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The mechanism of action of platinum anti-tumor drugs

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Abstract. A discussion is presented on the mechanism of action of platinum amine compounds, used as antitumor drugs. The relationship between biological acitivity and structure of the compounds is discussed, with special attention to the nature of the ligands coordinated to platinum. The primary biological target appears to be the interaction with nucleic acids, and therefore studies of the interaction of platinum amine compounds (both active and inactive ones) with nucleic acids and nucleic acid fragments are of great interest. Studies on mononucleotides have made clear that a strong preference exists for platinum binding at guanine-N7 sites. Investigations on oligonucleotides have shown that, when two neighboring guanines are present, chelation of the cis-$Pt(NH_3)_2$ unit (abbreviated cisplatin) is strongly preferred above all other possibilities. Studies on DNA (*in vivo* and *in vitro*) have made clear that similar binding modes occur in DNA and oligonucleotides, and that - after cisplatin binding - the DNA structure is distorted in the same way as double-stranded oligonucleotides.

INTRODUCTION

History

During the last decades it has become clear that platinum amine coordination compounds are very interesting from a medical biological point of view. Rosenberg and his group (ref. 1) initiated the renewed interest in these classical compounds, by studying the growth of *E.coli* bacteria under the influence of an electric field. This field was generated between two "inert" platinum electrodes and aqueous NH_4Cl was used as an electrolyte. The bacteria showed a strong filamenteous growth (ref. 2) which initially was not understood. However, subsequent experiments by the same group soon made clear (ref. 3) that the filamenteous growth was not caused by the electric field, but by the presence of small amounts of dissolved Pt(II) and Pt(IV) compounds in the - corroding - NH_4Cl solution. Detailed investigations showed that the compounds present in solution were, among others, *cis*-$PtCl_2(NH_3)_2$, *trans*-$PtCl_2(NH_3)_2$ and some Pt(IV) compounds. In subsequent microbiological studies the cis-Pt(II) species - now called cisplatin - turned out to be the most active in causing filament formation.

After these findings, tests were undertaken (ref. 4) to study the growth-reducing capacity of cisplatin on animal tumors, such as Sarcoma 180 in Swiss white mice. The results were so positive - in many cases total regression of the tumors was observed - that clinical trials were soon thereafter scheduled and performed. The first studies (ref. 5), demonstrated a remarkable anti-tumor activity for cisplatin. However, large-scale applications had to wait for some more years, since toxic side effects were severe. To be mentioned are: nausea, vomiting, bone marrow toxicity, neurotoxicity and renal toxicity (ref. 5,6).

The clinical application of cisplatin has increased enormously (ref. 6-8), mainly as a result of improved administration procedures and its use in combination therapy, *i.e.* the simultaneous application of a variety of synergistic anti-tumor drugs. Nowadays, usually a dosage of about 100 mg of cisplatin per m^2 body surface area, dissolved in saline, is given intravenously, *e.g.* every month, by standard protocols.

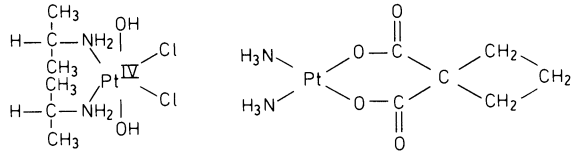
Final approval in the USA in 1979, has led to an increased number of applications of cisplatin and as a result it has become the leading and most widely used anticancer drug (30000 patients are cured each year in the USA). The drug is also registered widely in many other countries. Complete remissions are obtained for testicular cancers in more than 85% of all treated patients, whereas high effectivity in the treatment of ovarian and bladder cancer and considerable activity in osteogenic sarcoma, head and neck cancer, endometrial and cervical cancer and non-small cell lung cancer have been reported (ref. 9).

It should be noted that the corresponding trans isomer of cisplatin exhibits no anti-tumor activity, even though it has comparable chemical and binding properties to DNA (vide infra). The working mechanism of cisplatin has been of great interest for a variety of research groups, involving chemists, biochemists, biologists and medical researchers. The first studies already made it clear that reaction of platinum compounds with nucleic acids plays an important role in the biological effect. The present report deals with the status of the mechanism of action, with special attention to platinum-DNA interactions.

Several reviews have appeared (ref. 5-10) during the last decade, describing a wide variety of aspects of platinum-anti-tumor compounds and of binding of metal compounds to nucleic acids and fragments (ref. 8,10-14). The present paper focusses mainly on the molecular aspects of the mechanism.

Derivatives

Soon after the first reports about the biological activity of the classical compound cis-$PtCl_2(NH_3)_2$, an extensive investigation was started searching for analogs with similar activities and - hopefully - improved properties as a drug. Studies by several groups have resulted in a large variety of analogs with similar biological activities; some of these are used in extensive clinical trials in many countries (ref. 7,9,15). Indications are available that some of these analogs have superior properties in the treatment of tumors in patients who do not respond to cisplatin. Some of the most promising compounds, considered today, are $Pt(NH_3)_2$(CBDCA), (CBDCA = 1,1-dicarboxylatocyclobutane), now also called paraplatin, and CHIP (cis-,cis-,trans-dichlorodihydroxobis(isopropylamine)platinum(IV).) Some examples are presented in Figure 1. Especially the CBDCA derivative is promising because of its low toxicity.



CHIP PARAPLATIN

Fig.1. Schematic structure of derivatives of cisplatin, used in clinical trials and tumor treatment.

The studies with a large number of Pt(II) and Pt(IV) compounds, prepared during the last decade, have made clear that Pt compounds with anti-tumor activity have to fulfil all of the following structural requirements:

1. The two amine ligands in the Pt-compound should be in a cis-orientation. In a didentate chelating ligand, this geometric requirement is automatically fulfilled. The general formulae should be cis-$Pt(II)X_2(Am)_2$ and cis-$Pt(IV)Y_2X_2(Am)_2$. However, the number of variations studied in the case of Pt(IV) is still limited.

2. The ligands X, usually anions, should consist of groups that have intermediate binding strength to Pt(II), or that are - for other reasons - easily leaving (i.e. by enzymatic action). Examples are: Cl^-, SO_4^{2-}; citrate(3-), oxalate(2-) and other carboxylic acid residues. For the Pt(IV) compounds, the Y group is often OH^- (with the two Y ligands in trans orientation).

3. The amine ligands, either monodentate or didentate, should have at least one N-H group, i.e. possess a hydrogen-bond donor function (ref. 16). All compounds with both amine ligands lacking such a H-bond donor property, were found to be inactive. The role of this N-H group in the biological activity, however, is far from being understood. It could be either kinetic (i.e. play a role in the approach of the DNA), or thermodynamic (e.g. give an additional (de)stabilization after binding to the biological target DNA; vide infra). However, also steric effects and/or a role in transport through the cell wall cannot be excluded.

Knowledge of these basic requirements for an active drug, narrows the area in which one could - successfully - search for new anti-tumor active platinum compounds. Up till now, too little is known about the origin of the sometimes severe side effects. These effects cannot yet be predicted on the basis of molecular geometry of the Pt(II) compounds and even the targets (blood, organs, tumor tissue) show a different sensitivity to the drug. Much work remains to be done and is ongoing, e.g. to improve the administration protocol. Research towards new compounds is also focussing on readily soluble derivatives; some of them being Pt(IV) compounds, and have been described in detail (ref. 16-19).

MECHANISM

Hydrolysis and binding targets

The interactions that may occur in living tissue and during the transport of drugs through the body are of great importance to understand the mechanism of action. Most of the present knowledge is based on the behaviour of the classical compound cisplatin in aqueous solution, which may be hydrolysed depending upon the Cl^- concentration and pH in the medium. The hydrolysis reaction scheme of Martin (ref. 20) has been redrawn in Fig. 2.

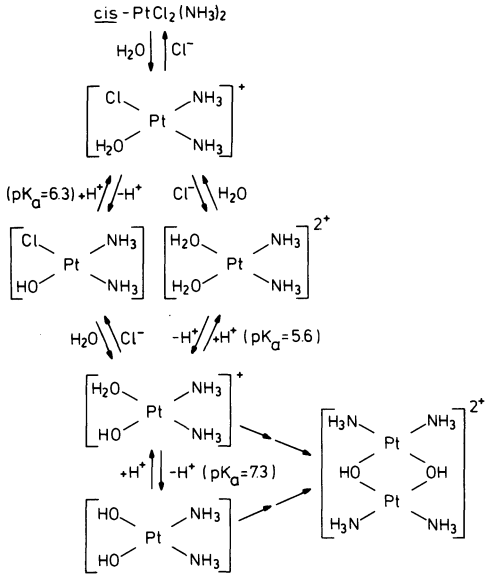


Fig. 2. Hydrolysis reaction scheme of cisplatin.

Under conditions with a high Pt-concentration and high pH values, formation of dinuclear and trinuclear hydroxo-bridged Pt-species does occur (ref. 7,20). However, these species are unlikely to be formed under biological conditions. In aqueous solution cisplatin will loose Cl^-, and aqua or/and hydroxo (at high pH) species are formed. In the body, the Cl^- concentration outside cells is rather high, (about 100 mM), and therefore hydrolysis is largely prevented there, but inside cells the Cl^- concentration is about 4 mM, allowing the hydrolysis process. According to calculations by Martin, the aquated species in the body fluids is present only for a few percent, whereas it amounts to about 50% of the total present inside the cell membrane (ref. 20).

The kinetics of these processes in vivo are not known in detail, but some information is available from in vitro studies. In these investigations cisplatin and analogs were reacted with e.g. nucleotides (such as 5'-GMP). As one would expect, the aqua-species, $[cis\text{-}Pt(NH_3)_2(H_2O)_2]^{2+}$, reacts much faster (ref. 21) with nucleic acids than the dichloro species. Quantitative kinetic information about these processes is still lacking, however. Generally, dilute solutions with a concentration of the Pt compound of about 0.1 - 1 mM in the dichloro form, need at least 24 hours for completion of the reactions with nucleic acid fragments under ambient conditions (pH 7, room temperature), whereas in case of the diaqua form, the reactions may be completed within a few hours (ref. 21-24). Preliminary investigations with analogs of cisplatin have shown (ref. 24), that in the case of carboxylate ligands as leaving groups, reactions with nucleic acids in vitro proceed much more slowly, although these compounds are good drugs (ref. 7,24).

Much less is known about the kinetics of the Pt(IV) drugs, such as CHIP, although evidence is accumulating (ref. 25,26) that binding to DNA only occurs after reduction of Pt(IV) to Pt(II) species. It is evident that much more experimental knowledge in this analytical-physical chemistry area is required.

It is generally accepted that DNA is the most important intracellular target of cisplatin. However, DNA is by far not the only target. Binding to proteins and RNA also occurs, as has been shown by many investigators, (ref. 5-7). In addition, it cannot be excluded that damage to cell membranes is also relevant, although no detailed information is as yet available. The possibilities concerning the cisplatin binding to DNA are schematically depicted in Figure 3. Even though there is good evidence for DNA being the critical target (ref. 2,3,27-31), this does not necessarily prove that this type of binding is the only reaction leading to killing of the tumor cell and the observed side effects in patients treated with cisplatin. It is evident that on the route to the tumor cells, the Pt

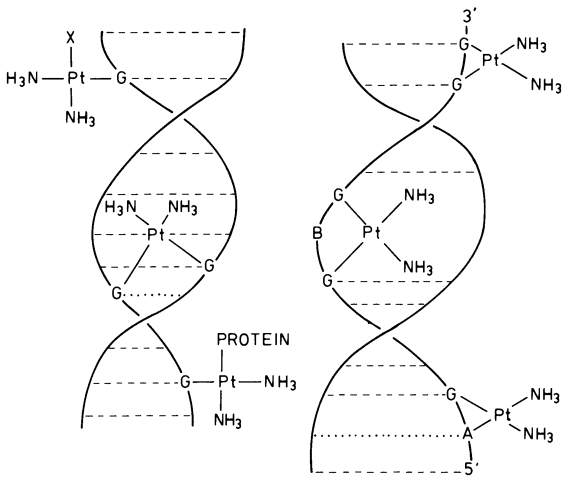


Fig. 3. Schematic binding possibilities for cisplatin to double-stranded DNA.

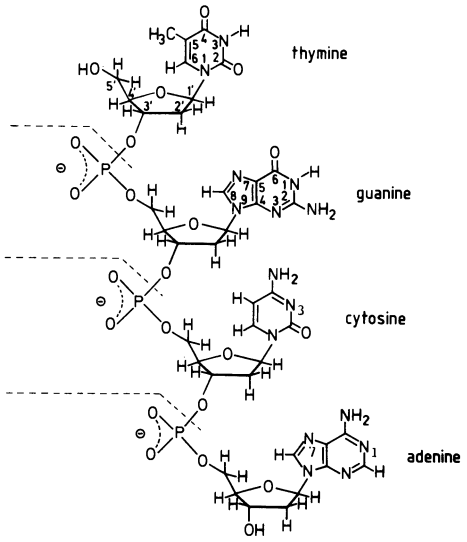


Fig.4. Tetranucleotide showing all the four nucleobases with the used ring numbering.

compounds can interact with e.g. blood proteins and with cell-wall components. These reactions might also contribute to the toxic side effects observed upon treatment of patients with cisplatin and analogs. However, these reactions have hardly been studied and therefore will not be discussed here.

Binding of platinum compounds to nucleobases, nucleosides and nucleotides

The recognition that binding of cisplatin to DNA is important, has directed many studies towards the area of metal-nucleobase interactions. For details the reader is referred to numerous reviews (ref. 5,7,12-14,32). In Figure 4 a tetranucleotide with the four common nucleobases of DNA is drawn with the recommended numbering system.

Metal binding can occur in principle at all sites having a lone pair of electrons. However, in practice metal binding occurs only to a relatively small number of sites, i.e. cytosine-N3, guanosine-N7, adenosine-N1 and adenosine-N7. Binding of metal ions to the sugar ring oxygen atoms has rarely been observed in nucleotides and nucleosides. Binding of metal ions to the phosphate groups in nucleotides is quite common, especially for class A metal ions such as Mg(II), Ca(II), but also binding of Zn(II), Cu(II) and Ni(II) has been observed. In case of Pt(II) - a class B metal -, however, only binding to the nitrogen atoms of the nucleobases has to be considered, although Pt-phosphate interactions do occur in certain cases (ref. 33,34). The role of phosphate under physiological conditions could be only secondary for coordination, but quite important for hydrogen bonding.

Especially the various studies that make use of NMR techniques have contributed a lot to a better understanding of the manner in which DNA fragments react with metal ions. High-field NMR spectroscopy is a very useful tool to determine the platinum binding sites in nucleic acid fragments, since crystal-structure determinations have been reported only for a limited number of cases. The proton NMR evidence for platinum binding to nucleobases can be summarized as follows:

- A downfield chemical shift of about 0.2 - 1.0 ppm for a nearby proton.
- The absence of shifts due to (de)protonation proves platination at that site, i.e. when a metal ion is coordinated at e.g. N7 of guanine, protonation cannot occur anymore at this site, resulting in the absence of a protonation pK_a at about pH = 2. Metal binding can also alter pKa's of sites where no metal is bound, e.g. the decrease of the pK_a at N1 for N7-platinated gunanine residues.
- A characteristic $^{3}J(^{195}Pt-^{1}H)$ coupling, manifested as "satellites" around H8 (34% of isotope ^{195}Pt) is often observed at low magnetic fields. At high fields (i.e. above 1.5 Tesla) this coupling disappears because a scalar chemical shift anisotropy relaxation becomes important (ref. 35).

In biological systems, all kinds of possibilities are present, but only the kinetically preferred ones are expected to be selected. It should be realised that in case of platinum binding, the kinetic effects dominate the binding, since ligand-exchange reactions of Pt(II) are quite slow. In other words: when platinum is attached to a certain site, it will take a long time (hours, days) before the Pt-nucleobase bond is broken. From competition studies, it became apparent that the guanine-N7 binding is strongly preferred under a variety of conditions, followed by adenine-N7, adenine-N1 and cytidine-N3. In addition, the presence of a 5'-phosphate group in a mononucleotide results (ref. 23) in a faster reaction than in the presence of a 3'-phosphate group.

In the past the role of the oxygen atom of guanine, O6, has been the subject of much debate. It had originally been assumed that an intrabase chelate with cis-$Pt(NH_3)_2^{2+}$ and the N7-O6 groups of guanine (see Figure 4) was an important end product, possibly explaining the difference between the cis- and trans-isomers. However, this binding is now generally accepted to be highly improbable and irrelevant, though a hydrogen-bond accepting role of the O6 group has been demonstrated from several X-ray studies of solid products.

An important observation, reported by Cramer and by Marcelis (ref. 23,36), was that in certain cases the rotation about the Pt-N7 bond is slow on the NMR time scale, as a result of steric hindrance in the coordination sphere of platinum. From the fact that DNA reacts with the two available coordination sites of cisplatin, the species formed upon the first binding step should be able to undergo fast rotation about the Pt-N bond in a "search" for a second binding position on the DNA. The fact that with small amine ligands on platinum, i.e. the anti-tumor active compounds, such a rotation is always fast, agrees with the earlier observation (vide supra) that amine ligands with at least one N-H group yield active compounds. Inactive compounds with bulky amine groups not only have different dynamics (rotation about the Pt-N bond), but also yield different structures of the formed adducts (ref. 23,36).

Binding to single stranded oligonucleotides

The knowledge that cisplatin has two binding sites available and that binding to neighboring nucleobases in DNA may occur, initiated investigations on Pt-binding to dinucleotides. In these compounds the second step of the binding (chelation) becomes possible within the same molecule. Of all 16 possible combinations of nucleobases (GG, AG, GA, CG, GC, TG, GT, AA, AC, CA, AT, TA, CC, CT, TC, TT) and other variations (i.e. ribose or deoxyribose; with or without a terminal 3'- or 5'-phosphate group), only a few have been studied in great detail. In addition to dinucleotides, larger oligonucleotides of defined sequences (up to dodecamers) have been used to study the binding of cisplatin, and other compounds.

Important questions addressed in these studies are:

- Is there a kinetic preference for any of these combinations (i.e. will a certain sequence be recognized by cisplatin)?
- What are the binding sites at the nucleobases?
- Are products with different sugar conformations or base orientations (isomers) formed?
- Can a detailed structure be determined, either in the solid state or in solution?
- What is the effect of the binding on the sugar ring?
- What is the effect of phosphate groups on the kinetics and structure of the formed chelate?

Work in this area is still ongoing in many research groups and will only be briefly summarized here. A very interesting observation, first made by Chottard et al. (ref. 37), deals with the occurrence of conformational isomers after chelation of cisplatin to a number of dinucleotides. In certain cases these isomers are rapidly interconverted, but in other cases they can easily be separated by chromatographic techniques, thus allowing characterisation by NMR techniques. Apart from NMR, other techniques - such as CD and IR - may be used to study the structure and conformation of small oligonucleotides.
Using high-resolution NMR techniques it appeared possible to determine the most likely structure in solution for the adduct cis-$Pt(NH_3)_2(d(GpG))^+$. Complete assignment of the proton resonances, decoupling experiments and computer simulations yielded a structural model (ref. 38), depicted in Figure 5, with the following characteristics:

- The two bases, coordinated through N7, are oriented head-to-head with a dihedral angle for the bases of about 60°;
- The sugar ring at the 5' side of d(GpG) has changed to the N-conformer (compared to the S conformer in free d(GpG));
- The other conformational characteristics of d(GpG) are hardly changed upon chelation to cisplatin.

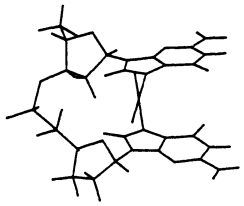


Fig. 5. Projection of a structural model for the $Pt(NH_3)_2(d(GpG))$ adduct, deduced from analysis of the NMR spectra.

From this model it was predicted that the distortion of the DNA structure, after chelation of cisplatin to a GG sequence, would be rather small, probably yielding a kink in the helix of about 40-70°.

Recently, Sherman et al. (ref. 39) have solved the X-ray crystal structure of the adduct cis-$Pt(NH_3)_2(d(pGpG))$. This species differs just one phosphate group with the above-mentioned adducts of d(GpG). The role of this phosphate group appears to be a stabilizing one, i.e. it is involved in a hydrogen bond with a NH_3 ligand at platinum. This could well be an important explanation for the observation that platinum anti-tumor drugs need an acid N-H group to donate a hydrogen bond. The O_3P-O--H-NH_2 interaction could stabilize and induce the distortion of the DNA, thereby interfering with replication processes (vide infra). The most important finding of this study, however, appears to be that the solid-state geometry is substantially the same as the one in solution. Part of the solid state structure is redrawn in Figure 6, based on the coordinates of Sherman et al. (ref. 39).

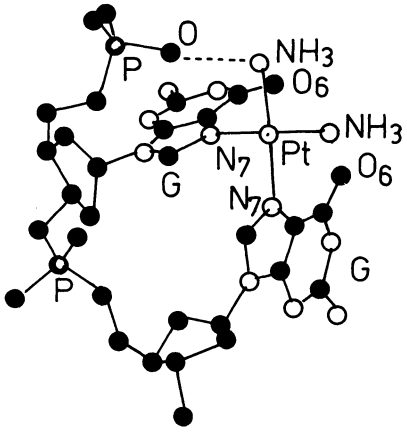


Fig. 6. Projection of the structure of cis-$Pt(NH_3)_2)(d(pGpG))$ as determined from single crystal structure analysis.(ref.39).

The analogy with Fig. 5 is clearly observed. Finally it should be mentioned here that such a distortion for a GG-chelate has also been calculated using molecular mechanics (ref. 40).

Four major questions have been adressed in studies dealing with trinucleotides containing:

1. What is the conformational effect of a third terminal base on the chelating properties of a -GG- unit?

2. Knowing the strong preference of cisplatin for guanine-N7 binding, does chelation occur to two guanines separated by a third base?

3. Knowing that chelation to AG-units - but NOT GA - occurs in vitro (see below), what are the products of reaction when GAG is treated with cisplatin?

4. Can chelation of the trans-isomer to GNG occur through both guanine-N7 atoms?

To study the first question, the trinucleotide d(CGG) was treated with cisplatin and the formed GG-chelate was investigated by X-ray diffraction techniques. It appears that again the 5'-G has a N-type sugar ring as a result of chelation, whereas the cytosine residue is involved in several hydrogen bonding and stacking interactions (ref.41); NMR studies had earlier indicated a similar structure for the GG-part (ref.42). The chelate formed with the G(2)G(3)-unit is roughly the same as the one found with d(GG) (see above). A structure projection is depicted in Figure 7. Our earlier findings (ref. 29) that cisplatin induces base-pair substitutions in -GCG- and -GAG- base sequences in bacterial DNA (E.coli) and has mutagenic properties, has led us to a study of cisplatin interactions with d(GCG), d(GTG), d(GAG) and d(pGGG). In all cases chelation through the guanines has been observed (ref. 43). Recent investigations (ref. 43) on d(GAG) have shown that cisplatin also has a tendency to form a AG-chelate (20%, compared with 80% of a chelate through both guanines).

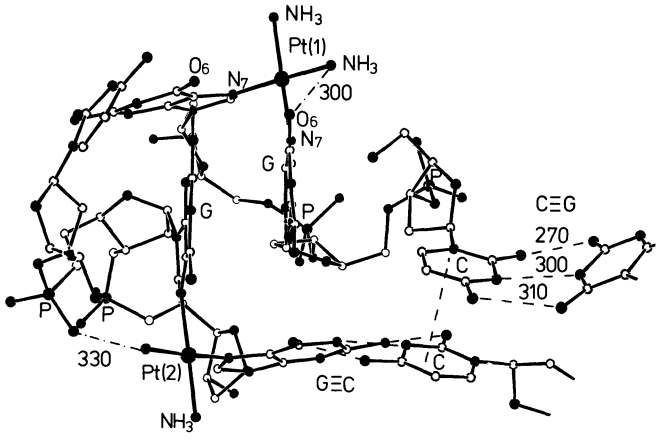


Fig. 7. Projection of one part of the X-ray structure of the adduct cis-$Pt(NH_3)_2$(d(CpGpG))

The first studies with larger single-stranded oligonucleotides in fact dealt with self-complementary (i.e. forming a duplex with itself) tetranucleotides and hexanucleotides, viz. d(CCGG), d(AGGCCT) and d(TGGCCA) (ref. 44). In all these studies it became immediately clear that the double helix is disrupted upon chelation of the cisPt unit at the GG site. Again NMR proved to be a very useful technique to study these reactions and their products. Another technique that can be applied to determine the binding sites of cis-$Pt(NH_3)_2^{2+}$ in oligonucleotides, is based upon the application of enzymes that digest the DNA (or oligonucleotides) to smaller fragments, some of which contain platinum species. We have first introduced this technique (ref. 45) for the analysis of cisplatin-treated salmon sperm DNA. Since then it has also been applied for the analysis of platinated oligonucleotides (ref.46-48). Because large oligonucleotides are needed to maintain a double-stranded structure after platination, more attention has been given recently to octa, deca, undeca and even larger oligonucleotides (ref. 49-54). Since these larger fragments are usually studied in connection with a (self)-complementary strand, their results will be treated in the next section.

Binding of platinum compounds to double-stranded oligonucleotides

As discussed above, the first investigations on oligonucleotides have made clear that - at least up to hexamers - platination results in disruption of the double-helical structure. One should realize, however, that in these self-complementary oligonucleotides 2 Pt groups will chelate. Therefore, we have undertaken a study with a duplex fragment, having a centrally located GG-unit in only one strand (ref. 49,50,53). The performed reactions are depicted schematically in Figure 8.

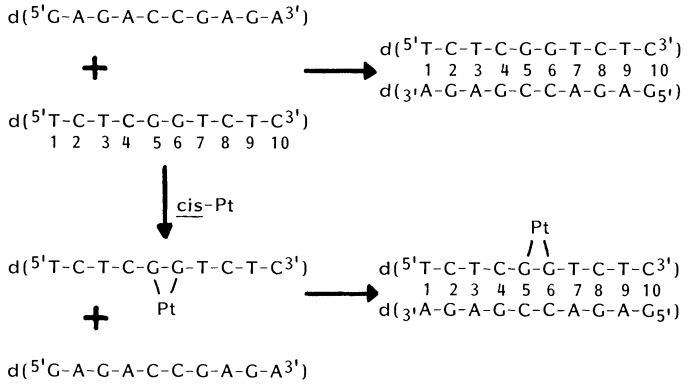


Fig. 8. Representation of the reaction of a decanucleotide with cisplatin.

Upon reaction of cisplatin with the strand d(TCTCGGTCTC) a high yield of the GG chelate is formed, as would be expected; subsequent addition of the complementary strand d(GAGACCGAGA) apparently results in duplex formation. Detailed analysis of proton and phosphorus NMR spectra and consideration of the CD spectra, has resulted in the following conclusions:

1) The double helix is somewhat destabilized, compared with the unplatinated duplex, as seen from the decrease in melting temperature of about 10-20°.

2) Observation of the imino-proton resonances - though somewhat shifted and broadening at low temperatures - of the central G:C base pairs, indicates that hydrogen bonding (Watson Crick type or related) remains possible after platination.

3) Careful analysis of the chemical shifts and coupling constants after platination, suggests a distortion of the double helix which is rather small and which can - at the moment - best be described as a kink in the helical axis of about 40-70°, without a large change in overall helical unwinding.
4) Comparison of CD spectra and ^{31}P spectra of platinated DNA (from several sources) and the platinated ds decanucleotide, strongly suggests similar distortions in both cases (ref. 50,55).

In an independent study, using the platinated octanucleotide d(GATCCGGC) and the complementary decanucleotide strand d(GCCGGATCGG), Chottard (ref. 51) arrived at similar conclusions. Studies with the adduct of cisplatin with d(GCCGGATCGC) complemented with d(GCGATCCGGC) clearly showed early metling of the inner G:C base pairs (ref. 54).

In a related piece of work we have platinated the undecamer d(TCTCGTGTCTC) and have concluded that a chelate using both guanosines (a GBG chelate) is again formed, despite the T in between them (ref. 52). Now addition of the complementary d(GAGACACGAGA) again results in duplex formation. However, the CD spectrum has significantly changed compared with the unplatinated duplex and this change is different from the change in the case of the decanucleotide (ref. 49). Detailed study of the proton NMR spectrum in the imino region indicates that two H-bridges between the bases have disappeared and that the helix melting temperature has decreased by about 25°C. Apparently the central T:A base pair cannot be formed at all, since T is bulged out as a result of chelation of the guanines to cisplatin. Addition of the almost complementary decanucleotide d(GAGACCGAGA) did not result in a better duplex formation (ref. 52).

Recently also Kidani (ref. 56) has shown - by using enzymatic digestion and HPLC - that cisplatin binding to self-complementary d(GGTCGACC) and to d(CGGATCCG) results mainly in cisPt-GG adducts.

From all the above-mentioned results the following general conclusions can be drawn:

1) When adjacent guanines are present in an oligonucleotide, cisplatin chelates to these dinucleotides at the N7 positions.

2) When GNG sequences are present in an oligonucleotide, cisplatin chelates also to both guanines; when the central base is adenine then also an AG chelate can be formed.

3) Double-stranded oligonucleotides may be formed with the above-mentioned platinated GG- and GNG-containing oligonucleotides (at least 8 units seem to be required).

4) The distortion of the double helix is rather small in case of GG-chelation by cisplatin. A larger distortion occurs in the case of GTG-chelation.

5) Melting of the double helix appears to start from the central part where the GG-Pt unit is located.

Binding of platinum compounds to DNA and RNA *in vitro*

As soon as it became apparent that the cisplatin-DNA interaction is an important event in the cytostatic process, investigations were started using DNA from several sources. In this short review only attention will be given to recent spectroscopic studies dealing with DNA and RNA binding, with special emphasis on the molecular details. Other aspects have been treated in earlier reviews (ref. 1,3,7)

Upon binding of cisplatin to DNA in vitro (either ss or ds) one may monitor the differences in spectroscopic properties. A very sensitive technique has been shown to be circular dichroism spectroscopy (ref. 37,57). In fact, early observations (ref. 57,58) already dealt with the enhancement of the ellipticity at 275 nm, indicating the occurrence of intrastrand cross linking. Our results on the decanucleotide show the same CD effects (ref. 49,53) after platination, suggesting similar distortions in the decamer nucleotide and in DNA.

More recently it has become possible to use ^{31}P NMR (ref. 50,55) to study DNA and - under comparable conditions - double-stranded oligonucleotides. As already discussed above, the first results indicate that similar distortions occur in DNA and oligonucleotides. It is even possible now (ref. 59), applying special proton NMR techniques, to use imino proton signals to study the distortions induced by cisplatin. This has been nicely shown for ribo-poly(I):poly(C) by Marzilli (ref. 59). In all these studies one should realize, however, that under in vivo conditions the loading of the DNA by cisplatin is much lower than in the DNA samples, which are suitable for spectroscopic studies. Therefore, one should be careful in extrapolating such results to in vitro mechanisms.

A different approach to study the cisplatin binding positions in DNA is based upon degradation of high-molecular weight DNA after platination, and analyzing the fragments - after separation - by a special technique, such as NMR. In our laboratories a method has been developed (ref. 45,60) to degrade cisplatin-treated DNA with enzymes to ((P1=98/0)) mononucleotides and Pt-containing mono- and dinucleotides. This mixture was subsequently separated on the basis of their net charge by anion-exchange chromatography. Four reaction products of cisplatin with salmon sperm DNA were isolated, together comprising at least 90% of the total platination. The structures of these adducts have been unambiguously proven by proton NMR spectroscopy and Pt-analyses with atomic absorption spectroscopy (AAS). In all cases the Pt was linked to the N7 atom of guanine or adenine and no indications have been found for binding at adenine-N1 or cytosine-N3 at the level of platination studied (up to 6 Pt atoms per 100 nucleotides; r_b = 0.06).

The formation of intrastrand crosslinks on GG and AG base sequences in DNA could be deduced from the presence of the adducts cis-$Pt(NH_3)_2$(d(pGG)) and cis-$Pt(NH_3)_2$(d(pAG)) in digests of platinated DNA. However, no indications for the presence of GA adducts could be found, and therefore crosslinks with a GA sequence are unlikely.

The formation of interstrand crosslinks in DNA, also demonstrated with alkaline elution techniques (ref. 5,31,61), could be related to the presence of the adduct cis-$Pt(NH_3)_2(dGMP)_2$. This product might originate from two guanines on different strands. The same Pt-digestion product, however, would also be obtained from intrastrand crosslinks on two guanines separated by one or more nucleobases. The occurrence of this type of crosslinks in DNA was deduced from mutation studies in bacteria (ref. 48), and proven in oligonucleotides (see above).

Already some years ago we observed (ref.45) that the results of enzymatic disgestion of cisplatin-treated DNA from several sources, result in larger amount of GG-adducts than would be expected from a random approach of the G-sites of the DNA by the cisplatin unit. In other words, when approaching the DNA, the cis-$Pt(NH_3)_2$ unit already "knows" which G is part of a GG-fragment (preferred) and which one is not. The molecular basis of this selectivity is not yet understood. It has also been observed under in vivo conditions.

Reactions of platinum amine compounds *in vivo*

Apart from the binding of cisplatin and analogs to DNA in the tumor cell - which is generally believed to be the origin of the antineoplastic activity - several other events will take place after administration of the drug into the body (usually through infusion in the blood stream). A large variety of reactions may take place during the transport through the body via the blood. As discussed above, hydrolysis reactions are severely suppressed because of the high Cl^- concentration in the blood. Nevertheless, reactions with e.g. blood proteins or peptides may occur, although systematic studies about the molecular basis of these reactions are not yet available. Likely candidates for binding may be the thiol and thioether groups of proteins and peptides, which are known to have a high affinity for platinum.

During the first 24 hours after the application of the drug, already 30-70% of the administered platinum is excreted from the body, mainly through the kidneys. Initially this was leading to a severe problem, because of the resulting kidney damage. Nowadays, the patients receiving cisplatin are simultaneously treated with a high dose of fluid that prevents this damage (ref. 7). Other toxic effects observed in the early days, when high doses of cisplatin were administered, were probably due to the formation of dinuclear hydroxo-bridged Pt-species (ref. 6,7). In later administration protocols, the platinum concentrations were lowered, so that only monomeric species are supposed to enter the body. With the new reagent paraplatin (see figure 1) higher doses seem possible (ref. 62).

The circulating platinum species is transported - most likely as a neutral species - into both tumor cells and normal cells, and then undergoes hydrolysis inside the cells (see Figure 2). Subsequently, the hydrolyzed Pt-species can react with all kinds of cell components, such as peptides, proteins, DNA and RNA.

Although the binding to DNA appears to be responsible for the ultimate cell killing, it cannot be excluded that reactions of the Pt species with other molecules are leading to toxic side effects. These effects can be classified as acute, subacute, and chronic. The molecular basis of these toxicities are far from understood, although medical research has resulted in protocols that reduce the effects enormeously; (e.g. the pretreatment hydration of the patient, which almost avoids nephrotoxicity).

Recently, it could be established that in living cells the same types of Pt-adducts are formed as in DNA treated with cisplatin in vitro (ref. 63,64). Also the strong preference of cisplatin to form intrastrand crosslinks on GG base sequences is found upon interaction with DNA in vivo.

The nature of the difference in response to Pt-chemotherapy between tumor and normal cells is still unknown. It is possible that rapidly dividing (tumor) cells are more susceptible, because of the inhibitory effect of Pt-DNA adducts during the DNA replication - a prerequisite for cell devision. Another explanation may be a difference in the capacities of the various cell types to remove Pt-DNA adducts. The influence of DNA repair systems on the survival of cisplatin-treated cells has clearly been demonstrated in E.coli bacteria (ref. 29) and in cultured human cells (ref. 31).

The question which of the induced Pt-DNA adducts is (are) responsible for the antitumor activity is still unanswered. Many investigators have tried to correlate interstrand DNA and DNA-protein crosslink formation with the cytotoxic action of cisplatin, but conflicting results were obtained (ref. 8,31). Untill now a biological role inside the cell has been found for only one type of Pt-DNA adduct. Brouwer et al. showed (ref. 29) that cisplatin can induce base-pair substitutions in E.coli bacteria at GAG and GCG base sequences. This strongly suggests that the intrastrand crosslink of cisplatin on GBG is responsible for this effect.

Studies are ongoing to establish not only the induction but also the repair of the various Pt-DNA adducts, because it is assumed that incorrectly repaired and non-repaired, persistent, lesions will be responsible for the antitumor effects of the drug. For the study of the Pt-adduct formation and repair in DNA of cells treated with biologically relevant doses, e.g. from patients treated with cisplatin, very sensitive detection methods are required. For this purpose immunochemical techniques have been developed with which, at the moment, femtomol (10^{-15} mol) amounts of the Pt-adducts in DNA can be detected with anti-Pt-adduct antibodies (ref. 63,65,66).

Differences between *cis* and *trans* isomers and the role of hydrogen bonding

It has been known for some time that also trans-$PtCl_2(NH_3)_2$ binds to DNA (ref. 5,8). It is likely that the first binding step does not differ very much from that of cisplatin. With mononucleotides also trans-$PtCl_2(NH_3)_2$ easily forms 1:2 adducts, and these differ hardly from those derived from cisplatin. Also the kinetic differences of the cis- and the trans-isomer hardly differ. The biological effect of trans-$PtCl_2(NH_3)_2$ is quite interesting and DNA binding studies deserve detailed study. Since it is clear that the binding affinities to the nucleobases are quite comparable, the different anti-tumor activity must originate from differences related to the bifunctional binding. Comparative studies with trans- and cis-platin on single-stranded and double-stranded oligonucleotides are beginning to contribute to the understanding of these differences. It has recently been shown (ref. 67) that also trans-$Pt(NH_3)_2^{2+}$ can chelate to GNG sequences through both guanine N7 atoms. The distortion of DNA after such a trans binding is likely to be very large. This has put forward the hypothesis that repair enzymes recognize and remove the trans compound easier than the cis compound. Replication, however, would not proceed when a cis-$Pt(NH_3)_2^{2+}$ unit is attached to a GG unit.

The above mentioned influence of the N-H group in the amine ligand of the Pt-compound may have an indirect role in the binding to nucleic acid fragments and to DNA. On one hand it could have a directing effect on the approach to the DNA, by having a H-bond interaction with guanine-O6; this possibly explains the kinetic preference of platinum compounds for guanine-N7. On the other hand, after formation of a bis-nucleotide adduct, it may have a H-bond interaction with a phosphate group, as has recently been found (ref. 39) in a crystal structure determination of cis-$Pt(NH_3)_2$(pGG-N7,N7) and was expected (ref. 40) from molecular mechanics calculations. Our work (ref. 41) on the structure of the adduct with CpGpG conforms this strong influence of hydrogen bonding. Several crystal structures of mononucleotides have shown earlier the great importance of hydrogen bonds in the solid state (see e.g. ref. 13,14,32).

REMAINING QUESTIONS

The progress made in the understanding of how cisplatin operates during the last decade, i.e. the period that covers the first clinical trials and the world-wide application of cisplatin and derivatives, is impressive. Nevertheless, the number of important questions that is still unanswered remains large, and much work needs to be done by combined efforts of chemists, pharmacologists, and biomedical researchers. Topics that are likely to be important for medical application, will deal with:

- the mechanism of action and side reactions in vivo of cisplatin and the origin of discrimination by the compound - if any - between a tumor cell and a normal cell.
- the differences in response between the various tumor types.
- the resistance of certain patients for this type of chemotherapy, already present at the onset of the treatment, or rising during the treatment procedure.
- the understanding of the different behaviour for related compounds.

Some other important questions that need an answer, are:

- Is there any discrimination by cisplatin between the cell wall of a tumor cell and a normal cell? I.e. can cisplatin recognize or select (certain) tumor cells?
- What is the relevance - if any - of cisplatin binding to other molecules than DNA (small and large ones) present in the cell?
- Does a molecular basis exist for synergism of cisplatin with other drugs?
- What kind of processes are involved in deplatination of the DNA, i.e. what are the mechanisms leading to repair of the DNA-cisplatin binding?
- What is the effect of the binding of cisplatin on DNA processing and cell division?
- What is the influence of DNA-platination on transcription of DNA and on translation of the RNA to produce proteins?

These questions will generate a large amount of exciting research problems for inorganic coordination chemist, especially in the area of structure and kinetics.

ABBREVIATIONS USED

Cisplatin	cis-$PtCl_2(NH_3)_2$	Am	amine ligand
CHIP	$PtCl_2(OH)_2$(isopropylamine)$_2$	ss	single stranded
CBDCA, paraplatin	cis-$Pt(C_6H_6O_4)(NH_3)_2$	ds	double stranded
G	guanine and guanosine	N	any nucleobase
GMP	guanosine monophosphate	C	cytidine and cytosine
B	any nucleobase except A	T	thymine and thymidine
A	adenine and adenosine		

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