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Reaction of Platinum(II) Antitumor Agents with Sulfhydral Compounds and the Implications for Nephrotoxicity*

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Abstract

Cisplatin, *cis*-diamminedichloroplatinum(II), is a square-planar Pt(II) complex which finds extensive use as an anticancer agent. This use has been limited by toxicity to the kidney. Cisplatin reacts by undergoing nucleophilic substitution of one or both chloride ligands. Water is a ubiquitous nucleophile. Hydrolysis was examined by observing the change in the UV spectrum of solutions of cisplatin and two congeners with less nephrotoxicity, carboplatin and iproplatin. Cisplatin was found to hydrolyze with a half-life of approximately 9 h at room temperature. Hydrolysis was inhibited by 0.1 M NaCl. Neither carboplatin nor iproplatin appeared to hydrolyze in 24 h. Since sulfhydral groups are important for renal tubular ion transport, in addition to serving many other functions in the cell, the reactions of Pt(II) compounds with cysteine, glutathione and dithiothreitol were examined by observing the evolution of a broad band in the UV spectrum. Reaction of RSH with cisplatin was approximately an order of magnitude more rapid than with carboplatin; NaCl inhibited the reaction. Iproplatin did not appear to react. It is concluded that the nephrotoxicity found with cisplatin may be related to reaction with renal tubular –SH-containing enzymes, that the slower reaction with carboplatin and iproplatin explains their lower toxicity, and that the protective effect of a simultaneous chloruresis with cisplatin administration is explained by Cl^- inhibition of hydrolysis and/or reaction with –SH.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), $(NH_3)_2Pt(II)Cl_2$) has become one of the most widely used antitumor compounds in oncology. Nephrotoxicity was seen in the first trials [1] and became the limiting factor in dose escalation. This nephrotoxicity involves damage to the proximal renal tubule and is similar to that induced by other heavy metals such as mercury [2]. The other principal toxicity of cisplatin has been to the inner ear (damage to the organ of Corti) [3]. It is interesting to note that a neurotoxicity has recently been observed at high doses of cisplatin [4] which may be similar to that which has been known for years to accompany mercury toxicity. These toxicities are exacerbated by the concurrent administration of the aminoglycoside antibiotics and appear to be irreversible.

There have been two approaches in attempting to overcome cisplatin nephrotoxicity. The first involves inducing a brisk urine flow across the proximal renal tubule (a diuresis) by hydrating the patient extensively [5]. More recently, this has been accomplished with normal saline, inducing a chloruresis, which appears to offer additional protection [6]. The second approach has been to develop analogs of cisplatin which retain efficacy yet are not as nephrotoxic. Two compounds, a Pt(II) compound carboplatin (CBDCA, *cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II), $(NH_3)_2Pt(II)(C_6H_6O_4)$) and a Pt(IV) compound iproplatin (CHIP, *cis*-dichloro-*trans*-dihydroxy-bis(isopropylamine)platinum(IV)), have emerged from clinical trials as having promise in this regard.

The primary reaction of cisplatin and other Pt compounds is by nucleophilic substitution of a chloride or equivalent ligand. This has been well studied for Pt(II) compounds [7] but less so for Pt(IV) compounds. Water is a ubiquitous nucleophile and the current hypothesis for the reaction of cisplatin with the intracellular target DNA is via a prior hydrolysis step. Sulfur, appearing as the sulfhydral residue of the essential amino acid cysteine, is an excellent nucleophile for Pt(II). In this preliminary study, the hydrolysis of cisplatin, carboplatin and iproplatin and their reaction with the sulfhydral compound cysteine (CYS) were studied in addition to the reaction of cisplatin and carboplatin with the biologically important compounds

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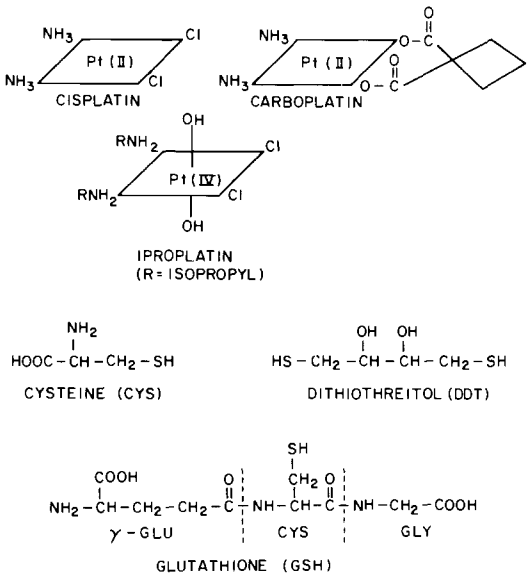

Fig. 1. Structures.

reduced glutathione (GSH) and dithiothreitol (DTT). DTT serves as a model for the many dithiol-containing enzymes of the cell. These reactions were examined in an attempt to gain further insight into their relevance to the nephrotoxity, ototoxicity and, possibly, the antitumor action of Pt anticancer compounds.

Experimental

Cisplatin, carboplatin and iproplatin were the kind gifts of Bristol-Myers, Inc. Cysteine, glutathione and Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)) were obtained from Sigma, and dithiothreitol was obtained from Aldrich. Structures are shown in Fig. 1. All other chemicals were of reagent grade. Spectrophotometric studies were done on a Beckman DU-50 spectrophotometer equipped with a Peltier temperature controller and a Kinetics Pack. Concentrations of sulfhydral compounds were determined by the method of Ellman [8]. A small aliquot (50 or 100 μl) of reaction mixture was mixed with an excess of DTNB in water and the pH adjusted to 7.3 with phosphate buffer. Concentration was determined from the absorbance at 412 nm. A standard curve gave an extinction coefficient of 13 900 for the RSH + DTNB adduct.

The kinetics of cisplatin hydrolysis were measured by measuring the disappearance of the absorption band at 300 nm, A_{300}, which has an extinction coefficient of 127, and evolution of the absorbance of the monoaqua product at 270 nm which has an extinction coefficient of 125. In addition, the change of A_{290} and A_{252} were monitored for cisplatin, and for carboplatin and iproplatin under similar conditions.

When sulfhydral compounds are reacted with Pt(II) compounds, a broad absorption appears in the UV. The kinetics of reaction between Pt compounds and sulfhydral compounds were determined by measuring the evolution of A_{270}.

Results

Hydrolysis of Pt Compounds

Fresh solutions of cisplatin (0.5 to 2.0 mM) were prepared in water or 0.01 phosphate buffer, pH = 7, in the presence and absence of NaCl. Increase of A_{270} was rapid at 40 °C with a $t_{1/2}$ of less than 2 h and slower at 25 °C with a $t_{1/2}$ of about 9 h in general agreement with the more precise measurements by other authors. (This method is relatively imprecise due to the difficulties in measuring small changes in absorbance.) NaCl (0.1 M) completely inhibited the reaction. An increase in absorbance at A_{252} was noted at 24 h for cisplatin in phosphate buffer *vs.* water. These results suggest that cisplatin will readily undergo hydrolysis to the monoaqua compound at physiological pH and temperature in the absence of Cl^- but that 0.1 M Cl^- is sufficient to completely suppress this hydrolysis. Also, phosphate may slowly react either with cisplatin or the hydrolysis product. While the rate of reaction is slow at room temperature, aqueous solutions of cisplatin are unstable.

There is much less change in A_{270} of carboplatin at 40 °C and none at 25 °C over 24 h, suggesting that that there is little hydrolysis of carboplatin as compared with cisplatin. In addition, the rate of reaction of a freshly made solution of carboplatin with CYS was identical to the rate for a solution which had aged for 24 h, whereas hydrolyzed cisplatin reacts more rapidly (see below) supporting the conclusion that carboplatin undergoes hydrolysis only slowly. The spectrum of iproplatin undergoes almost no change over 24 h, suggesting that hydrolysis is negligible for this compound also.

Stoichiometry of the Reaction of Cisplatin with CYS

The reaction of –SH-containing compounds is not straightforward and the results presented here are preliminary. Concentrations of CYS and GSH (0.1 to 2 mM) were reacted with 0.5 mM cisplatin and carboplatin in phosphate buffer at pH 7 at 65 °C (water bath) for 4 h. Under these conditions, cisplatin + DTT form a precipitate. The change in A_{270} is shown in Fig. 2. The concentration of unreacted –SH for reaction of 0.5 mM cisplatin with increasing concentrations of CYS, GSH and DTT is shown in Fig. 3. From this one can conclude that the stoichiometry of the reaction of CYS with cisplatin is two S atoms per Pt in the presence of excess –SH.

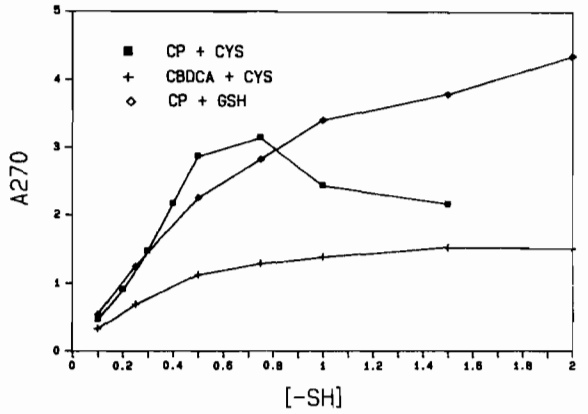

Fig. 2. Absorbance at 270 nm after reaction of 0.5 mM cisplatin (CP) or carboplatin (CBDCA) with CYS or GSH at 65 °C for 4 h as a function of $[-SH]_0$.

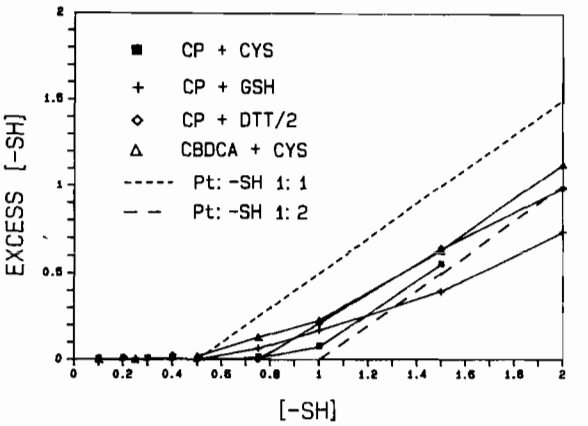

Fig. 3. Excess [–SH] after reaction of Pt(II) compounds (0.5 mM) with RSH at 65 °C for 4 h as a function of $[-SH]_0$.

However, it appears that the broad UV absorbance develops principally with binding of the first CYS. There may actually be a decrease in A_{270} with further binding of CYS beyond 1:1. This is not seen with GSH or DTT. There is little change in A_{270} over 24 h at room temperature after reaction at 65 °C indicating that the product is stable to further hydrolysis. GSH (1-*gamma*-glutamyl-1-cysteinylglycine) contains three amino acids but only the central cysteine contains a sulfhydral residue which is the presumed nucleophile (Fig. 1). However, in contrast to reaction with CYS, A_{270} continues to rise after reaching the value of 3.0 for two CYS per Pt determined above. This rise may represent reaction wherein either the glutamate or glycine residue of GSH also bind to the Pt. Analysis of residual –SH with DTNB, as for CYS above, suggests that two GSH bind per Pt which is surprising given the steric restraints on the bulky GSH. Reacting cisplatin with DTT yielded cloudy solutions after 1 h, but it is presumed that both sulfurs of this ligand are bound to the Pt. Analysis of unreacted DTT supports this conclusion (Fig. 3). Reaction of CYS with carboplatin did not give the same initial increase of absorbance as seen with cisplatin (see Fig. 2). There was little measurable reaction between CYS and iproplatin as ascertained either by change in absorbance in the UV or by consumption of CYS as measured by DTNB.

Because of the difficulties in obtaining consistent results between compounds, in the kinetic studies reported below an extinction coefficient at 270 nm of 3.0×10^3 was arbitrarily used for the product of Pt(II) and two –SH groups (assuming a final product of $Pt(II)(NH_3)_2(-SR)_2$). This allows one to obtain comparative rates of reaction but not rate constants.

Reaction Rates of Pt Compounds with CYS, GSH and DTT

When cisplatin is reacted with CYS, GSH or DTT, there is evolution of a broad band in the UV spectrum. In the presence of excess nucleophile, the time course of this evolution is not coincident with that expected for a pseudo-first-order reaction as shown in Fig. 4. After an initial slow rise, there is a long and variable linear period followed by a tailing off. This behavior is not a result of various amounts of the hydrolysis product as might be expected if solutions of cisplatin had been used at various times after preparation, as can be seen from Fig. 5 where the reaction of CYS with fresh cisplatin and hydrolyzed cisplatin are compared (hydrolyzed cisplatin was prepared by letting a solution of cisplatin in water age at room temperature for 24 h). This behavior coupled with the finding from the stoichiometry studies that the absorbance only changes with the binding of the first sulfur suggests

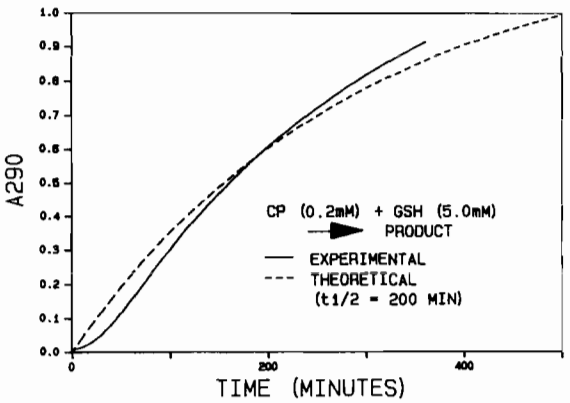

Fig. 4. Comparison of experimental and theoretical evolution of absorbance at 290 nm for the reaction of cisplatin (CP) with GSH at 40 °C. Theoretical absorbance calculated for pseudo-first-order reaction.

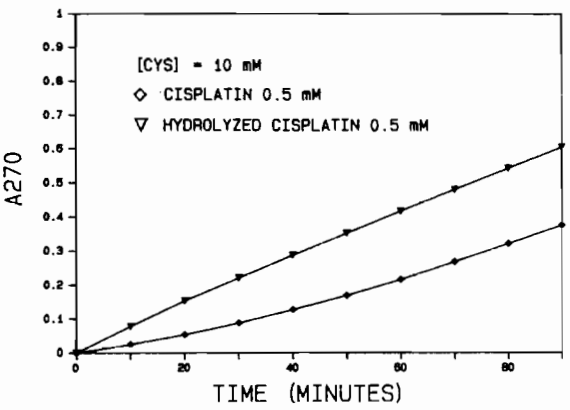

Fig. 5. Comparison of the evolution of absorbance at 270 nm for freshly prepared cisplatin *vs.* hydrolyzed cisplatin (see text) reacting with CYS.

TABLE I. Rates of Reaction of RSH with Cisplatin and Carboplatin (CBDCA)

Pt(II)[a]	RSH	[NaCl] (M)	Rate[b] (mol l^{-1} s^{-1})	Ratio to cisplatin + CYS
Cisplatin	CYS	0.0	1.2×10^{-8}	1.0
Cisplatin	CYS	0.1	7.5×10^{-9}	0.62
Cisplatin	GSH	0.0	4.1×10^{-8}	3.4
Cisplatin	GSH	0.1	2.4×10^{-8}	2.0
Cisplatin	DTT	0.0	9.6×10^{-8}	8.0
Cisplatin	DTT	0.1	8.3×10^{-8}	6.9
CBDCA	CYS	0.0	1.3×10^{-9}	0.11
CBDCA	GSH	0.0	1.3×10^{-9}	0.11
CBDCA	DTT	0.0	5.6×10^{-9}	0.47

[a]Conditions: [Pt(II)] = 0.5 mM; [–SH] = 10 mM; T = 40 °C; pH = 7 (0.003 M phosphate buffer); A_{inf} at 270 nm of product = 3.00 (see text for discussion of A_{inf}). [b]Rate determined from linear portion of absorption *vs.* time curve as described in text.

that the mechanism of reaction of sulfhydral compounds with cisplatin is not simple.

An apparent rate of reaction can be obtained for comparison purposes by measuring the change of the species absorbing in the UV at A_{270} during linear portions of the curve using A_{inf} as calculated from the stoichiometry studies above and assuming all the Pt compound is converted to the product Pt(II)-$(NH_3)_2(-SR)_2$. Rates were calculated by this method for the reaction of cisplatin and carboplatin with CYS, GSH and DTT; and cisplatin with RSH at 40 °C, pH = 7, in the presence and absence of NaCl. Results of these calculations are given in Table I.

Discussion

While the nephro- and ototoxicities of cisplatin have been known for years, there has been no identification of a molecular target responsible for the damage. Since both proximal renal tubular cells and hair cells in the organ of Corti are involved in ion transport, it is possible that the origin of the toxicity is in a reaction of cisplatin with enzymes involved in this transport. The decreased toxicity of carboplatin and iproplatin would be due to a decreased rate of reaction with this target.

The drug cisplatin reacts by nucleophilic substitution of a chloride ligand(s). By analogy, carboplatin reacts by substitution of the bidentate dicarboxylato chelating ligand (Fig. 1). Because of the bidentate nature of this ligand, it would be expected to be more resistant to substitution. Iproplatin was found to be resistant to both hydrolysis and substitution, at least using the methods employed here. This suggests that this drug may need to be reduced intracellularly to a Pt(II) compound before further reaction with intracellular targets. It should be noted that reduction of iproplatin and loss of the apical –OH groups would yield a *cis*-compound quite similar to cisplatin. Attempts to reduce iproplatin chemically and then demonstrate reaction with RSH compounds in our laboratory have so far not met with success.

Clinically, carboplatin and iproplatin appear to have minimal reactivity with the targets in the renal tubular cells and the organ of Corti. That is, administration of these drugs to patients has resulted in minimal nephro- and/or ototoxicity [9, 10]. The demonstration here that the rate of hydrolysis of carboplatin and its reaction with the sulfhydral nucleophile is slower than the hydrolysis and reaction of cisplatin (Table I) appears to offer an explanation for the decreased toxicity of this compound. Furthermore, as discussed above, iproplatin appears to be unreactive in its Pt(IV) form.

Both the renal tubule and the organ of Corti are sites of extensive ion transport. It has recently been demonstrated by Ross and coworkers that, at least in the proximal renal tubule, disulfhydral compounds are intimately involved in this transport [11]. Inactivation by reaction with DTT of the sulfhydral groups exposed to the lumen of the tubule (presumably on the exterior of the plasma membrane) inhibits cation transport. Given the avidity of cisplatin for sulfhydral groups, as demonstrated here, and the much slower reaction of carboplatin and iproplatin, one can postulate that reaction with these sulfhydral-containing enzymes is a central mechanism of renal toxicity for cisplatin. Sulfhydral groups have been noted to be depleted in kidneys after cisplatin administration [12].

In conjunction with this, the demonstration that this reaction can be inhibited by the presence of Cl^- (Table I) offers an explanation for the protective effect of a chloruresis both experimentally [13] and for patients receiving high doses of cis-

platin. We have previously shown that patients being hydrated with normal saline have an increase in [Cl^-] in their urine and decreased nephrotoxicity [14].

The intracellular target of cisplatin in proliferating cells is presumed to be DNA. This requires a prior hydrolysis step. The extensive research that supports this hypothesis has been reviewed [15]. The data presented in this paper suggest that sulfhydral groups in the cell are also prime targets of Pt(II) compounds. Reaction with these groups does not require prior hydrolysis. Indeed, the rate of reaction of cisplatin with CYS appears to be only slightly more rapid for hydrolyzed cisplatin as opposed to freshly prepared cisplatin (Fig. 5).

The intracellular target of carboplatin in proliferating cells is again presumed to be DNA [16] although reaction of carboplatin with this molecule is also dependent on its rate of hydrolysis which, as has been shown here, is extremely slow. In fact, it is difficult to explain the efficacy of this drug in a quantitative manner if one must assume the prior hydrolysis/activation step. The kinetics of the reactions of cisplatin and carboplatin demonstrated here bring into question whether DNA is the primary intracellular target of these drugs in tumor cells.

Laboratory investigations over the years have often implicated reaction of cisplatin with sulfur-containing nucleophiles. Investigation of the metabolism of platinum [^{14}C]ethylenediamine dichloride in the rat [17] showed that the drug was localized mainly in the cytosol in the form of low molecular weight complexes. The biological activity of cisplatin can be inhibited by thiourea and l-methionine [18], and thiourea was found to reverse cisplatin DNA crosslinks [19]. In a finding that has implications for measuring the pharmacokinetics of free *versus* total platinum species, cisplatin was found to bind to serum proteins over time rendering it inactive [20, 21]. This is presumably a reaction of cisplatin with cysteine residues on serum proteins and is consistent with the half-life of approximately 200 min for reaction of cisplatin with GSH (Fig. 4). Several agents have been used in an attempt to 'rescue' patients from the nephrotoxicity of cisplatin. Diethyldithiocarbamate, a disulfur nucleophile with some similarities with DTT, reacts with cisplatin avidly and has been demonstrated to protect kidneys from cisplatin damage yet not decrease its antitumor effect [22]. Sodium thiosulfate has received extensive investigation by Howell and Taetle as an agent to be given intravenously in conjunction with intraperitoneal cisplatin, effectively preventing nephrotoxicity [23]. It can be demonstrated that serum cisplatin is inactivated by the co-administration of sodium thiosulfate. Finally, mesna (2-mercaptoethane sulfonate) did not appear to decrease nephrotoxicity although it does decrease cytotoxicity [24].

Sulfhydral groups are avid nucleophiles for Pt(II). Since sulfhydral groups are found on the amino acid cysteine, they are ubiquitous in the cell. The ease of the reversible oxidation/reduction of disulfhydral groups to the disulfide makes this reaction important in intracellular metabolism. Possible sulfhydral targets in proliferating cells include GSH, ribonucleotide reductase [25], the reductive enzymes thioredoxin [26] and glutaredoxin [27] which are associated with ribonucleotide reductase, glutathione reductase, transcription factor IIIA [28], and even a newly discovered dithiol compound in the mitochondrial respiratory chain [29]. How cisplatin, carboplatin and, possibly, iproplatin reduced to a Pt(II) compound might alter cellular metabolism and/or proliferation by interacting with these targets remains to be delineated.

It has been demonstrated that elevations of GSH are present in cells resistant to cisplatin [30] and, conversely, depletion of GSH by blocking glutathione synthetase with the compound BSO (buthionine sulfoximine) leads to extreme cisplatin sensitivity [31]. The respective intracellular concentrations of cisplatin and GSH are too disparate (rough estimate of $<5 \times 10^{-5}$ M for cisplatin and $>1 \times 10^{-3}$ M for GSH) to allow the direct reaction of cisplatin with GSH and subsequent depletion of GSH to account for these effects. It is more likely that these drugs react with a disulfhydral-containing enzyme which is in low concentration. Cisplatin has been shown to bind avidly to the sulfhydral-containing metallothionein in Ehrlich cells [32]. Investigations are continuing in our laboratory to identify such enzymatic targets.

Acknowledgement

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