Biomolecular Targets for Platinum Antitumor Drugs

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Abstract: *Cis*-diamminedichloroplatinum(II) (cisplatin) is widely used for the treatment of testicular, ovarian, and other forms of cancer. Several second generation platinum centered antitumor drugs have been approved or undergoing phase-3 clinical trial. Cisplatin arrests the cell cycle at the G2 phase by a mechanism commonly known as apoptosis. At the molecular level, it is generally believed that the anticancer properties of these compounds are due to the covalent binding to DNA. In addition to DNA binding, the platinum drugs bind and interact with proteins and enzymes. The toxic effects of the drugs have been usually attributed to protein binding. However, a growing body of work points to much more complex anticancer mechanisms involving direct and indirect interactions of platinum compounds with proteins and enzymes. In this review, a discussion on the strength and weaknesses of DNA binding mechanism followed by enzymes and protein interactions with the drugs are presented for the comprehensive understanding of apoptosis. The purpose of this review is to encourage researchers to explore metallobiochemistry of platinum drugs focusing attention to cellular and molecular events beyond DNA binding.

The discovery of the anticancer activity of *cis*-diamminedichloroplatinum(II) over some thirty years ago [1a] has generated intense interest in exploring the metallobiochemistry of this soft metal center [1b]. This platinum compound is the choice for treatment of various cancers especially testicular and ovarian cancer [2-4]. Carboplatin and iproplatin, two second generation drugs, show reduced toxicity in most cases over the original compound. A new oral drug, JM-216 has currently attracted considerable interest as well [5]. Structures of some highly active platinum compounds are shown in Figure **1**.

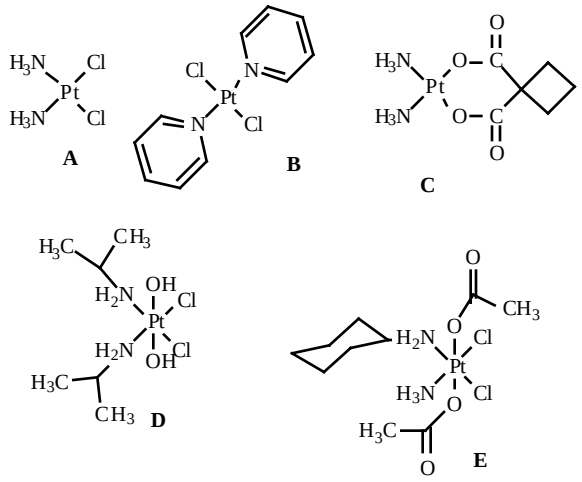

Fig. (1). Structural formulas of some representative platinum(II) and platinum(IV) anticancer drugs: A, cis-diamminedichloroplatinum(II) (cisplatin); B, trans-dichlorodipyridineplatinum(II); C, cis-diammine(1,1-dicarboxylatocyclobutane)platinum(II) (carbopatin); D, cis,trans,cis-dichloro- dihydroxo-di(isopropylamine)platinum(IV) (iproplatin); E, trans-diacetato-(ammine)- cis-dichloro-(cyclohexaneamine)platinum(IV) (JM-216).

Cisplatin attests the cell cycle at the G2 phase [6]. Apoptosis has been shown to be the key cellular event responsible for exhibiting the anti-cancer activity of cisplatin. Covalent binding to DNA is believed to be a key molecular mechanism [7], however, mechanisms based on binding to other biomolecules have yet to be ruled out [8]. Although significant progress in understanding the nature of platinum-DNA interactions has been made, a complete understanding of cellular and molecular biochemistry is yet to be achieved. In fact, the DNA binding model does not adequately explain many experimentally observed cellular and molecular events. In this review article, a brief description of the strength and weaknesses of the DNA binding model is presented first, followed by a few alternative mechanistic models with supportive evidence are also discussed. Finally, some key issues are brought to light for pointing future directions of research.

1. THE ACTIVE FORM OF CISPLATIN

The platinum anticancer drug is administered to patients through intravenous injection. In the extracellular environments, the platinum compound experiences high chloride concentration (~100 mM) and does not undergo appreciable hydrolysis. Under this condition, substantial loss of platinum due to reactions with proteins and extracellular enzymes take place. In fact, many toxic effects of this compounds are attributed to binding to enzymes. When cisplatin passes the cell membrane and experiences reduced intracellular chloride concentration (~10 - 20 mM), the compound undergoes some hydrolysis. In aqueous solution at physiological pH, the fate of cisplatin [9a] can be depicted schematically in Figure **2**. Since the monoaqua-

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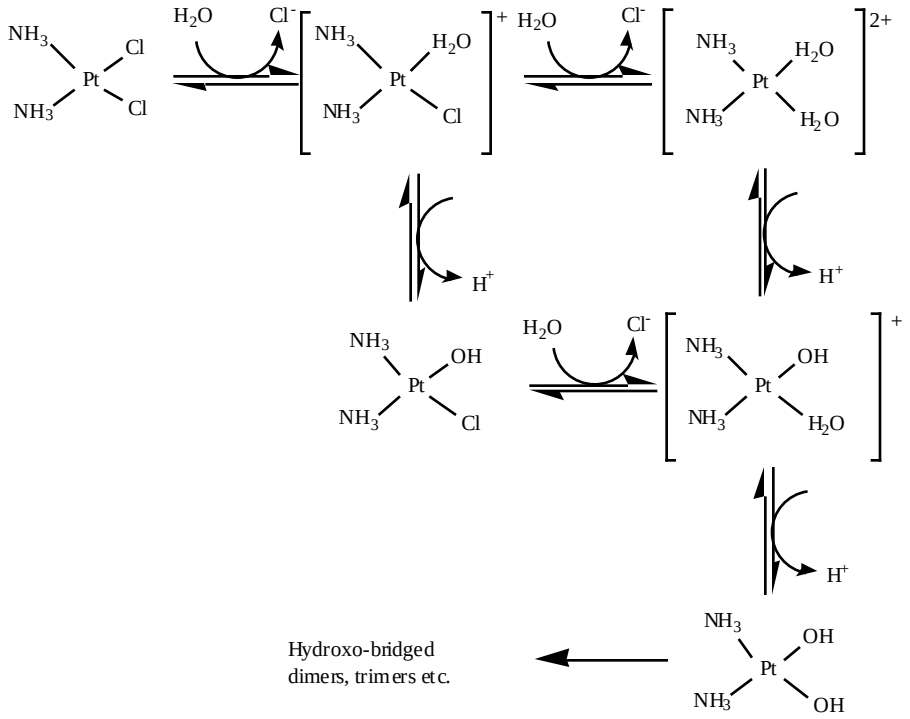

Fig. (2). Schematic representation of the fate of cisplatin metabolism in aqueous solution at physiological pH.

chloroplatinum(II) is highly reactive even toward poor nucleophile, it is highly unlikely that the diaqua-species is ever formed in the cellular milieu. However, many studies have been conducted based on the assumption that diaqua-platinum complex is the reactive species. Since the reactivity and selectivity of dichloro, chloro-aqua, and diaqua toward various biomolecules are quite different, conclusions reached from the use of the last mentioned platinum complex may not be extended to cellular milieu. Once the biomolecule(s) reacts with the monoaqua(or hydroxo)-chloro species, the second chloride is replaced either via a second aquation process or direct reaction with the chloro species based on the reactivity of the nucleophiles. Figure **3** shows a general scheme for the anticipated pathways of the reactions with cellular molecules.

The second platinum drug, carboplatin, undergoes much slower hydrolysis than cisplatin [9b]. Since the DNA reactions are primarily limited by the hydrolytic pathways, the reaction between carboplatin and DNA is extremely

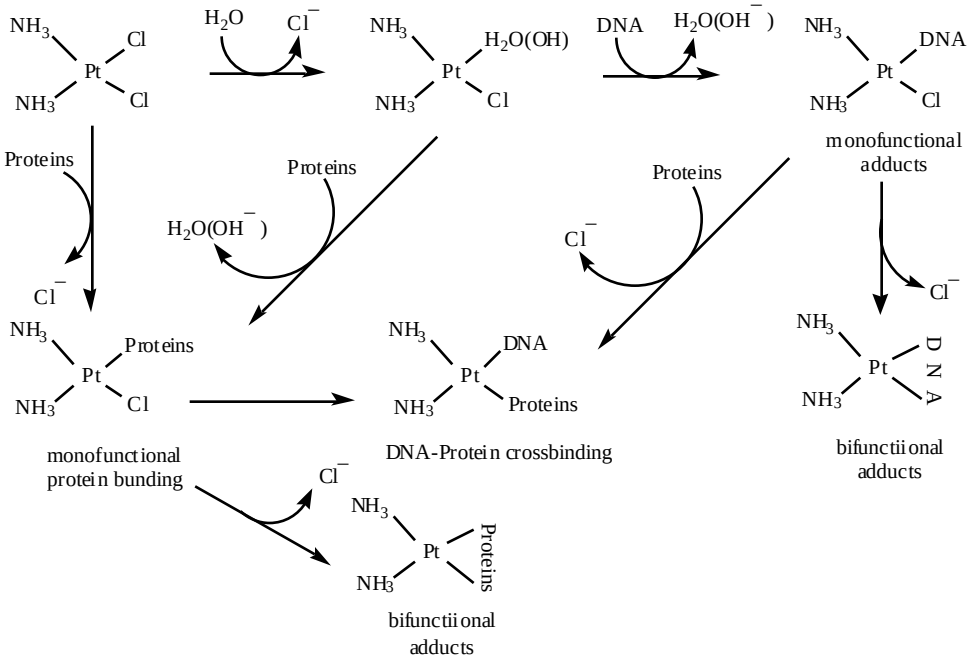

Fig. (3). Representation of various reaction pathways for forming DNA and protein adducts.

slow. For example, the half life of carboplatin reaction with DNA is estimated to be 21 days. The third platinum drug, iproplatin, is believed to undergo reduction first by cellular reducing agents such as ascorbate, glutathione, and cysteine to generate active platinum(II) compounds with analogous structural features to that of cisplatin [10,11]. In particular, it is assumed that such reductions afford *cis*-dichloro-di(isopropylamine)platinum(II).

2. DNA BINDING MODEL

A great deal of efforts has been made to pinpoint the biological target(s) for cisplatin. An initial hint for DNA as a possible target came from the original experiments by Rosenberg and co- workers [1] in which these authors observed filamentous growth of DNA, commonly observed by DNA damaging agents. Subsequently, through a classic experiment, LeRoy *et al.* [12] followed the fate of cisplatin in Hela cells. These authors have isolated both DNA- and protein-bound platinum species. However, based on the quantitative arguments, these authors concluded that DNA binding is the key lesion since more platinum was bound to DNA than protein. Although the conclusions reached by these authors are ambiguous as discussed below, most workers in this field has accepted the conclusion that the DNA is the target for cisplatin [7,13,14].

Since it is generally accepted that DNA is the cellular target, extensive *in vitro* experiments have been carried out to pinpoint the binding sites in DNA [15], kinetics of DNA reactions [16,17], structural changes associated with such binding [18-21], and consequences of DNA binding on replication and repair machineries [22-25]. These experiments revealed a variety of binding modes in DNA and proteins which are shown in Figure **4**. These binding modes include inter- and intra- strand bifunctional binding to DNA, DNA-protein cross binding, monofunctional adducts etc [12, 26,27]. Among the various DNA binding modes, intrastrand binding to adjacent guanine bases is accounted for 60 - 70% DNA binding. The second most abundant binding (10-20%) is through the adjacent guanine and adenine bases. Other binding modes, including interstrand binding through the two G residues, intrastrand binding through two G bases intervened by a third a nucleotides, have been observed.

It is generally believed that Ptd(GpG) lesion is the key binding mode for exhibiting cytotoxicity. This intrastrand binding mode severely distorts the DNA double helix

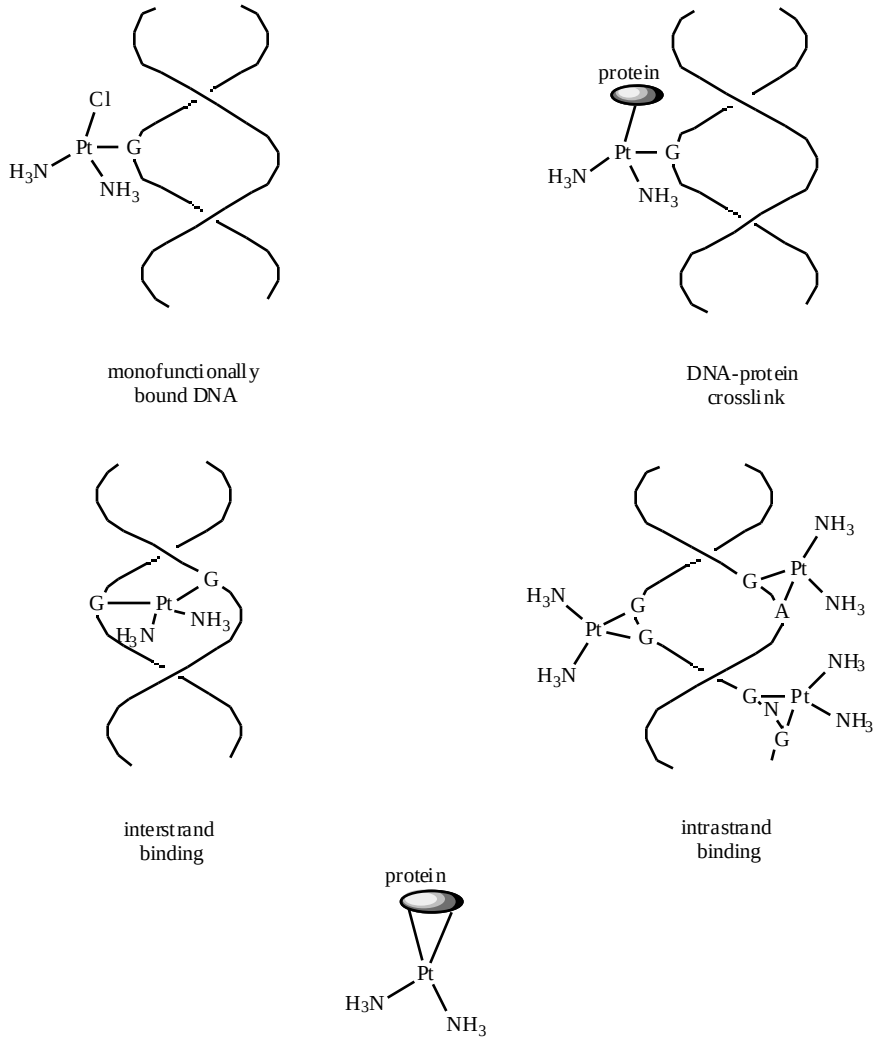

Fig. (4). Various binding modes of cisplatin to DNA and proteins.

[18,19]. In particular, this distortion creates a large kink in the B-form DNA as demonstrated by X-ray structural and NMR studies [18,19]. Furthermore, it has been shown that platinated DNA with this particular binding feature was not recognized by DNA polymerases isolated from cytosolic cell extract [28], human and calf thymus polymerase-α [29,30], and polymerase-ε [31]. It is, however, difficult to pinpoint that Ptd(GpG) is the only lesion responsible for the anticancer activity since other binding modes are present in DNA adducts. For example, the interstrand binding modes with two G bases also creates a severe structural distortion. Also, the binding modes such as Ptd(ApG) is also expected to create similar distortion that has been observed for the adjacent guanine binding. The roles of the monofunctional and 1,3 bifunctional adducts have yet to be addressed. This is primarily based on the fact that several new platinum complexes exhibit antitumor activities that can bind the DNA only in a monodentate binding mode [32].

It was initially believed that *cis*-configuration for the platinum complex was a requirement for exhibiting antitumor activities due its ability to severely distort DNA structures by forming bifunctional adducts. The *cis*-configurational requirement for exhibiting cytotoxicity can no longer be substantiated since *trans*-dichloro-di(pyridine)platinum(II) is shown to exhibit very good antitumor activities [33,34]. Also, *trans*-platin does exhibit antitumor activity at higher concentrations. Furthermore, the requirements for the bifunctional DNA-adducts may not be justified. Several platinum compounds bind DNA through one binding site and exhibit effective anti-tumor activities as stated above. How these complexes induce structural distortions of DNA is yet to be understood.

To explain the differential responses of *cis*- and *trans*-isomers, Lippard and co-workers and others [23,35,36] have postulated a repair mechanism that is applicable for the *trans*-platinated DNA adducts but not for the *cis*-platinated counterparts. In particular, the former authors [23] have hypothesized that cisplatin-DNA adducts are tightly bound by structure specific recognition proteins (SSRP). The implication here is that the SSRP camouflages the *cis*-platinated DNA from being exposed to repair machinery. However, the involvement of a repair mechanism *in vivo* is yet to be demonstrated. Furthermore, a cautionary note by the lead author [37] "we do not know for sure that HGM-domain proteins are the key to cisplatin's anticancer activity" clearly indicates that further work is needed to unveil the anti-tumor mechanism. Moreover, the hypothesis based on differential binding of *cis*- and *trans*-platinated-DNA to shielding proteins can not be substantiated by experimental observations. For example, Moggs *et al.* [38] have shown that cell extracts devoid of shielding proteins did not alter the repair rates of intra-strand platinated DNA.

Kinetic studies have been conducted to understand the preference of adjacent G bindings. The N7 site of the guanine base in the purine ring, in particular, is shown to be most reactive site. The reactions of DNA is largely governed by consecutive kinetic processes mainly limited by the aquation processes as shown below. Once the platinum compound experiences reduced chloride environment, release of one chloride ion take place crating an aqua(chloro)platinum(II) species which reacts with DNA quickly. This DNA-chloro-platinum complex undergoes the second hydrolysis followed by the formation of bifunctional adducts. The rate of second aquation largely depends on the nature of the existing coordinated ligand as well as incoming ligand. For example, for the reaction between dGpG and cis-platin, the second rate of aquation was observed to be 6 x 10^{-5} s^{-1} while for the corresponding dApG, the rate constant was evaluated to be 3 x 10^{-5} s^{-1} [39]. Usually, the second aquation is slower than the corresponding first aquation reaction.

Considering the biphasic nature of the kinetic processes and that DNA binding is a kinetic rather than thermodynamic controlled process, one would expect that all G bases be almost equally reactive at the initial binding step. Therefore, bifunctional adducts containing G and a second base would be more abundant that what had been observed. The selectivity of cisplatin toward the two adjacent G is perhaps due to initial hydrogen bond formation between the phosphate moiety and coordinated ammine ligand giving more accessibility to those phosphate that are exposed, suggesting a specific structural motif recognition. In fact, a wide range of rate constants have been reported for reactions of cisplatin with various oligonucleotides containing G bases with diverse sequence [16,17].

3. HOW SOUND IS DNA BINDING MODEL?

Both *cis*- and *trans*- isomers form intra-strand cross-linking with DNA, yet the *cis*-isomer is the more effective antitumor drug. It should be noted that both *cis*- and *trans*-platinated DNA templates are not recognized by polymerases [28]. Secondly, DNA polymerase-β, a repair enzyme, bypasses the platinated DNA during the chain extension reaction indicating that platinum binding to DNA can not inhibit the replication cycle [30]. The ability of polymerase-β to continue DNA synthesis speaks against the DNA being the target. The involvement of structure specific recognition protein in camouflaging the cisplatin-DNA but not the transplatin-DNA adducts also suffers a criticism that both Chinese hamster and monkey cells did not exhibit selective repair of transplatin- DNA adducts [40] de-emphasizing the argument that there exists a repair mechanism for the *trans*-platinated DNA but not for the *cis*- counterpart. Thirdly, several platinum complexes that form monofunctional adducts with DNA and do not cause much structural changes of the nucleic acids are also active anticancer drugs. Fourthly, based on the comparative reactivity of carboplatin and cisplatin toward DNAS, >200 fold of more carboplatin is required to exhibit equal binding to the nucleic acid [41,42], yet only 4 -20 fold higher dose of carboplatin is required to menifest equal cytotoxicity [43,44]. Furthermore, mechanisms based on proteins and enzymes binding have yet to be ruled out. For example, we have shown that the activities of DNA polymerases such human DNA polymerase-α [29], *E. coli* polymerase I [45], and polymerase T7 [39], are significantly inhibited due to the direct binding to these enzymes. Also, the reactions of *cis*-platin with these enzymes are much faster that those for DNA. Finally, the binding to proteins including some key

transcription factors which are involved in signal transduction pathways for inducing apoptosis is yet to be evaluated.

4. THE EXTENT OF DNA BINDING IN CELLULAR MILIEU

Platinum(II) is known to be a soft metal center that has tremendous affinity for binding to sulfur donors, especially thiols, compared to nitrogen donors. The intra-cellular concentrations of sulphydryl groups including cysteine and glutathione could be as high as 10 mM. It is therefore desirable to estimate the extent of DNA binding in the presence of thiol containing small amino acids and peptides. Recently, we have estimated the extent of DNA binding by a HPLC method by monitoring the concentration of unbound bases in the presence and absence of biological thiols. The HPLC data indicate that the DNA coordination to cisplatin is minimal in the presence of biological thiols. An upper 1estimate of ~2% can be placed for the cisplatin-DNA binding [46]. The concentrations of these thiols used in our experiments are similar to those found in the cellular milieu. Eastman [3a] has also estimated that ~1% of administered cisplatin binds the genomic DNA.

5. THE ROLE OF GLUTATHIONE AND PLATINUM-GLUTATHIONE COMPLEXES

The role of glutathione toward the cytotoxicity of cisplatin and other platinum anticancer drugs appears to be dual. The tripeptide both deactivates and activates the anti-cancer activity. cisplatin induces an enhancement of glutathione concentration in the cell [47,48]. The cellular response to increase the level of glutathione is certainly understandable since the tripeptide is major detoxifying agent both for exogenous and endogenous toxins. In one study, the ineffectiveness of cisplatin to several different ovarian cells has been correlated with the increased level of glutathione [49]. In another study, the platinum glutathione complex has shown to arrest the protein synthesis by blocking the translational activity [50]. The higher effectiveness of cisplatin has also been demonstrated by co-administering cisplatin and glutathione in patients. It is not clear whether this increase in effectiveness is due to the reduced toxicity or due to the modification of the platinum drug by covalent binding to the metal center. Finally, in the case of platinum(IV) drugs, glutathione produces active platinum(II) complexes by reducing the tetravalent metal center. It is commonly believed that this reduction process generates cisplatin analogs. Unfortunately, results obtained from our laboratory do not support such a hypothesis. Instead, the reduction of glutathione produces glutathione coordinated platinum complexes rather than diaminedichloroplatinum(II) species [51].

The reactivity of glutathione toward various platinum(II) complexes has been measured in the presence and absence of DNA [52]. These reactions are much faster than those of DNA and that both the parent *cis*-platin and its monohydrolyzed product react with the peptide. Among the various modes of binding, monoglutathione complex by replacing one chloride, bis-glutahione and mono(glutathione) chelate complexes by replacing both chloride, and a bis-chelate complex through the replacement of ammonia and chlorides are known.

6. ROLES OF DNA POLYMERASES

Human DNA polymerases have been classified in several categories based on their roles. Polymerase-α is in the heart of the replisome and considered to be the main replication enzyme [53]. This enzyme exhibits several functions including hydrolysis of dNTP , polymerase activity, and in some cases, exonuclease activities. This enzyme is mainly responsible for the accurate synthesis of genetic information. Human polymerase-β, on the other hand, is mainly engaged in DNA repair. The structure of human polymerase-α is unknown at this point; however, both the NMR and X-ray structural characterizations of the polymerase-β have been reported [54]. The primary sequence of human polymerase-α reveals the presence of several cysteine residues near the carboxy termius [55]. The sequence homology and other features of the carboxy terminus strongly indicate the presence of a zinc finger motif in this domain [56]. This domain is considered to be involved in binding DNA before initiating replication.

Inhibitions of polymerase activity of several polymerases by cisplatin have been reported [28-31,39,45,57,58]. The mechanism of inhibition of DNA polymerase activity can be envisaged in two possible pathways. The platination of DNA might lead to the lack of recognition by the polymerases as a template [28,30,31,57,58]. Alternatively, direct covalent platinum binding to the enzyme may rendered irreversible inhibition due to either structural changes of the enzyme or loss of active sites [29,39,45]. In fact, the inhibition studies reported fall under two categories. In the first category, platinated DNA or oligonucleotides were used as templates and the polymerase activities then measured. This strategy is based on the assumption that DNA is the cellular target. Except polymerase-β [30], all polymerases do not show activities when platinated DNA is used as a template. Note that polymerase-β bypasses the platinated DNA and effectively synthesize a strand complementary to the primer strand. Also, the preparations of platinated DNA often utilizes conditions which are not usually encountered in the cellular reactions [57].

In the second category, the polymerases are incubated with platinum antitumor drugs up to two hours and then the activity of these enzymes was measured. In fact, complete inhibition of the enzyme activity of E. coli polymerase-I, polymerase T7, and human polymerase-α was observed in two hours [39,45]. The extent of inhibition was directly correlated with the enzyme platinum complex formation. Detailed kinetic analysis revealed that the inhibition kinetics is zero order with respect to platinum and first order with respect to the enzyme implying a large binding constant for the platinum-enzyme complex. This inhibition process was not due to platinum-DNA binding since a small inhibition of polymerase activity (<5%) was registered when similar

experiments were conducted by incubating DNA with platinum complexes for up to two hours.

The source of polymerase- α inhibition was examined in some details. For example, experiments dealing with human polymerase-α reveal that Zn(II) was released from the enzyme as cisplatin reaction proceeded [29]. In fact, the platinated enzyme failed to show any polymerase activity. Although, the structure of human polymerase-α is not known at this point, based on amino acid sequence homology, it is postulated that the enzyme contains a zinc-finger motif. The NMR structure of this zinc-finger motif has just been solved from our laboratory [56]. The structure reveals striking similarities with many DNA binding Zn-finger domain. Based on the observation of zinc release and structural features of the zinc finger domain, we concluded that platinum binding to this domain has severely distorted the structural domain which is responsible for binding DNA before initiating DNA synthesis. This hypothesis is further supported by the fact that the NMR structure of the platinated zinc finger motif show remarkably different structure from that observed for the native domain [56]. In particular, loss of helicity and an unwinding of the DNA binding loop were observed in the platinated zinc finger domain.

Figure **5** shows schematic representation of the effect of cisplatin binding on human polymerase-α and its consequences for not recognizing the template DNA for initiating DNA replication. Not all polymerases, however, contain zinc finger domain, although they all have DNA binding motifs. For example, the DNA binding domain of E.coli polymerase-I lies in a cleft between two helices in which several cysteine residues are located [59]. The inhibition of other polymerases can also be explained by similar structural distortions due to platinum binding to cysteine residues. The coordination by platinum to these cysteine residues might modify the structural domain to the extent that it can not tightly bind the template nucleic acid.

In addition to polymerases α through ε, other polymerases are involved in the replication process. Roles of several newly discovered polymerases have been emerging at this point. For example, polymerases in the UmuC/DinB superfamily are now thought to be involved in the replication process when cells sensed damaged DNA [60, 61]. These polymerases do not replicate the genes with the same degree of fidelity that is observed for polymerase-α. Note that these new polymerases have zinc finger domains like polymerase-α and might also be affected by platinum complexes.

7. ROLE OF TRANSCRIPTION FACTORS

The involvement of a variety of transcription factors is quite apparent from the observation that cisplatin induces apoptosis at the G2 phase [6]. In this phase of the cell cycle, transcription processes are primarily involved before cells prepare for division. The inhibitions of apoptosis involving several transcription factors have already been addressed to understand the cisplatin resistance in certain cancer cell lines. For example, an overexpression of Bcl2 [62, 63], and activation of p21 [64] contribute significantly to platinum drug resistance presumably by inhibiting apoptosis. These two transcription factors are regulated by p53, an important transcription factor that primarily functions in the G1 phase of the cell cycle. Duckett *et al*. [65] have shown that human

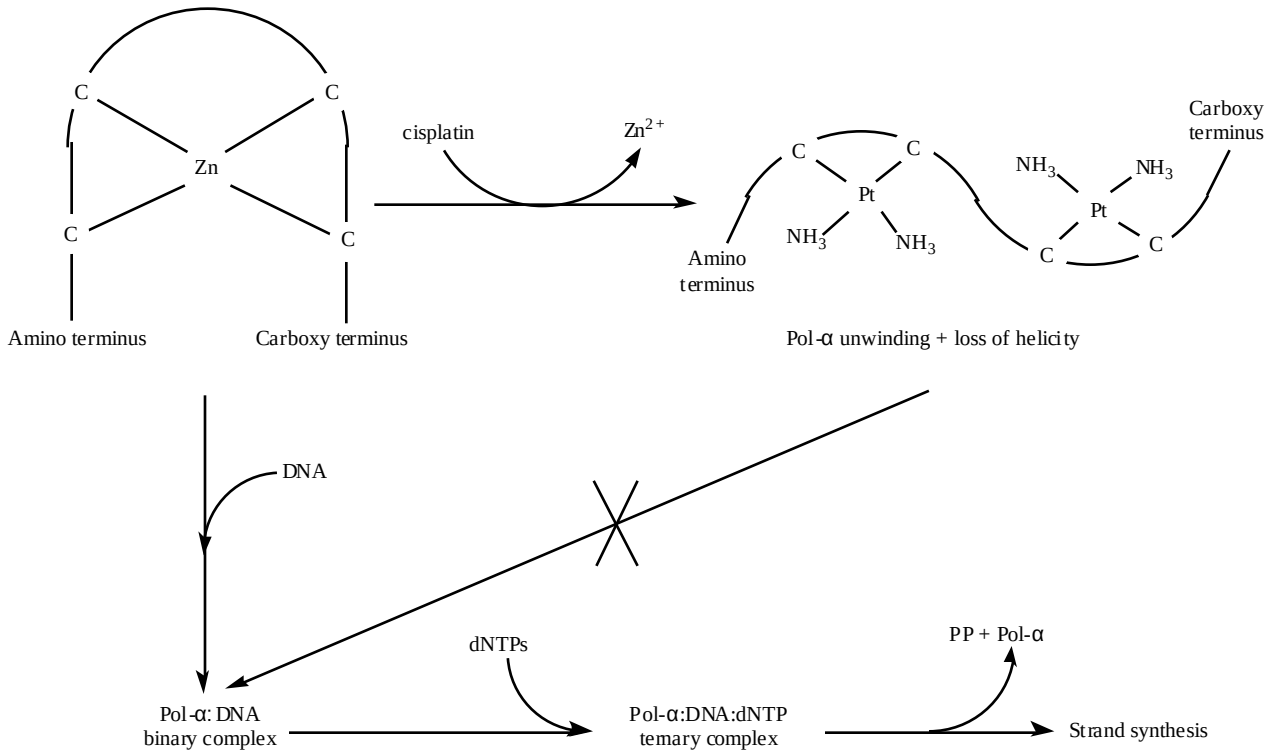

Fig. (5). Proposed mechanism of polymerase inhibition by direct binding to cisplatin.

IAP like protein also inhibits the apoptosis induced by cisplatin. Recently, Tsang and coworkers [66] reported an extensive study of expression of anti-apoptotic proteins by treating cisplatin sensitive and cisplatin resistance cell lines. A down regulation of XIAP (X-linked inhibitor of apoptosis) transcription factor was observed by cisplatin sensitive but not resistant cells. An over-expression of these transcription factors contributes significantly to the resistance mechanism. It should be noted that all these IAP proteins contain one or multiple zinc finger domains.

It is interesting to note that many transcription factors contain zinc clusters either in the form of zinc finger or RING finger domains in which cysteine and histidine residues are coordinated with the metal center. Since we have shown that the zinc finger domain of polymerase-α undergoes facile reaction with the release of zinc, such domains in transcription factors might also be affected in a similar fashion by direct binding to platinum ion. At this point direct interactions of platinum drugs with transcription factors have not been explored. Therefore, future work should be directed to unveil the interactions between platinum drugs and transcription factors.

8. CONCLUDING REMARKS

Since platinum compounds react indiscriminately with many different biomolecules including nucleic acids and proteins, effects of platinum binding to each type of biomolecule need to assessed separately and collectively for comprehensive understanding of anticancer mechanisms. The reactivity and the binding modes are determined by the nature of the ligands attached to the platinum compounds. Certainly, DNA binding by platinum is one of the possible mechanisms. We have shown an alternative mechanism based on polymerase binding that explains the anticancer activity of platinum compounds without invoking differential repair mechanisms. Furthermore, mechanisms based on transcription factor binding need to be explored. It is interesting to note that RNA polymerases which control the transcription processes also contain zinc finger domains, the domains that are susceptible to undergo facile reaction with platinum compounds. It is the view of this author that platinum drugs render their anticancer properties through several parallel pathways. The relative contributions are perhaps modulated by structure of the platinum compounds and their reactivity toward biomolecules. Likewise, several pathways are operative for deactivating these compounds. An intricate balance between the activation and deactivation processes decides the fate of these compounds in determining the efficacy or resistance to different types of tumors.

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