft [54] (*Fig. 4*). In the *head-to-head* experiment using the CH1*cis*R xenograft (*Fig. 4*), AMD473 showed comparable activity by the i.p. or oral route and, moreover, markedly greater activity than either i.v. administered cisplatin or carboplatin or orally administered JM216 [54].

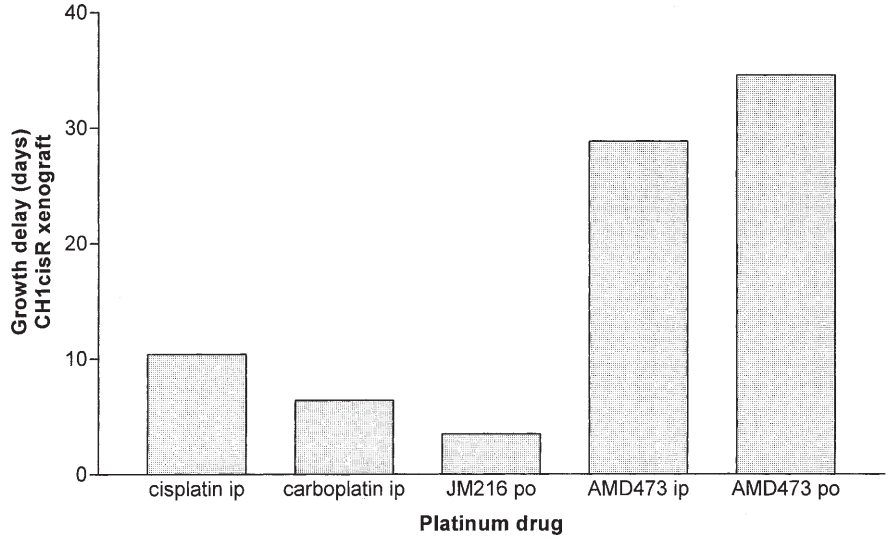

Fig. 4. *Antitumor activity in growth delay* (time taken in days for treated *vs*. control tumors to reach twice the volume at the start of drug treatment) *for i.p. administered cisplatin* (4 mg/kg q7d×4), *carboplatin* (80 mg/kg q7d×4), *oral JM216* (90 mg/kg q7d×4), *i.p. administered AMD473* (35 mg/kg q7d×4) *and oral AMD473* (400 mg/kg q7d×4) *in mice bearing the CH1*cis*R acquired cisplatin-resistant human ovarian advanced-stage s.c. xenograft*

Toxicology, Pharmacology and Metabolism. The dose-limiting toxicity of AMD473 in mice (and rats) is myelosuppression (leukopenia and thrombocytopenia). No renal-, liver- or neuro-toxicity has been observed [54]. Platinum pharmacokinetics following i.v. administration to mice (20 mg/kg) showed a biexponential decay in plasma with a rapid distribution ($t_{1/2\alpha}$ of 24 min) followed by a slow elimination ($t_{1/2\beta}$ of 44 h). Following oral dosing, platinum absorption was rapid (T_{max} of 0.5h) with a bioavailability of 40% [54]. Platinum accumulated mainly in the liver, kidney and spleen [54].

Biotransformation studies involving incubations in human plasma, exposure to tumor cells and dosing to mice have shown that AMD473 is detectable up to 6 h *post* administration (i.p. or oral) to mice and is mainly biotransformed to aquated activation products [55]. Notably, and in contrast to results obtained for JM216 [29], only AMD473 itself was detectable within human ovarian carcinoma cells; no GSH adduct was formed [55].

AMD473 Summary

Based on the above described properties of

- Reduced reactivity toward sulfur-containing soft nucleophiles relative to cisplatin, including glutathione;
- *In vitro* circumvention of acquired cisplatin resistance due to decreased transport, increased glutathione and enhanced DNA repair;
- Unique DNA-binding properties compared to cisplatin;
- Significant *in vivo* antitumor activity by both the intraperitoneal *and* oral routes against acquired cisplatin-resistant human ovarian-carcinoma xenografts;
- Toxicological properties reminiscent of carboplatin with myelosuppression being dose-limiting; less complex metabolism than JM216,

in 1996, AMD473 was selected for Phase-I clinical trial under the auspices of the *UK Cancer Research Campaign*. The initial, single-dose intravenous administration, Phase I of AMD473 began in November 1997. Subsequently (April 1998) the drug has been licenced to *Zeneca* for continuing clinical development.

C(5)-OHP-Cl

Oxaliplatin ((1,2-diaminocyclohexane)oxalatoplatin(II), *Eloxatin*®) is the lead molecule of the 1,2-diaminocyclohexane class of platinum complex, originally developed on the basis of exhibiting circumvention of acquired cisplatin resistance in murine L1210 leukaemia models [56]. Oxaliplatin is now undergoing numerous Phase-II/III trials and is registered for use in France for patients with 5-fluorouracil refractory colorectal cancer [57]. *Kidani et al.* [58][59] have attempted to obtain orally active derivatives of oxaliplatin by adding axial carboxylate ligands ranging from acetate to octanoate. Initially, on testing by intraperitoneal administration to mice bearing the L1210 leukemia, the bis(valerato) (C(5)) complex (C(5)-OHP) was most active. This complex also showed some marginal activity in this model when

administered by the oral route (maximum of T/C% of 148 using 20 mg/mouse daily for 5 days). Compared to JM216, C(5)-OHP was reduced much more slowly by 5 mM ascorbate (0.8 h for JM216 *vs.* 50 h) [58].

In addition to preparing the bis-carboxylate complexes they also explored reactions to obtain the mono-carboxylate compounds. These were obtained either by reacting the bis-carboxylate with the appropriate acid or reacting the *trans*-dichloroplatinum(IV) complex with silver carboxylate. In both cases chromatographic purification of the product was necessary [59]. Four complexes with an axial carboxylate ligand of butyrate, valerate, caproate and heptanoate were synthesised. One complex, the valerate (C(5)-OHP-Cl), exhibited oral antitumor activity against the subcutaneous M5076 reticulosarcoma model; C(5)-OHP was inactive in this model. The plasma total and ultrafilterable platinum AUC for C(5)-OHP-Cl was 4–6 times greater than for C(5)-OHP, suggesting improved absorption [60]. To date, no toxicological properties for C(5)-OHP-Cl have been reported.

Summary

The goal of allowing platinum-based chemotherapy to be administered by the oral route has been achieved. This was realised through the synthesis of a new class of ammine/amine Pt^{IV} complexes possessing lipophilic axial carboxylate ligands. A large number of this class of complex exhibited oral antitumor activity in the ADJ/PC6 plasmacytoma murine model. JM216 was selected on the basis of possessing both good oral antitumor activity, including effectiveness against a panel of human ovarian carcinoma xenografts, favourable toxicological properties in rodents and relatively low emetogenic properties in the ferret model. JM216 (BMS 182751) is now undergoing Phase-II evaluation. In contrast to possible concerns of severe emesis with oral platinums, this has not been a significant clinical issue with JM216. However, saturable absorption leading to non-linear pharmacokinetics was observed in the single-dose Phase I resulting in daily-for-5-days schedules being introduced. The dose-limiting toxicity with JM216 is myelosuppression; some evidence of antitumor activity has been observed in patients with hormone refractory prostate cancer.

At the preclinical level, oral antitumor activity has been observed with two other classes of platinum compound, the Pt^{IV} monocarboxylate, C(5)-OHP-Cl, and the first Pt^{II} complex to exhibit oral activity, the sterically hindered AMD473. Now licenced to *Zeneca*, AMD473 exhibited promising circumvention of acquired cisplatin resistance against both *in vitro* and *in vivo* preclinical models. Together with the drug's favourable pharmacokinetic and toxicology profile in rodents (myelosuppression was dose-limit-

ing with no nephrotoxicity) AMD473 has been selected for phase I clinical trial under the auspices of the *UK Cancer Research Campaign*, initially as an intravenous formulation. The Phase-I intravenous study began in November 1997.

The drug development programme leading to the discovery of JM216 involved a collaboration between *Johnson Matthey* (Sonning, UK, and West Chester, Pennsylvania), the *Drug Development Section/CRC Centre for Cancer Therapeutics* at the *Institute of Cancer Research* and *Bristol Myers Squibb*. A subsequent collaboration between *Johnson Matthey/AnorMED* and the *CRC Centre for Cancer Therapeutics* resulted in the discovery of AMD473. Numerous colleagues/collaborators have contributed including chemists at *Johnson Matthey/AnorMED* (*Barry Murrer*, *Chris Barnard*, *Mike Abrams*, *Chris Giandomenico*, *Geoff Henson*), biologists at *ICR* under the Directorship of *Ken Harrap* (*Prakash Mistry*, *Florence Raynaud*, *Sarah Morgan*, *Mervyn Jones*, *Phyllis Goddard*, *George Abel*, *Frances Boxall*, *Swee Sharp*, *Ciaran O'Neill*, *Melanie Valenti*, *Grace Poon*), Ph.D. students (*Kirste Mellish*, *Jeff Holford*), clinicians at *ICR/Royal Marsden Hospital* (*Ian Judson*, *Mark McKeage*, *Philip Beale*) and scientists at *BMS* (*Anna Marie Casazza*, *Bill Rose*, *John Schurig*, *Al Crosswell*).

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Methods for Screening the Potential Antitumor Activity of Platinum Compounds in Combinatorial Libraries

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The success of cisplatin and carboplatin in treating cancer, combined with the intrinsic and acquired resistance of many tumors to traditional platinum chemotherapy, has generated considerable interest in developing next-generation platinum drugs. The advent of combinatorial chemistry has had a major impact on drug discovery, but has not yet facilitated the search for new cisplatin analogues. For new platinum drugs to be discovered by a combinatorial approach, there must be improved inorganic synthetic methodologies and protocols to identify 'hits' from platinum libraries. This chapter evaluates standard and novel screening methods for speed, convenience, accurate prediction of cytotoxicity, and compatibility with high-throughput technologies. There are promising new *in vitro* methods to assess the interaction of platinum-modified DNA with mechanism-based targets such as HMG-domain proteins. Cell-based screening methods include high-throughput cytotoxicity assays and new reporter-gene assays that measure the biochemical effects of platinum complexes. Practical considerations generally preclude *in vivo* screening of libraries of platinum complexes.

Introduction

The success of cisplatin and carboplatin in treating cancer, combined with the intrinsic and acquired resistance of many tumors to traditional platinum chemotherapy, has generated considerable interest in developing next-generation platinum drugs. Since the discovery of the antitumor activity of cisplatin, researchers have reported the synthesis, characterization, and antitumor activity of thousands of platinum compounds [1][2]. The previous two chapters in this section describe the promising activity of novel multinuclear Pt^{II} and orally active Pt^{IV} complexes [3][4].

The advent of combinatorial chemistry has had a major impact on drug discovery [5–7], but has not yet facilitated the search for new cisplatin an-

Cisplatin. Edited by Bernhard Lippert

alogues. To date there is only one published report of a molecular-diversity approach to platinum drug discovery [8]. One reason for this situation is that the field of combinatorial chemistry has evolved almost exclusively from organic chemistry [6][9–11]. Methods for combinatorial synthesis of inorganic compounds, with the exception of solid-state materials, have been developed only in a few laboratories [8][12]. It should be possible, however, to apply the advances made by organic chemists in parallel synthesis, automation and chemoinformatics to the generation of libraries of platinum complexes.

In order for new platinum drugs to be discovered by a combinatorial approach, there must be suitable screening protocols to identify 'hits' from platinum libraries [5][7]. The best screening methods accurately predict activity, are fast and simple, and can be run in a high-throughput environment. A mechanism-based screen that detects the interaction with a target molecule is ideal, but as *Part 3* of this volume shows, mechanistic studies of platinum drugs have not yet identified a single critical target. Aside from general agreement that DNA binding is required for cisplatin activity, there is little consensus about the biologically relevant interactions that cause tumor cell death. It has, therefore, been a challenge for researchers to develop a rational strategy for the synthesis of platinum compounds having better anticancer activity than cisplatin.

There are three major approaches to assessing the potential activity of a chemotherapeutic compound prior to human clinical trials. *In vitro* assays, either solution- or solid-phase, are often used early in the screening process, especially when a biologically relevant molecular interaction has been identified. Studies in cultured mammalian cells are employed to predict the activity of a compound. If a compound shows promise in such *in vitro* and cell-based assays, then it is tested extensively in animals before proceeding to humans. The remainder of this chapter discusses these three methods as potential routes to identifying active platinum compounds from combinatorial libraries.

In Vitro Screening Methods

In vitro methods are not widely used to predict the antitumor activity of platinum compounds. Until it is determined what factors render a specific platinum-DNA adduct cytotoxic, the *in vitro* screens for platinum drugs will be limited. Because a good platinum drug must form DNA adducts, preliminary screening methods have been employed which measure DNA binding alone as a measure of potential antitumor activity. In one such study [13], the DNA binding of palladium and platinum complexes having inter-

calative ligands was monitored by the quenching of ethidium bromide fluorescence [14]. Upon metal-DNA binding, an intercalative ligand displaces ethidium from DNA, causing a decrease in fluorescence. From the change in fluorescence, DNA-binding constants were determined for a series of fourteen Pd^{II}/phenanthroline/amino acid complexes. There was a weak correlation between DNA binding and both *in vitro* cytotoxicity and *in vivo* antitumor activity for the compounds tested. The results are summarized in the *Table*.

Another report reveals, however, that *in vitro* DNA-binding assays are insufficient to predict platinum antitumor activity [15]. Primer extension (*Fig. 1*) was used to identify specific adducts formed by platinum complexes on DNA in HeLa cells. The DNA adduct profile correlated well with *in vivo* antitumor activity for *cis*- and *trans*-DDP, $Pt(en)Cl_2$, and two acridine-tethered platinum complexes. When the complexes were allowed to react with purified DNA in solution, there were no substantial differences in adduct profiles between active and inactive compounds. This result demonstrates that cell-based assays can be better predictors of *in vivo* activity than *in vitro* assays, particularly when the *in vitro* screen does not require a unique, mechanism-based molecular interaction.

Table. *Comparison of DNA-Binding, Cytotoxicity, and Antitumor Activity of a Series of Pd^{II} Compounds* (data from [13])

Complex	Pd-DNA Binding constant $K \times 10^{-6}$ (l/mol)	Cytotoxicity index[a])	Antineoplastic ratio[b])[c])
[Pd(phen)(leu)]Cl	5.10	96.7	n.d.
[Pd(phen)(lys)]Cl	7.96	96.5	56
[Pd(phen)(met)]Cl	5.16	95.0	n.d.
[Pd(phen)(ser)]Cl	2.93	93.8	n.d.
[Pd(phen)(arg)]Cl	4.52	90.3	50
[Pd(phen)(his)]Cl	1.45	87.6	n.d.
[Pd(phen)(asn)]Cl	5.84	86.9	n.d.
[Pd(phen)(trp)]Cl	3.35	83.7	n.d.
[Pd(phen)(gln)]Cl	2.25	82.8	n.d.
[Pd(phen)(pro)]Cl	1.00	78.3	48.2
[Pd(phen)(tyr)]Cl	4.90	77.9	n.d.
[Pd(phen)(ala)]Cl	1.01	73.8	n.d.
[Pd(phen)(gly)]Cl	1.67	70.2	n.d.
[Pd(phen)(asp)]	0.75	37.8	n.d.

[a]) Cytotoxicity index measures the percent dead MCF-7 cells counted by using the trypan blue dye exclusion assay.
[b]) Pure 615 mice implanted with sarcoma 180 were treated for nine days with 20 mg/kg/day. The ratio refers to the percent increase in lifespan, relative to untreated controls.
[c]) n.d.: Not determined.

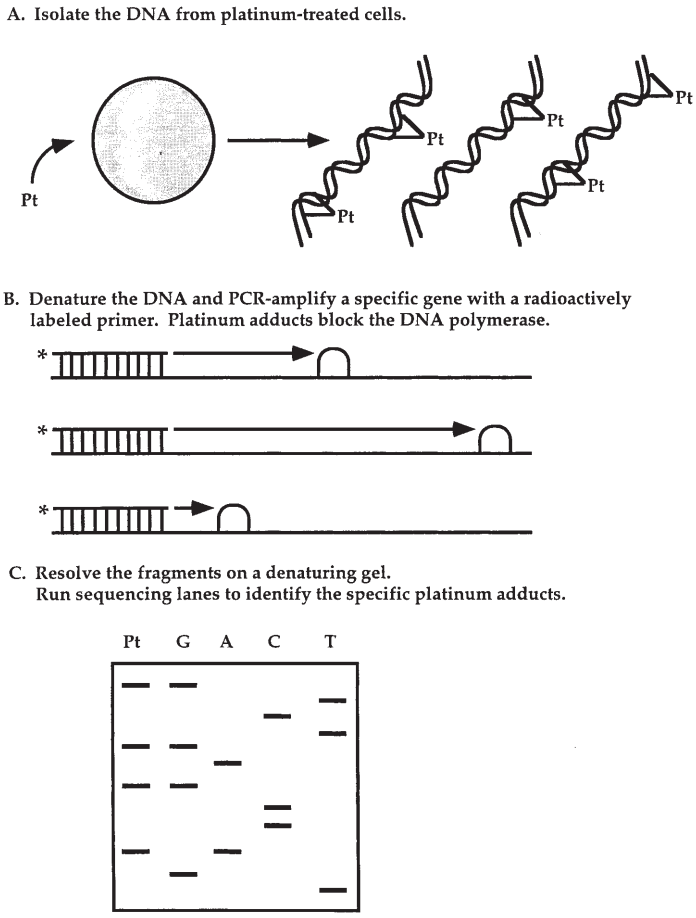

Fig. 1. *Identifying platinum-DNA adducts by primer extension*

Proteins that recognize cisplatin-modified DNA may effect the cytotoxicity of the drug [16]. In particular, high-mobility group (HMG) domain proteins, which specifically bind 1,2-intrastrand platinum-DNA cross-links, can mediate cisplatin cytotoxicity by shielding the adducts from repair. One strategy for screening potential platinum drugs, therefore, would be to evaluate a compound based on its ability to form DNA adducts that bind specifically to HMG-domain proteins. This approach was evaluated by screening a mixture of platinum-amino-acid complexes for their ability to bind DNA and, subsequently, HMG1 in a gel-mobility-shift assay [8]. The mixture was prepared by combining potassium tetrachloroplatinate with two equivalents of an equimolar mixture of 17 amino acids in water; the pH was maintained at 6 and, after several days, the product mixture was evaluated without pur-

ification. The mixture was combined with DNA to form platinum adducts, as confirmed by atomic absorption spectroscopy. As illustrated in *Fig. 2*, the mixture contained components that shifted the platinated DNA upon addition to HMG1 in a gel-shift assay.

In order to identify the platinum complex or complexes responsible for this behavior, a sublibrary synthesis and screening approach was employed. Five pools of four amino acids each were used to prepare new mixtures to be screened by the gel-shift assay. After three iterations of sublibrary synthesis and screening, the platinum-lysine complex Kplatin, shown in *Fig. 3*, was selected. Kplatin has *in vitro* DNA-binding properties similar to those of cisplatin. Its toxicity towards HeLa cells, on the other hand, is almost 100-fold lower than that of the parent compound. Subsequent studies with analogous *N*,*O*-chelated platinum-amino-acid complexes further revealed that the ability of a platinum compound to form a ternary Pt-DNA-HMG1 complex does correlate well with its toxicity towards HeLa cells [17]. The negative charge of the coordinated carboxy group diminishes the tendency for the platinum compound to bind to DNA.

Although the platinum-amino-acid complexes do not show much promise as cytotoxic agents, these results demonstrated the utility of *in vitro* screening methods to survey the DNA-binding properties of platinum compounds in a combinatorial manner. Assuming that HMG-domain proteins are involved in the cisplatin mechanism of action, then screening based on the Pt-

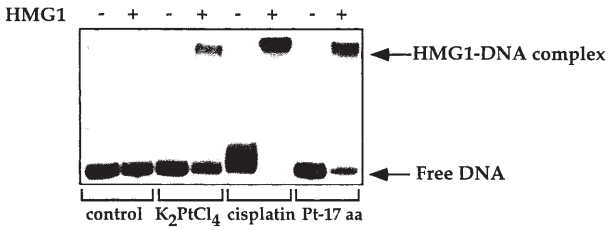

Fig. 2. *Native polyacrylamide gel demonstrating the specific binding of HMG1 to a radiolabeled, platinated 123-bp DNA fragment*. The DNA was modified with platinum compounds or mixtures as indicated.

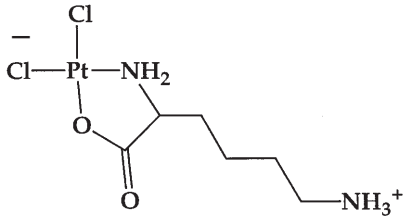

Fig. 3. *Structure of dichloro(*L*-lysine)platinum(II), or Kplatin.*

DNA-HMG1 complex formation would be mechanism-based. The gel-shift assay, however, is neither fast nor convenient, so alternative protein-DNA binding assays are required to adapt the method for high-throughput screening.

A better approach would be to screen the molecular libraries on solid-phase supports [6][10][11]. Solid phase methods offer several advantages, allowing compounds to be identified by immobilization and position in a binding assay. Solid-phase screening can also be performed with the aid of robotics to increase throughput [7]. As indicated in *Fig. 4*, a fluorescently labeled HMG-domain protein would facilitate the search for Pt-DNA-HMG binding by solid-phase methodologies.

Accordingly, the Pt-DNA-HMG binding screen was implemented in the solid phase by constructing a fusion of HMG1 with the green fluorescent protein (GFPuv) [17]. As illustrated in *Fig. 5*, the fusion protein HMG1-GFPuv binds specifically to cisplatin-modified DNA covalently attached to a nylon membrane. The binding is easily monitored by using a fluorescent microplate reader. The fluorescence readout, corresponding to bound HMG1-GFPuv, correlates well with cytotoxicity for a series of closely related *N*,*O*-chelated platinum-amino-acid complexes, which again reflects DNA-binding ability. The solid-phase Pt-DNA-HMG1 binding method, or

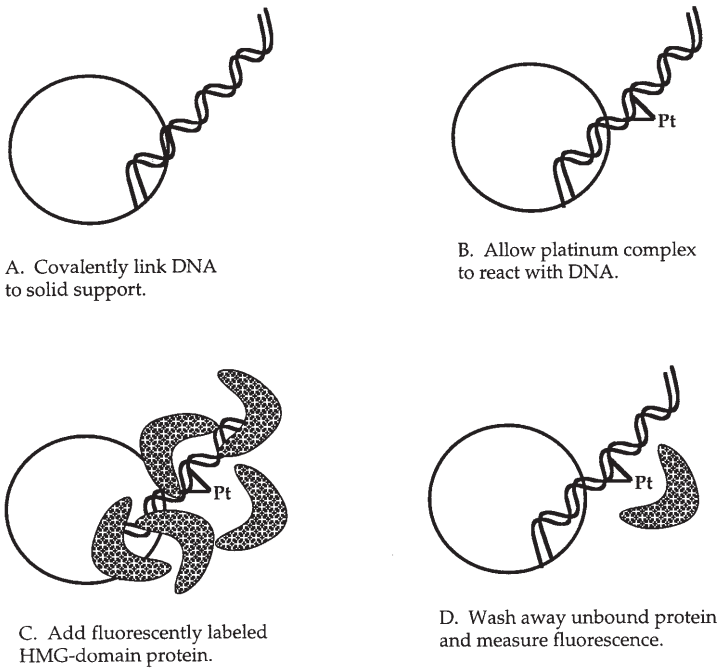

Fig. 4. *A solid-phase approach to screening platinum compounds for specific Pt-DNA-HMG interactions*

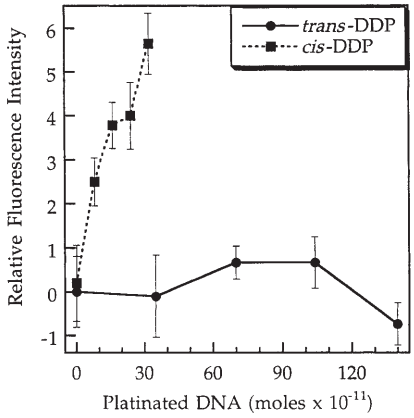

Fig. 5. *Results of the solid-phase assay for Pt-DNA-HMG1 binding*. A 19-bp DNA duplex was modified with *cis*- or *trans*-DDP and covalently linked to a nylon membrane. The membrane was incubated with HMG1-GFPuv and washed extensively. Bound protein was determined by measuring fluorescence retained on the membrane.

similar methods using other proteins that recognize platinated DNA, could be adapted to a high-throughput setting for the purposes of mechanism-based screening of potential platinum antitumor drugs.

Cell-Based Assays

Cell-based assays, particularly those using human tumor cell lines, provide a relatively fast and inexpensive way to assess the *in vivo* activity of new compounds. The success of a cell-based assay depends in part on the nature of the chosen cell line. The NCI anticancer drug screening program tests compounds against a panel of 60 human tumor cell lines [18]. For practical reasons, individual laboratories have generally selected only a few relevant cell lines for screening. There is no general agreement about which cytotoxicity assay is the best predictor of anticancer activity.

In a clonogenic, or colony-counting, assay, cells are treated with a prospective anticancer compound, and their viability is determined by measuring the resulting colonies. The clonogenic assay is widely used, partly because the ability to prevent cell division is taken as an essential property of anticancer drugs [18]. The disadvantage of the clonogenic assay is that it is time- and labor-intensive. It often takes more than one week for visible colonies to form, and manual colony counting is quite tedious [19]. Although computer-assisted colony-counting methods are available, the clonogenic assay is not particularly suited to high-throughput screening. Aside from

time considerations, in order to see subtle differences in percent survival, at least 100 cells must initially be seeded in an area large enough for individual colony formation. Thus, the clonogenic assay cannot be miniaturized to the extent necessary for conveniently screening large libraries of platinum compounds.

Several rapid, convenient cytotoxicity assays have been developed as alternatives to the clonogenic assay. The neutral red assay measures uptake of a dye into cells, with only viable cells binding the dye [20]. The microtetrazolium (MTT) assay correlates cell count with the reduction of the yellow tetrazolium salt to purple formazan, a reaction which occurs in the mitochondria of viable cells only [19][21]. Sulforhodamine B (SRB), which is used in the NCI anticancer drug-screening program, is an anionic aminoxanthene dye that measures cell count by binding to cellular proteins [21]. In one study MTT and SRB assays yielded quite similar cytotoxicity profiles for cisplatin and other anticancer drugs in seven human tumor cell lines [21].

All three of the foregoing rapid cytotoxicity assays are suited to high-throughput measurements by microplate reading, but it is necessary to wait several days following treatment in order to measure cell viability. Moreover, these assays measure short-term, 2–3 day, delays in cell growth, rather than the inhibition of cell division. In one report, there was little correlation between results of the MTT and colony-counting assays for non-small-cell lung cancer cell lines treated with mitomycin C or cisplatin [18]. In another, very good correlation among the SRB, MTT and colony-counting assays was observed for 16 human ovarian carcinoma cell lines treated with cisplatin [19].

Screening platinum complexes for cytotoxicity is not a mechanism-based approach. Another cell-based screening method [22] is predicated on the observation that mismatch-repair-deficient cells are less sensitive to cisplatin than wild type cells [22][23]. A review of the role of mismatch repair proteins in cisplatin cytotoxicity reveals that tumors resistant to cisplatin sometimes have mutations in one or more genes encoding for such proteins [16]. A mismatch-repair-deficient cell line constitutively expressing GFP was mixed with a repair-proficient line and the heterogeneous population treated with DNA-damaging agents. Five days following exposure, both cisplatin and carboplatin enriched the GFP-expressing mismatch-repair-deficient cell population. The potential cytotoxicity of any platinum compound might therefore be assessed by measuring the enrichment of GFP-expressing, mismatch-repair-deficient cells in the population. This method has a direct fluorescent readout that could be readily adapted to high-throughput conditions, but the 5-day delay necessary to see population enrichment cannot be shortened. Because the method is based on a proposed mechanism of resistance to platinum antitumor drugs, it is an advance over methods that merely measure cell death.

Another, related method [24] uses a HeLa Tet-on cell line stably transfected with the enhanced green fluorescent protein (EGFP) gene under the control of the tetracycline-responsive element (TRE). Upon induction with doxycycline, the cells express EGFP. Treatment with cisplatin and other effective platinum complexes caused a dose-dependent decrease in EGFP expression. Treatment with *trans*-DDP and other DNA-damaging agents led to a sharp increase in EGFP expression. *Fig. 6* shows typical results of a 13.5-h co-treatment of HeLa cells with platinum and doxycycline, and *Fig. 7* plots platinum cytotoxicity *vs.* EGFP inhibition. The method exploits the apparent difference in cellular response to cisplatin adducts compared to other forms of DNA damage. Although the somewhat low levels of EGFP expression require cell lysis in order to quantitate fluorescence, with the use of improved fluorescent reporter-gene systems and better detection methods this approach could be adapted for high-throughput work. The advantage of the EGFP induction assay is speed; the results can be obtained overnight.

Another reporter-gene assay has also been used to screen platinum compounds. The fluorescent compound CCF2-AM, depicted in *Fig. 8*, emits green fluorescence when intact but blue light upon cleavage with β-lactamase [25]. The compound is readily taken up by cells and, because of the enzymatic amplification, very low concentrations of the β-lactamase reporter gene can be detected. *Fig. 9* reveals that *cis*-DDP, to a much greater ex-

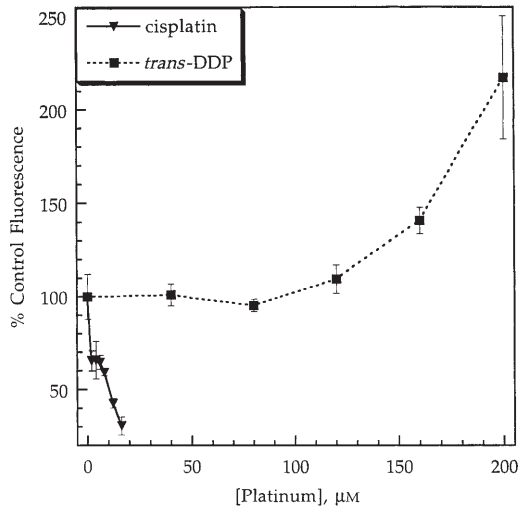

Fig. 6. *Effect of a 13.5-h* cis- *or* trans-*DDP treatment on doxycycline-inducible EGFP expression in HeLa Tet-on cells*. The fluorescence values were divided by total protein to correct for variations in cell count.

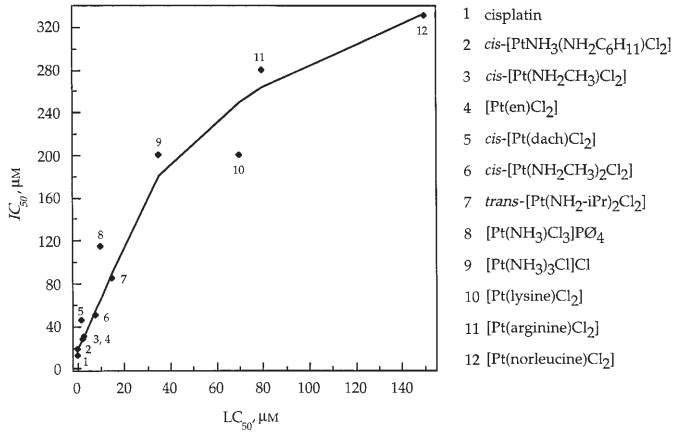

Fig. 7. IC_{50} vs. LC_{50} *values for HeLa cells treated with a series of platinum complexes.* LC_{50} was determined by a colony-counting assay. IC_{50} was the platinum concentration at which EGFP expression was reduced to 50% of control.

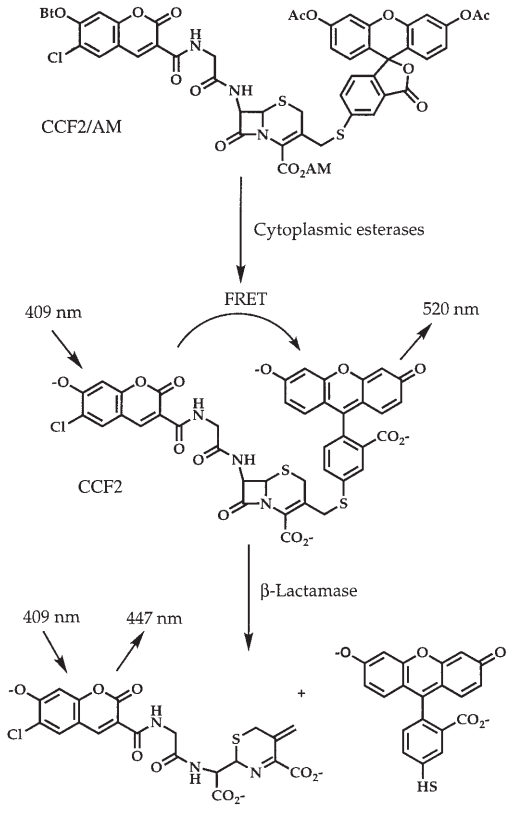

Fig. 8. *Cleavage of the fluorescent reporter CCF2-AM in cells expressing β-lactamase*

tent than *trans*-DDP, inhibits inducible β-lactamase expression as measured by CCF2 cleavage in a Jurkat cell line. This assay is performed in an automated high-throughput setting, and the results can also be obtained overnight [24].

These new reporter gene cell-based techniques represent the beginning of a new era in platinum-drug screening in cells. Instead of merely measuring cytotoxicity, the new methods screen compounds for cellular effects specific to biochemical changes induced by platinum antitumor drugs. The methods are also convenient and readily adaptable to high-throughput settings. In both the mismatch-repair and reporter-gene-induction assays, the major disadvantage is that stable cell lines expressing reporter genes must be constructed. Because it can take up to several months to obtain stably transfected cell lines, it is necessary to choose cell types judiciously before proceeding with screening.

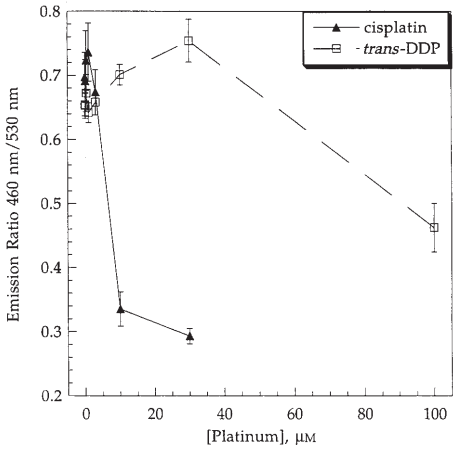

Fig. 9. *Effect of an 20-h* cis- *or* trans-*DDP treatment on carbachol-inducible β-lactamase expression in Jurkat cells*. The gene expression was determined by measuring the cleavage of the fluorescent dye CCF2-AM.

In Vivo Screening Methods

Animal models are frequently employed to assess the antitumor activity of new platinum compounds. Often, mouse tumors such as mammary, lung or colon carcinomas are used. In other cases human solid tumor xenografts are grown in immune-deprived mice for drug screening. Although it can be argued that the efficacy of a compound against tumors in mice may

not predict its activity in humans, the pharmacokinetic and toxicity properties of a new compound must be evaluated in animals before proceeding with human clinical trials [26].

Although animal models can provide important information regarding the bioavailability and pharmacology of potential anticancer drugs in mammals, they are not always accurate predictors of activity against human tumor cells. In one report [27], the activity of a series of isomeric [1,2-bis(difluorophenyl)ethylenediamine]dichloroplatinum(II) compounds was evaluated in MXT murine mammary carcinomas *in vivo*; the same compounds were also tested against several human cell lines in culture. The *in vivo* screen revealed a 2,6-difluoro-substituted compound to be the most active, whereas the 2,4-difluoro-substituted compound was most active against the human breast-cancer cell lines. It was concluded that the mouse mammary carcinoma is not an appropriate model for human breast cancers. Extreme caution must be employed when animal tumor results are used to predict activity in human tumors.

Xenografts in mice of human cancer cell lines can serve as good models for human tumors. In one report [28], a panel of eight human ovarian carcinoma cell lines and companion xenografts were used to screen cisplatin, carboplatin, tetraplatin, and iproplatin. Overall, there was good correlation between the cell-based and xenograft assays. This report not only demonstrates that xenografts can be good models for human tumors, but also that human cell-line assays can predict the activity of a platinum compound approximately as well as xenograft studies. In most cases, it would seem prudent to use human cell-line assays as preliminary screens of platinum compounds before moving on to animal studies.

Although it is essential to test promising compounds in mice and other animal models prior to human trials, it is economically, ethically and often scientifically preferable to use cell-based and *in vitro* approaches to eliminate inactive compounds before commencing animal trials. Clearly, animal models are not an appropriate screen for combinatorial libraries of platinum complexes; they should be used to study further the promising leads identified by high-throughput methods.

An alternative *in vivo* approach to screening platinum complexes was recently described [29]. A series of diamineplatinum(II) complexes was applied to cucumber or maize roots; the root-growth inhibition indicated the cytotoxicity of the compound. Because the root-growth inhibition results correlated with the antitumor activity of the complexes in mice, it was proposed that plant roots would afford inexpensive and rapid screens for platinum compounds. Although the plant-based screening method is unique and inexpensive, it is not an ideal approach to identifying platinum drugs to treat human cancers. Given that even the selection of human tumor cell lines can

affect the results of a cytotoxicity assay, it seems unlikely that any plant-cell line would suitably model human cancer. This method could be used as a very preliminary screen for general cytostatic activity, but it is more laborious than any of the high-throughput cell-based assays; the root length must be measured by hand daily for three days.

Conclusions

Although the field of medicinal chemistry has been revolutionized by the advent of combinatorial synthesis and high-throughput screening, application of these methodologies for platinum-drug discovery and lead optimization is in its infancy. In part, new methods for inorganic combinatorial synthesis must be devised. This process can be accelerated by exploiting the recent advances in organic small-molecule combinatorial chemistry. Also, new mechanism-based methods for screening platinum compounds must be developed with a focus on speed and automation. The three reporter gene assays described in this chapter [22][24], as well as the Pt-DNA-HMG1 binding assays [8][17], represent the beginning of a new trend toward mechanism-based screens for platinum compounds. It would be especially useful to have more solution- or solid-phase *in vitro* approaches to predicting platinum cytotoxicity. In order to develop screening methods, however, a greater understanding of the biochemistry of platinum antitumor activity is essential. When there is compelling evidence for the role of a specific platinum-DNA-protein interaction in platinum cytotoxicity, for example, then a high-throughput, mechanism-based screening method will surely follow.

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Computational Studies on Platinum Antitumor Complexes and Their Adducts with Nucleic-Acid Constituents

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Molecular-orbital calculations performed on platinum antitumor complexes and related species, and force-field calculations carried out on their adducts with nucleic acids are reviewed. The aim of the author is to point out the methodological difficulties encountered in these calculations, and to comment on the (sometimes problematic) results which they have yielded.

Introduction

Platinum(II) complexes have played a major role in the development of fundamental principles of coordination chemistry. Cisplatin, *cis*-diamminedichloroplatinum(II), one of today's most successful antitumor drugs, happened to be one of the classical compounds serving to demonstrate these principles. Together with its *trans*-congener, it was among the first inorganic compounds in which the phenomenon of isomerism has been observed [1–3]. This couple of isomers belonged to the prominent examples used by *Alfred Werner*, the founder of coordination chemistry, in his '*Beitrag zur Konstitution anorganischer Verbindungen*' [4]. Pt^{II} compounds are parade representatives for the so-called *trans*-effect [5] and *trans*-influence [6], and their electronic structure has been a topic of interest and controversy. Early theoretical investigations of the electronic properties of platinum complexes included calculations based on ligand-field theory [7–10], extended *Hückel* (EH) [11][12] and intermediate-neglect-of-differential-overlap (INDO) [13] methods, as well as $X\alpha$ calculations [14]. The discovery of the antitumor activity of cisplatin has provided an additional impetus to these quantum-chemical investigations. Moreover, the finding that DNA is the

Cisplatin. Edited by Bernhard Lippert

crucial biological target of the platinum drugs prompted a number of structural studies of adducts formed between platinum complexes and DNA or its constituents. Molecular-orbital (MO) calculations were used in studies of the smaller model compounds, and force-field (*i.e.*, molecular mechanics) calculations for larger systems.

The aim of the present contribution is to critically review computational work related to platinum antitumor drugs, published prior to 1998. After a section devoted to molecular-orbital calculations on platinum antitumor complexes and related compounds, we address force-field calculations on platinum adducts with DNA constituents that have been used (mainly in combination with NMR spectroscopy) to evaluate the structure of the adduct. A brief outlook concludes this chapter.

Molecular-Orbital Calculations

Rosenberg's discovery that cisplatin displays antitumor activity [15] prompted several groups to examine the electronic structure of cisplatin and of some related complexes by means of semiempirical [16–20] or Xα [21][22] molecular-orbital calculations. The molecular-orbital schemes derived by *Dimoglo et al.* [16] and by *Carsey* and *Boudreaux* [17] featured unreasonably small HOMO-LUMO gaps. For instance, for *cis*-[$PtCl_2(NH_3)_2$], *Dimoglo et al.* calculated a HOMO-LUMO gap of 1.19 eV, whereas *Carsey* and *Boudreaux* found an orbital-energy difference of 1.36 eV. These values contradict the experimental observation of the lowest-energy electron transition near 3 eV [18]. On the other hand, *Krogh-Jespersen* and co-workers presented a fairly convincing interpretation of luminiscence, absorption, MCD, and NQR spectra based on extended *Hückel* molecular-orbital (EHMO) calculations [18–20]. Although no excited-state calculations were performed in these studies, the transition-energy estimates from simple orbital-energy differences followed the expected trends and allowed for a plausible assignment of the spectral bands. However, the relativistic correction terms used in these calculations did not include spin-orbit coupling, which has been criticized by *Boudreaux* [23].

Xα Calculations involve the straightforward possibility of evaluating ionisation energies by calculating the *Slater* transition state [24]. In their Xα studies, *Barber et al.* [21] as well as *Zuolaga* and *Arratia-Pérez* [22] compared the calculated ionization energies for *cis*-[$PtCl_2(NH_3)_2$] with results from ESCA measurements. The agreement was rather poor, in spite of the attempt by *Zuolaga* and *Arratia-Pérez* [22] to improve it by adding a constant value to the calculated ionization energies. This disagreement prompted *Boudreaux* [23] to express doubts on the capacity of the *Slater*

transition-state method to correctly account for the relaxation accompanying ionization [23]. *Boudreaux* also questioned the finding of *Zuolaga* and *Arratia-Pérez* [22] that, in *cis*-[$PtCl_2(NH_3)_2$], a 'Cl-Cl bonding region' and 'an almost neutral ligand Cl_2 molecule' exist in the complex, a conclusion which lacks any experimental evidence and casts doubt on the meaningfulness of the calculations.

'Platinum pyrimidine blues', formed upon the reaction between aquated cisplatin and pyrimidine bases, have attracted the attention of chemists for their potential as antitumor drugs [25], as well as because of their unique spectroscopic properties [26]. A structural analysis of a related blue compound with α-pyridone revealed a tetrameric structure [27]. An EHMO calculation on one half of the α-pyridone-blue cation in the reduced Pt^{II} form was performed by the *Patterson* group [28], and an attempt was made to extrapolate the results to the whole tetrameric cation having an average platinum oxidation number of 2.25. A complete assignment of the single-crystal optical spectrum based on calculations of *Slater* transition states in which spin-unrestricted relativistic SCF-Xα-SW calculations were used, taking into account the polarization and, semi-quantitatively, the intensity of the transitions, was achieved by *Ginsberg et al.* [29]. These calculations have shown that the HOMO containing the unpaired electron is Pt-Pt σ^* in character and is delocalized over all four platinum atoms, allowing α-pyridone blue to be characterized as a *Robin-Day* class III-A compound [30].

Somewhat more loosely related to platinum antitumor drugs were EHMO calculations performed on heterobimetallic Pt-Pd complexes with a bridging methylcytosinate anion by *Mealli*, *Randaccio*, *Lippert*, and coworkers [31][32]. The calculations served to elucidate the metal-metal and metal-ligand binding interactions, and yielded a qualitative interpretation of the ^{195}Pt-NMR chemical shifts.

Several semi-empirical studies of platinum-nucleobase complexes [33–37] were inspired by the idea that the antitumor activity of different platinum complexes could correlate with some fundamental molecular property of the drug itself or of its DNA adduct. Thus, the fact that cisplatin is antitumor-active whereas transplatin is not, was related to *a*) different admixtures of platinum orbitals into the HOMO [33], *b*) the change in atomic charges induced in the platinated nucleobase [36], or *c*) the hypothetical bidentate binding of the *cis*-$(NH_3)_2Pt^{II}$ moiety to the N(7)/O(6) atoms of guanine [34]. In the same spirit, it was found that the antitumor activity of *cis*-[$PtCl_2$(amine)$_2$] complexes correlates with the propensity to bind the N(3) atom of 1-methylcytosine [37]. Obviously, these authors largely underestimated the complexity of the tumor-inhibiting mechanism.

A more extended search for structure-activity relationships was attempted by *Abdul-Ahad* and *Webb* [38], who used one- and two-variable regres-

sions relating the toxicity and anti-tumor activity of a series of *cis*-[$PtCl_2$(amine)$_2$] complexes with fundamental molecular properties such as the electrostatic potential, polarizability, electric field, frontier electron densities, and binding energy, calculated by means of the INDO-SCF method. One prominent and seemingly plausible result was the finding that the toxicity of the compounds correlates with the calculated binding energy, apparently indicating that the less stable the molecule, the higher its toxicity. However, the binding energy was defined as the difference between the total energy of the molecule and the sum of energies of the isolated constituent atoms, which is quite unrelated to the stability of a platinum complex in solution. Thus, the identified correlation is probably rather fortuitous.

Krauss and co-workers [39], and later *Kozelka et al.* [40], performed *Hartree-Fock* (*HF*) calculations on complexes of the formula $[Pt(NH_3)_3B]^{2+}$, where B is a heterocyclic nitrogen base (*e.g.*, pyrimidine, guanine, adenine), using relativistic pseudopotentials and rather limited orbital basis sets, in order to determine force-field parameters for platinum binding to nucleobases, such as the force constant for the bending of the Pt-N bond out of the plane of the heterocyclic base [39a][40], Pt-N(B) stretching force constant [39b] or atomic charges [39b][40]. Recently, *Kozelka* and *Bergès* [41] have questioned the results from these previous calculations, showing that a good description of the electron density for a platinum complex requires extended basis sets and the inclusion of electron-correlation effects. Nevertheless, the energy curve for the out-of-plane bending of the Pt-N(guanine) bond, calculated with the B3LYP hybrid exchange-correlation functional and a relatively extended basis set including polarization functions [42], was very similar to those calculated previously for the Pt-N(pyrimidine) [38] and Pt-N(adenine) [40] bond bending using the limited basis set *HF* calculations, showing that this out-of-plane bending energy is well reproduced already on the *HF* level. The agreement between *HF* and density functional (DF) calculations in this point can be viewed as an endorsement for the recent work by *Chval* and *Šíp* [43], who addressed the pertinent question whether two adjacent purines crosslinked by platinum retain their planar geometry under the constraints imposed by the covalent crosslink on one hand, and the stacking forces on the other hand. The authors used pseudopotential *HF* calculations to show that the purine bases are likely to undergo significant puckering. They suggested that in molecular-mechanics calculations, instead of modeling this puckering explicitly, reduced force constants could be used for the corresponding out-of-plane bending.

Basch et al. [44] used pseudopotential *HF* calculations for a determination of dissociation energies for the Pt-ligand bonds in *cis*-[Pt(OCH_3-*O*)(CH_2OH-*C*)(NH_3)$_2$]. This hypothetical compound models the antitumor-active complexes *cis*-[Pt(ascorbate)(dach)] (dach = cyclohexane-1,2-di-

amine) [45], in which the platinum atom is coordinated by two nitrogen, one oxygen, and one carbon atom. The differentiation between the Pt-N bond *trans* to O (E_{diss} = 42.5 kcal/mol) and that *trans* to C (E_{diss} = 27.5 kcal/mol) followed the trend expected from the order of *trans*-influences (C > N) [46]. However, the absolute E_{diss} values coming from these uncorrelated *HF* calculations should be considered with caution, since they may be underestimated [47].

Molecular-mechanics modeling of oligonucleotides crosslinked at a GG sequence by a *cis*-$(NH_3)_2Pt^{II}$ moiety revealed the possibility of hydrogen bonding between one NH_3 ligand of platinum and the phosphodiester group 5' to the platinated GG dinucleotide [48–50]. Such a hydrogen bond was later found in the crystal structure of *cis*-[$Pt(NH_3)_2${d(pGpG)}] [51]. In aqueous solutions, both direct and water-mediated NH···OP hydrogen bonding is conceivable. *Krauss et al.* [52] investigated these two possibilities by comparing the energetics of a $[Pt(NH_3)_4]^{2+}\cdots H_2PO_4^-\cdots H_2O$ cluster in configurations with direct and through-water ammine-phosphate hydrogen bonding. They concluded that both modes are energetically competitive. This conclusion received support from a combined NMR/molecular-modeling study of a double-stranded decanucleotide-*cis*-$(NH_3)_2Pt^{II}$ adduct [53]. A correlation of the NMR spectra with molecular models indicated an equilibrium between conformations featuring direct NH···OP hydrogen bonding and those where one or more water molecules separated the ammine and phosphodiester groups.

Recently, *Carloni* and co-workers [54][55] have started studies of the chemical bonding in cisplatin and related complexes, using plane waves to expand the electronic wave function. Density-functional calculations [56] with the *Becke-Perdew* [57][58] or *Becke-Lee-Yang-Parr* [59] gradient-corrected exchange-correlation functionals were used to optimize the molecular geometries and to calculate vibrational frequencies. Whereas periodic boundary conditions were used in [54], the calculations done later [55] employed non-periodic boundary conditions [60]. The latter approach yielded for cisplatin a HOMO-LUMO gap of 3 eV, in agreement with the absorption spectra [18], whereas the calculations with periodic boundary conditions gave a somewhat smaller gap (2.3 eV). Another calculation of this type was devoted to the crystal of an adenine-thymine base-pair modified with *trans*-$(CH_3NH_2)_2Pt^{II}$ [61]. The agreement between calculated and experimental platinum-ligand bond lengths and stretching frequencies was fairly good. These calculations were intended as the first step towards *ab initio* molecular-dynamics calculations on cisplatin-oligonucleotide interactions (see *Conclusion* and *Outlook*).

Molecular-Mechanics Calculations

General Considerations

In molecular-mechanics calculations, the atoms are considered to move in a force field defined by an energy function based on classical (rather than quantum) mechanics. Thus, the energy of a given molecular conformation is not calculated in an iterative SCF procedure, as in quantum-chemical approaches, but rather uses an analytical formula based on effective potentials.

Although the principal utility of molecular mechanics lies in its capacity to predict the conformations and dynamic behaviour of macromolecules, some effort has also been expended in the development of force fields for smaller molecules. Rapid predictions of conformations of small systems such as simple metal complexes can be useful in routine work. For deeper insights into the molecular structure, molecular-orbital calculations will usually do a better job. *Cundari et al.* [62] have recently appended the MM2 force field with parameters allowing for the modeling of simple platinum complexes. Among the compounds on which the force field was tested were cisplatin and several other antitumor platinum complexes. Pt^{II}, with its strong preference for square-planar coordination geometry is a 'well-behaved' metal ion, whose implementation into a classical force field is relatively easy. However, when a Pt^{II} complex is incorporated into the framework of a nucleic acid, problems different from those encountered in the modeling of small molecules arise. For instance, when modeling guanine-guanine crosslinks within a DNA double helix, it is necessary to carefully parameterize the force retaining the platinum atom in the guanine planes. This and other specific problems of the molecular modeling of transition-metal complexes with nucleic acids are discussed in [63], together with the presentation of some principal results arising from modeling work on platinum-DNA adducts prior to 1995. Another review on the modeling of Pt-DNA complexes has been written by *Hambley* [64]. A compilation of force-field parameters for the modeling of platinum-guanine complexes has been published by *Yao et al.* [65].

Early Modeling Studies. Comparison between a GG and an AG Adduct of cis-$(NH_3)_2Pt^{II}$

Among the first attempts to model the structural perturbation of DNA following complexation with platinum were those by *Jankowski* and co-workers [66–68], who represented each base, sugar, and phosphate of a DNA strand as a point. This representation ignores the specific energetics of stacking, base

pairing, and deoxyribose puckering. Accordingly, the insights into the structure of DNA-platinum adducts emerging from these calculations were rather limited.

Molecular modeling of the major cisplatin-DNA adduct, the GG intrastrand crosslink, using force-fields with atoms or united atoms (for CH_n groups) as elementary structural units has revealed the occurence of numerous minimum-energy conformations of similar energy [38][48–50]. To circumvent this problem of multiple-minima, *Herman et al.* [53] correlated the structural data from molecular models calculated for the double-stranded decanucleotide d(GCCG*G*ATCGC)·d(GCGATCCGGC) (**1**) crosslinked at the G*G* sequence by *cis*-$(NH_3)_2Pt^{II}$, with those determined from NMR spectra. This approach enabled the authors to conclude that the platinum crosslink bends the double helix towards the major groove (*Fig. 1*). This conclusion was in agreement with the results of electrophoretic mobility measurements on multimers of GG-platinated oligonucleotides [69]. A similar correlation between molecular mechanics models and NMR data was subsequently used by *Fouchet et al.* [70] for the structural analysis of the nonamer duplex d(CTCA*G*CCTC)·d(GAGGCTGAG) (**2**) bearing an A*G*-*cis*-$Pt(NH_3)_2$ adduct. The feature distinguishing the structures of **1** and **2** was the position of the base complementary to the 5′-platinated purine: whereas in **1**, the cytosine complementary to the 5′-guanine of the GG-Pt crosslink was found to oscillate between the positions stacked on the 5′-base ('Model C') and the 3′-base ('Model E') of the unplatinated strand, respectively (*Fig. 2*), the NMR data for **2** indicated that the thymine complementary to the adenine of the platinated A*G* dinucleotide remains stacked

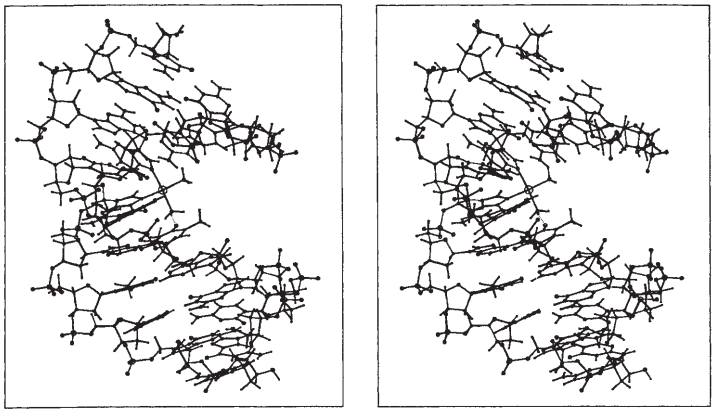

Fig. 1. *Stereoview of one of the model structures* ('Model C_{0N}') *proposed for the oligonucleotide d(GCCG*G*ATCGC)·d(GCGATCCGGC)* (**1**) *bearing a* cis-*$(NH_3)_2Pt^{II}$-G*G* adduct* (reproduced from [53] with permission)

on the 5′-branch (*i.e.*, corresponding to a 'Model E' structure). Evidence for this conclusion included the NOE connectivity linking the H(8)/H(6) base protons with the H(1′) proton of the adjacent base on the 3′-side (a B-DNA characteristic), which was found interrupted at two steps in **1** but only at one

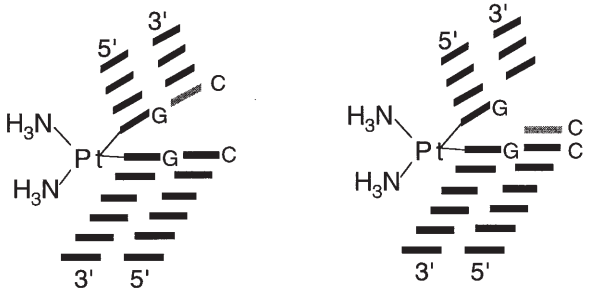

Fig. 2. *Schematic drawing of two possible stacking schemes for oligonucleotides featuring a GG-platinum crosslink*

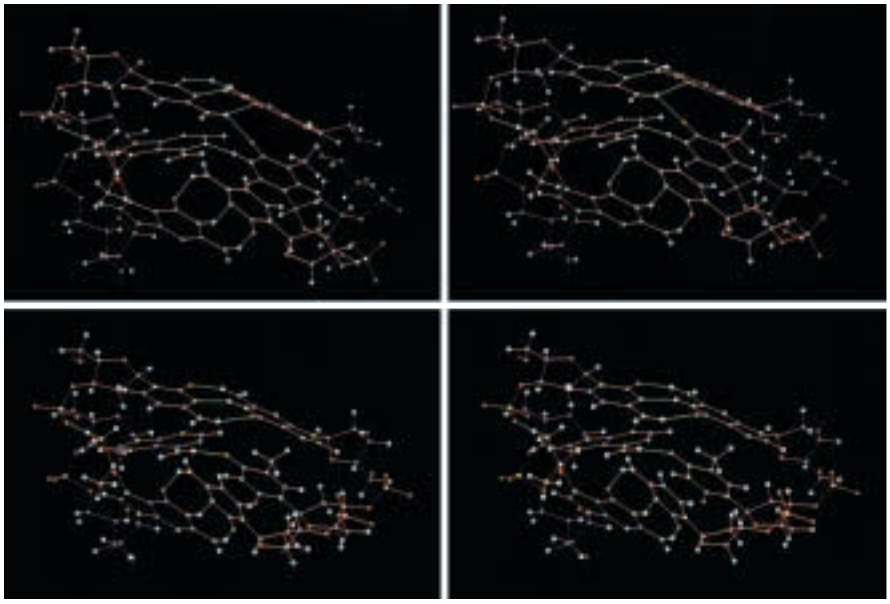

Fig. 3. *Stereoviews of the central trinucleotide d(CA*G*)·d(CTG) of the A*G*-platinated duplex d(CTCA*G*CCTC)·d(GAGGCTGAG)* (**2**). *Top*: energy-minimized model in which the thymine conserves its *Watson-Crick* imino hydrogen bond with A*, whereas the amino hydrogen bond is disrupted. *Bottom*: energy-minimized model in which the thymine retains its *Watson-Crick* amino hydrogen bond with A*, whereas the imino hydrogen bond is disrupted. The H(2′) proton of the cytidine of the platinated strand lies in the shielding cone of the five-membered ring of A* in both models, which accounts for the strong upfield shift observed for its NMR signal (reproduced from [70] with permission).

step in **2**. *Fig. 3* shows two possible arrangements of the central d(CA*G*)·d(CTG) trinucleotide of **2**. The main difference between the two structures shown in *Fig. 3* concerns the hydrogen bonding of the thymine base with its A* counterpart: whereas in the upper model, the *Watson-Crick* imino hydrogen bond is conserved while the amino hydrogen bond is disrupted, the opposite is true for the lower model. In both models, the thymine stacks on the adjacent cytosine, corresponding to 'Model E' (*Fig. 2*).

Problem of Multiple Minima. Combined Use of NMR Spectroscopy and Force-Field Calculations. Chemical Shifts as Structural Indicators

The so-called 'multiple-energy-minima problem' encountered during the early modeling work [38][48–50] and well-known as a classical obstacle in the modeling of macromolecules, has led to the establishement of computer programs allowing for the use of experimental structural data – typically from NMR measurements – as a feedback for model building and refinement. Examples of structures of double-stranded platinum-oligonucleotide adducts solved by means of such programs will be mentioned in the following paragraphs. The article by *L. G. Marzilli* and co-workers in this book brings a detailed account of structural studies on platinum-nucleotide and platinum-oligonucleotide adducts based on NMR techniques.

An NMR/molecular-modeling study of the double-stranded decamer d(CCTG*G*TCC) · d(GGACCAGG) (**3**), crosslinked at the G*G* sequence by *cis*-$(NH_3)_2Pt^{II}$ was accomplished by *Yang et al.* [71]. From 1942 distance constraints from NOE crosspeak integrals it was concluded that the structure could be described by a model similar to one of the interconverting structures ('Model C') proposed for **1** (*Fig. 2*). The same group investigated three palindromic oligonucleotides with 8–12 bases, containing platinum-crosslinked G*G*-sequences in different positions with respect to the middle of the palindrom [72]. The dodecamer $d(GACCATATG*G*TC)_2$ (**4**), bearing a *cis*-$(NH_3)_2Pt^{II}$ moiety on both strands, was shown to form an intact Z-shaped duplex at 2 °C, for which a family of converged models of the 'C'-type were generated using 2236 NOE crosspeak integrals (*Fig. 4*). In a different self-complementary oligonucleotide, $d(ATGG*G*TACCCAT)_2$ (**5**), containing a (en)Pt^{II}-G*G* adduct, which was investigated by *Marzilli* and co-workers [73], the platinum crosslink promotes the formation of a hairpin structure.

One problem with molecular-mechanics and molecular-dynamics calculations using NOE-derived distance constraints arises from the $1/r^6$ dependence of the NOE cross-peak integrals. If the molecule forms an equilibrium between, say, two conformations, one featuring a short and the oth-

er a long distance r_{AB} between the protons A and B, the time-averaged r_{AB} value will be much more similar to the short than to the long one. In such a case, an NOE-based distance constraint will bias the structure towards that with the shorter distance, and energy-minimized models obtained from the trajectory of a molecular-dynamics simulation using this distance constraint will probably cluster around the structure with a short r_{AB}. The other component of the equilibrium, featuring the long r_{AB} distance, may not appear in the obtained selection of models at all. One possibility to circumvent this problem is using time-averaged distance constraints [74]. Alternatively, or in addition, other structural data such as torsion angles derived from *J*-couplings or information obtained from anomalous chemical shifts can be included in the model building procedure. For instance, torsion angles from *J*-couplings were incorporated into the distance-geometry calculations (a strategy of conformational analysis where a distance matrix for the construction of an initial model is subsequently refined by means of force-field calculations [75][76]) used for the modeling of the hairpin-like adduct **5** [73], in the form of distance constraints.

Fig. 4. *Steroview of an energy-minimized model proposed for the doubly-G*G*-platinated oligonucleotide d(GACCATATG*G*TC)*$_2$ (reproduced from [72] with permission)

Our group has demonstrated the utility of the analysis of chemical shifts in the modeling of the *cis*-$(NH_3)_2Pt^{II}$ adducts **1** and **2**, whose NMR spectra featured unusually shielded H(2′)-signals of the nucleotide 5′ to the platinated G*G* of A*G* dinucleotide, respectively. This large shielding could be explained only by the 'E-type' model (*Fig. 2*), which allows the H(2′) proton in question to penetrate the shielding cone of the 5′-platinated base. On perusal of the chemical shifts of the duplex adducts **3** and **4** measured by *Wang*, *Reedijk*, and co-workers [71][72], we observe that in **3**, the H(2′)-signal of T(3) is strongly shielded, as in **1** or **2**, whereas the corresponding proton in **4**, H(2′) of T(8), resonates in the normal range. This lack of shielding in **4** supports the proposed assignment of a 'C-type' structure to the two platinum crosslinks of this adduct. On the other hand, we believe that the shielding of H(2′)[T(3)] in **3** indicates the involvement of the 'E-type' structure in the solution equilibrium. Of particular interest for structural studies of oligonucleotides containing guanine-guanine or adenine-guanine platinum crosslinks are the chemical shifts of the H(8) protons of the platinated purines. These shifts reflect the mutual position of the two bases [77]. Very challenging in this respect are the intrastrand d(GpG) adducts of the *Farrell* compounds $[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}H_2N(CH_2)_nNH_2\}]^{2+}$ (n = 2–6), whose H(8) chemical shifts of the coordinating guanines (Table 1 of [78]) vary with n. For instance, in the d(GpG) platinum chelate of the n = 2 compound, the H(8) resonances are: 5′-G, 8.70; 3′-G, 8.81 ppm, whereas for n = 3, the values are: 5′-G, 8.08; 3′-G, 8.45 ppm. Clearly, the mutual position of the guanines must be completely different in the two cases. Concurring with this conjecture, the ^{31}P chemical shifts, sensitive toward changes in the conformation of the phosphodiester group, are also very different, –3.06 ppm for n = 2 and –4.61 ppm for n = 3. A model has been presented for the n = 6 adduct [78], but the dependence of the chemical shifts on n has not yet been addressed.

Molecular-Modeling Studies on Platinum-Oligonucleotide Adducts Containing Crosslinks of Non-adjacent Guanines

Ptak and co-workers [79][80], and *Van Garderen* and *Van Houte* [81] investigated double-stranded G*XG*-*cis*-$Pt(NH_3)_2$ intrastrand adducts with X = C and X = T, respectively. Whereas in the models proposed for the G*CG* adduct the central cytosine bulged out, in the structure derived from the (more detailed) NMR analysis of the G*TG* adduct, the central thymine was positioned in the minor groove. *Prévost et al.* [82] carried out an internal coordinate molecular modeling study of a double-stranded G*TG* adduct with *trans*-$(NH_3)_2Pt^{II}$. Such adducts have been shown to rearrange to

interstrand G*C* crosslinks [83]. A structural study of such a G*C* interstrand chelate is still missing. On the other hand, an interstrand adduct with a *cis*-$(NH_3)_2Pt^{II}$ moiety crosslinking the two guanines of a central $d(GC)_2$ dinucleotide within a duplex was investigated by two groups [84][85]. Very similar results were found for two different decanucleotides: the *cis*-$(NH_3)_2$-Pt^{II} residue lies in the minor groove, the cytosines complementary to the platinated guanines are extrahelical, and the double helix is locally reversed to a left-handed form, unwound and bent toward the minor groove. *Fig. 5* shows a stereoview of the interstrand adduct as modeled by *Huang et al.* [84].

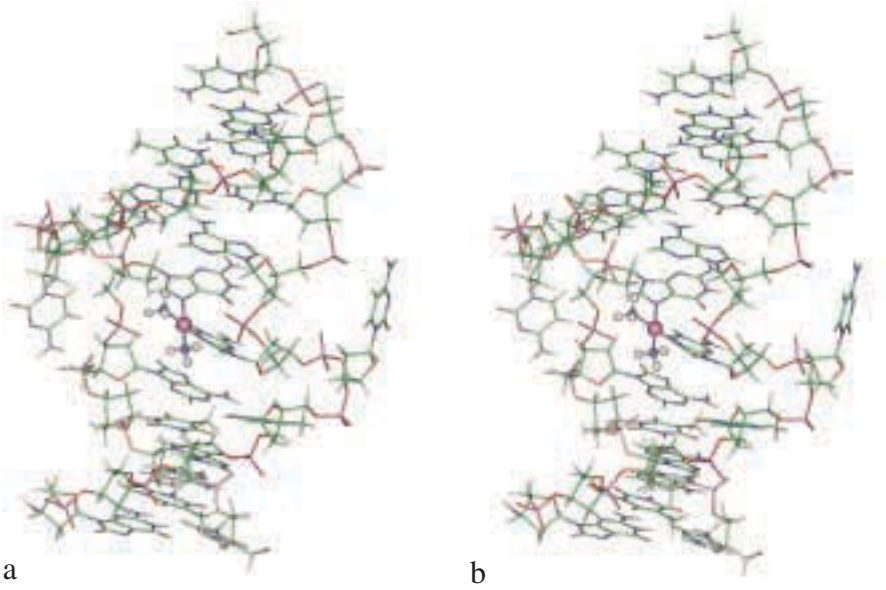

Fig. 5. *Stereoview of one energy-refined model structure proposed for the G*G*-interstrand-crosslinked duplex d(CATAG*CTATG)*$_2$ (coordinates were kindly supplied by Prof. *Gary Drobny* [84])

Yang et al. [86] analyzed the self-complementary octanucleotide $d(CATG*CATG)_2$ with the two G* guanines of the central $d(G*C)_2$ sequence crosslinked by the dinuclear complex $[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}H_2N(CH_2)_4\text{-}NH_2\}]^{2+}$. The nitrate salt of this bis-platinum compound synthesized by *Farrell* and co-workers exhibits strong cytotoxicity against cisplatin-resistant cancer cells [87] and is efficient in forming G-G DNA interstrand crosslinks [88]. The above interstrand adduct features an interesting dumbbell structure.

Platinum-Dinucleotide Complexes

Dinucleotide-platinum complexes have been frequently used as simple models for platinum crosslinks between two nucleobases of DNA. These single-stranded adducts are considerably more flexible than adducts of duplexes and exist in solution usually as equilibria between different conformations. Modeling of such adducts leads to a number of structures of very similar energy. *Kozelka* and co-workers employed molecular-mechanics [77] and molecular-dynamics [89] calculations in order to get insights into the structural details of dinucleotide complexes of *cis*-$(NH_3)_2Pt^{II}$. It was shown that these intrastrand adducts can adopt, in principle, two *head-to-head* (*HH*) and two *head-to-tail* (*HT*) conformation-types. For instance, the NMR spectrum of *cis*-[$Pt(NH_3)_2${d(TpG)-*N3(1),N7(2)*}] shows the co-existence of two slowly interconverting conformers. Correlation of the NMR spectra with the trajectories of molecular-dynamics simulations allowed the two conformers to be unambiguously assigned to two different *HH* structures [89]. The related adduct with d(GpC), *cis*-[$Pt(NH_3)_2${d(GpC)-*N7(1), N3(2)*}]$^+$, features four sets of peaks, indicating that all the four (two *HH* and two *HT*) conformational families interconvert slowly on the NMR scale and have similar energies [90]. On the other hand, the NMR spectra of the *cis*-$(NH_3)_2Pt^{II}$ adducts of the ribo- and deoxyribodinucleotides r(GpG) and d(GpG) consist of single sets of peaks [91][92]. Molecular-dynamics (MD) simulations of these adducts were carried out by our group in an attempt to correlate structural data extracted from NMR (atom-atom distances, torsion angles, atomic positions from the analysis of chemical shifts) with structural information obtained from the MD trajectories [93]. Two obstacles have so far impeded a definitive structural assignment: First, the conformational changes are apparently rapid enough to yield one averaged set of NMR signals, but not fast enough to allow a statistical evaluation from a 1-ns long MD simulation. Second, the number of structural constraints obtained from the NMR data do not unambiguously define a preponderant three-dimensional structure. In an attempt to increase the amount of structural parameters obtainable from NMR measurements, *Dunham* and *Lippard* [94] have used a spin-labeled analog of cisplatin, *cis*-[PtClI(NH_3)(4-aminoTEMPO)] (TEMPO = 2,2,6,6-tetramethylpiperidinyloxy), which forms with d(GpG) two isomeric adducts of the formula *cis*-[$Pt(NH_3)$(4-aminoTEMPO) {d(GpG)}]$^+$. The structure of the 5′ orientational isomer was investigated by means of a 200 ps molecular-dynamics simulation, using long-range distance constraints obtained from the measurement of paramagnetic relaxation effects, and dihedral-angle constraints determined from *J*-couplings measured on the analogous diamagnetic compound with the nitroxyl radical reduced to a hydroxylamine group. This approach relies on the assump-

tion that the reduction of the ligand does not modify the overall structure of the adduct. Since the gross features (*e.g.*, helicity) of the d(GpG) and r(GpG) adducts with *cis*-$(NH_3)_2Pt^{II}$ have been shown to be extremely sensitive to small changes, such as replacing the riboses by deoxyriboses or (de)protonation of the guanines [77][92], it is not *a priori* clear whether, upon the nitrosyl-to-hydroxylamine reduction of the TEMPO ligand, the adduct will stay in the same conformation. The question, therefore, arises, whether the paramagnetic distance constraints and the diamagnetic torsion-angle constraints were measured on the same structure. A dinucleotide complex, with its large variety of energetically accessible conformations, might have been rather a delicate test-case for this original and promising strategy.

Molecular Modeling of Reaction Intermediates

Molecular-mechanics modeling has also been used in the search for clues to the kinetic selectivity of cisplatin. Cisplatin is known to bind preferentially to DNA sequences with two or more adjacent guanines [95][96], forming a GG platinum chelate. 5′-AG-3′ Sequences are also platinated to a minor extent, whereas 5′-GA-3′ chelates occur very rarely. *Laoui et al.* [97] used the AMBER program [98], supplied with *ad hoc* parameters for platinum binding, to model the pentacoordinate intermediate for the platination of the dinucleotides d(GpG), d(ApG), and d(GpA) with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. It was concluded that the platination of both guanines of d(GpG) is favored by hydrogen bonding between one NH_3 ligand of platinum with the N(7)/O(6) atoms of the adjacent guanine. The apparent accord between the differences for the activation energies calculated for A platination in d(ApG) *vs.* 5′-G platination in d(GpG), and for A platination in d(GpA) *vs.* 3′-G platination in d(GpG), with those determined from the measured pseudo-first-order rate constants [98], turned out to be fortuitous. The rate constants for 5′-G and 3′-G platination of d(GpG) had been assigned erroneously (*i.e.*, inversed), as was revealed later by a rigorous kinetic analysis [99]. *Hambley* [100] subsequently pursued a similar approach constructing models for the pentacoordinate intermediates of the *chelation* step at d(ApG) and d(GpA) sequences within a mini double-helix. The observed unfavorable interaction between one NH_3 ligand of platinum and the exocyclic adenine amino group within the GpA intermediate was interpreted as a rationale for the binding preference for ApG over GpA. We believe that this interpretation applies only to the case where an AGA sequence is platinated at the central guanine and the selection between AG and GA cross-linking takes place during the chelation step. This is not likely to be the only factor determining the ApG over GpA selectivity, however, since CGA

and TGA trinucleotides, once platinated at G, should finally yield GpA chelates. ApG sequences are probably favored in the platination step already; when seeking the rationale for this preference, one should model the reaction intermediate for the *platination* step.

The main problem with predictions of kinetic preferences based on force-field calculations of relative stabilities of reaction intermediates arises from the fact that the energies of the competing intermediate structures usually differ by amounts smaller than the accuracy of the energy calculations. These calculations are inherently inexact, since the force-field parameters for the intermediates are mostly unknown (high-level *ab initio* calculations would be needed to determine them). Moreover, the probability that a reaction passes *via* a given intermediate depends not only on its enthalpy, but also on entropy; thus, a dynamic description of the *solvated* intermediate would be required. Finally, even if we knew the structure of a reaction intermediate perfectly, it would always remain an *approximation* for the geometry of the transition state, whose free energy is the real determinant of the reaction kinetics.

Concluding Remarks and Outlook

Molecular-Orbital Calculations

Molecular-orbital calculations on compounds containing third-row transition elements are complicated by three factors. First, the large number of electrons can usually not be treated in an all-electron representation. The approximations used include the effective core potential (ECP) approach [101][102] and the frozen-core approximation [103]. Second, relativistic effects, whose contributions to the orbital energies of the outer electrons are roughly proportional to the square of the nuclear charge [104], are expected to be important. Third, Pt^{II} compounds are characterized by the relatively low energy of d-d excitations (typically 30,000–40,000 cm^{-1}) [105] so that significant mixing of excited states into the ground state and hence a non-negligible impact of electron correlation is expected. We have recently attempted to quantify the impact of electron correlation and of relativistic corrections on the optimized geometry and the calculated dipole moment of a polar platinum complex [41]. Both effects proved to be crucial. Density-functional calculations [56] appear to be a promising alternative to the considerably more expensive classical correlated methods such as the *Møller-Plesset* perturbation theory [106]. From this and other [107] studies, it becomes clear that quantum chemistry on platinum complexes must include electron correlation and relativistic effects. An excellent com-

prehensive review of the challenges of computational chemistry of the heavier elements has recently been written by *Cundari et al.* [108].

Force-Field Calculations

Molecular-mechanics calculations on platinum-oligonucleotide adducts have to deal with problems related to nucleic acids as well as with those arising from the platinum coordination chemistry.

The probably most delicate aspect of nucleic-acid modeling is the treatment of long-range interactions. The particle-mesh *Ewald* method, introduced by *Darden et al.* [109][110] has yielded very promising results in a number of recent molecular-dynamics simulations of oligonucleotides [111].

Platinum(II)-nucleobase complexes can be, in principle, modeled within the framework of a classical force field, thanks to the strong preference of Pt^{II} for the square-planar coordination geometry. However, the parameterization of the interactions within the coordination sphere and of those of the coordination sphere with the surroundings requires some care. Two problems deserve special attention. The first is the calculation of the stretching and bending force constants, and torsion barriers describing the displacements of the nucleobase with respect to the platinum position. The second is the determination of meaningful atomic charges for platinum and its ligating groups. Both parameter groups can be derived using high-quality quantum-chemical calculations, *i.e.*, correlated *ab initio* methods including relativistic effects.

Ab Initio *Molecular-Dynamics Simulations*

Ab initio molecular-dynamics simulations, introduced by *Car* and *Parrinello* [112], have the ambition to model biological systems in laboratory-relevant conditions, *i.e.*, either in solution or in solid phase (see, *e.g.*, [113–115]). Recently, *Carloni et al.* [116] applied this method to a study of the first hydrolysis step of cisplatin. They were able to reproduce satisfactorily the free energy of activation and provided a model for the transition state. Their preliminary results, which include a model of the transition state for the chelation step of the reaction between the diaqua form of cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, and d(GpG), seem to indicate that *ab initio* modeling of substitution reactions on heavy-metal centers may become possible in the near future. The main drawback of *Car-Parrinello* calculations – their considerable computer-time cost – can be expected to abate in the next years

thanks to the progress in the development of parallel program codes and in computer technology. This is good news for coordination chemists interested in reaction mechanisms in general, and for workers investigating platinum-oligonucleotide interactions in particular.

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