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CHARACTERIZATION OF THE REACTIONS OF PLATINUM ANTITUMOR AGENTS WITH BIOLOGIC AND NONBIOLOGIC SULFUR-CONTAINING NUCLEOPHILES*

PETER C. DEDON and RICHARD F. BORCH†

Department of Pharmacology and Cancer Center, University of Rochester, Rochester, NY 14642, U.S.A.

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Abstract—Substitution reactions with biologic nucleophiles appear to govern the antitumor and toxic properties of platinum complexes. In this paper we have characterized the reactions of several platinum antitumor agents with sulfur-containing amino acids, peptides, proteins, and nonbiologic nucleophiles. The rate constants for the reactions of *trans*-diamminedichloroplatinum(II) (*trans*-DDP), *cis*-diamminedichloroplatinum(II) (DDP), diammine (1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA) and *cis*-diisopropylamine-*cis*-dichloro-*trans*-dihydroxy platinum(IV) (CHIP) with cysteine (Cys), methionine (Met), and glutathione (GSH) were determined at 37°. A reactivity ratio of 1:1.5:22:6500 was determined for the reaction of GSH with CHIP, CBDCA, DDP, and *trans*-DDP respectively. The rate constant for the binding of DDP to DNA, 7.4×10^{-5} sec^{-1}, decreased to 5.9×10^{-5} sec^{-1} and 1.7×10^{-5} sec^{-1} in the presence of 0.5 and 5 mM GSH respectively. The products formed in the reaction of GSH with *trans*-DDP, DDP, and CBDCA were also examined. Under conditions of high platinum concentration (2–3 mM), CBDCA and DDP form large molecular weight species with GSH as indicated by ^{1}H-NMR and ultrafiltration experiments. The complex $[Pt(GSH)_2 \cdot 3H_2O]_n$ was isolated from the reaction of 3 mM DDP with 6 mM GSH. The product formed in the reaction of 3 mM *trans*-DDP with 6 mM GSH was not macromolecular in nature, and ^{1}H-NMR spectra revealed that platinum was bound to the Cys sulfhydryl group. Rate constants were determined for the reactions of these platinum complexes with diethyldithiocarbamate (DDTC) and thiosulfate, two agents known to reduce platinum-mediated nephrotoxicity. DDTC, but not thiosulfate, was shown to rapidly chelate platinum from $[Pt(GSH)_2 \cdot 3H_2O]_n$. The effects of DDP, CBDCA, and CHIP on the sulfhydryl-dependent rat renal proximal tubule membrane enzymes alkaline phosphatase (AP), γ-glutamyltranspeptidase (GGTP), leucine aminopeptidase (LAP), and the Na^+/K^+- and Mg^{2+}-adenosine-5′-triphosphatases (ATPases) were also investigated *in vitro*. The ability of platinum complexes to inhibit these enzymes parallels their reactivity with other nucleophiles. DDTC and thiourea were shown to restore activity to platinum-inhibited enzymes. Chloride ion was found to reduce platinum-mediated enzyme inhibition in an unpredictable manner, the greatest effect being observed with LAP and GGTP and the least with the ATPases. None of these renal enzymes was directly inhibited by DDP *in vivo*.

The biologic activity of platinum-based antitumor agents is governed by complex chemical reactions with a large number of biologic nucleophiles. The antitumor properties of these complexes are believed to result from the formation of bifunctional platinum adducts with DNA [1], a reaction probably dependent on the initial formation of reactive aquated Pt(II) complexes [2–4]. We and others have hypothesized that part of the nephrotoxicity, gastrointestinal toxicity, and possible bone marrow suppression induced by platinum-based antitumor agents may involve ligand exchange reactions of platinum by sulfhydryl groups with subsequent inactivation of essential enzymes and other proteins [2, 5, 6]. Gonias *et al.* [7] have demonstrated that DDP-induced inactivation of human α_2-macroglobulin occurs by cross-linking of subunit sulfhydryl groups. In terms of biotransformation, platinum complexes have been shown to react with plasma protein sulfhydryl groups [8] and with methionine; a bis adduct of the latter has been isolated from the urine of patients receiving *cis*-diamminedichloroplatinum(II) (DDP) [9]. It has also been demonstrated that GSH plays a role in the resistance of normal and tumor cells to DDP toxicity [10, 11]. Finally, a number of sulfur-based compounds have been exploited to reduce platinum toxicity. The organic thiophosphate, WR-2721, selectively protects against DDP nephrotoxicity when given just prior to DDP [12]. Howell *et al.* [13] have shown that thiosulfate reduces nephrotoxicity when it is administered concomitantly with DDP. We have demonstrated that systemic administration of diethyldithiocarbamate (DDTC) 1–4 hr after DDP inhibits platinum complex-induced marrow suppression, nephrotoxicity, gastrointestinal toxicity and emesis without interfering with the antitumor properties of DDP [14]. The mechanism of DDTC rescue appears to involve chemical selectivity for reversal of specific bonds between platinum and bio-

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† To whom reprint requests should be addressed.

logic ligands [2]. It is ineffective when administered prior to DDP and is the only agent effective when given after DDP.

In all of these examples, nucleophilic substitution reactions of the platinum complexes are responsible for the associated tumor and host toxicities and amelioration of toxicity. The kinetic aspects of these reactions, however, remain poorly understood. The importance of the kinetics of interaction of platinum complexes with biologic nucleophiles has been clearly demonstrated by Knox *et al.* [4]. They found that, while diammine (1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA) and DDP differ in their toxicity and spectrum of antitumor activity, the only difference in the mechanism of cytotoxicity is one of kinetics of interaction with DNA [4]. Given the importance of sulfur-containing species in the biologic activity of platinum complexes, we performed experiments aimed at clarifying the kinetics of reactions of several platinum complexes with different sulfur-containing amino acids, peptides, proteins, and nonbiologic nucleophiles.

MATERIALS AND METHODS

Materials. Leucine-*p*-nitroanilide, choline chloride, sodium diethyldithiocarbamate, oxidized glutathione, 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid (HEPES), morpholinopropanesulfonate (MOPS), glycine, and salmon testicle DNA were purchased from the Sigma Chemical Co. *Cis*-diamminedichloroplatinum(II), *trans*-diamminedichloroplatinum(II), disodium 1,1-cyclobutanedicarboxylate, methionine, cysteine, deuterium oxide, sodium perchlorate, sodium 1-heptanesulfonate, and tetrabutylammonium hydrogen sulfate were purchased from the Aldrich Chemical Co. Phosphate salts and acetonitrile were purchased from the Baker Chemical Co. Sodium thiosulfate pentahydrate and thiourea were purchased from the Fisher Chemical Co. Glutathione was purchased from ICN Nutritional Biochemicals. Diammine (1,1-cyclobutanedicarboxylato)platinum(II) and *cis*-diisopropylamine - *cis* - dichloro - *trans* - dihydroxy - platinum(IV) (CHIP) were obtained through Bristol Laboratories. Elemental analyses were performed by Galbraith Laboratories, Inc. Fisher 344 rats were obtained from Charles River Breeding Laboratories and were given food and water *ad lib.*

Instrumentation. Atomic absorption spectroscopy (AAS) was performed on an Hitachi Zeeman-Effect flameless atomic absorption spectrophotometer. UV-VIS spectroscopy was performed on a Beckman DU-8 spectrophotometer. Infrared spectra were obtained on a Beckman model IR-33 spectrophotometer. Proton NMR spectra were collected on an IBM WP 270-SY spectrometer with the following parameters: pulse width, 3 μsec; sweep width, 4000 Hz; acquisition time, 2 sec; 16k data points; and water suppression by gated decoupling. Trimethylsilylpropanesulfonate (TMPS) was used as reference.

Statistics. Least-squares linear regression analysis for the kinetic plots was performed with the MDCSTAT Statistics Package (Macro Data Collection, Inc., Novato, CA). All values with indicated errors are mean ± SD for specified number of experiments or replicates.

Rates of chloride release from DDP and CHIP. The rates of release of chloride ion from DDP and CHIP at 23° were determined using an Orion chloride-specific electrode as described previously [2]. Solutions of DDP and CHIP in 20 mM $NaNO_3$ (0.9–2.3 and 2–15 mM respectively) were analyzed for chloride ion. The first-order rate constants were determined from the initial rate of chloride release according to the following equation:

$$k_1 = \frac{(d[Cl^-]/dt)_0}{[DDP]_0}$$

where k_1 is the first-order rate constant. The rate of release of chloride was linear over 60 min ($r^2 = 0.995$).

Reaction kinetics of platinum complexes with sulfur-containing nucleophiles. The quantitation of DDP, *trans*-DDP, CBDCA and CHIP for kinetic analyses was achieved by reversed-phase solvent-generated cation exchange and ion-pair high-pressure liquid chromatography (HPLC). The basic system consisted of a Beckman 345 Ternary Liquid Chromatograph; a 2.1 × 30 mm Altech Direct Connect guard column with C18 reversed phase pellicular medium; a 4.6 × 250 mm Econosphere C18 reversed phase, 5 μm particle-size analytical column; a u.v. detector at 225 nm; and a Hewlett–Packard model 3390A integrator. For the reactions of DDP, *trans*-DDP, and CBDCA with Cys, Met, DDTC and GSH, the following mobile phase was employed: phosphate buffer (50 mM, pH 2.5, 23°) and 2.5 mM 1-heptanesulfonate at a flow rate of 1.5 to 2 ml/min [15]. The column was equilibrated with this mobile phase for 20 hr prior to use. For the reactions of DDP and CBDCA with thiosulfate and the reactions of CHIP with GSH and DDTC, the following mobile phase was employed: acetonitrile in a solution of 10 mM tetrabutylammonium hydrogen sulfate with pH brought to 3.5 with Na_3PO_4; flow rate, 1.5 to 2 ml/min [15]. Elution characteristics are indicated in Table 1. The reaction of DDTC with $[Pt(GSH)_2 \cdot 3H_2O]_n$, CBCDA, and DDP was monitored by following the appearance of $Pt(DDTC)_2$, which was quantitated by HPLC as described previously [16]. For the reaction of thiosulfate with $[Pt(GSH)_2 \cdot 3H_2O]_n$, the reaction mixture was filtered through a 10,000 dalton molecular weight cutoff Centricon 10 Microconcentrator. The filtrate was treated with 1 M DDTC at 100° for 15 min followed by extraction and quantification of $Pt(DDTC)_2$ as described above. The reaction of thiosulfate with $[Pt(GSH)_2 \cdot 3H_2O]_n$ was also monitored by following the appearance of GSH by the method of Hissin and Hilf [17]. Thiosulfate did not react with the *o*-phthalaldehyde reagent in the presence of free amines (serine).

Substitution reactions of square-planar Pt(II) complexes proceed by a two-term rate law [18]:

$$-\frac{d[\text{complex}]}{dt} = k_1[\text{complex}] + k_2[\text{nucleophile}][\text{complex}]$$

Table 1. Chromatographic retention data for kinetics experiments

1-Heptanesulfonate*		Tetrabutylammonium sulfate*		
Compound	k_r†	Compound	k_r†	% CH_3CN‡
DDP	0.17	CHIP	4.1	1
trans-DDP	0.67	GSH	3.0	1
Cys	1.2			
CBDCA	1.7	CHIP	0.73	10
GSH	5.5	DDTC	36	10
1,1-Cyclobutane-dicarboxylate	11	DDP	0.32	20
Met	13	Thiosulfate	2.2	20
DDTC	22			

* Mobile phase component; see Materials and Methods.

† $k_r = \frac{t_r - t_o}{t_o}$, where t_r = retention time of compound and t_o = retention time of void volume marker (H_2O).

‡ Vol:vol percentage of CH_3CN in 10 mM tetrabutylammonium sulfate.

where k_1 is the pseudo first-order rate constant for the reaction of solvent (water in these experiments) and k_2 is the second-order rate constant for attack by the nucleophile. The aquated platinum complexes go on to react with the nucleophile at rates at least 10-fold faster than the parent complex [18]. At concentrations of nucleophile in large excess over the platinum complex, the rate law becomes:

$$-\frac{d[\text{complex}]}{dt} = k_{obs}\,[\text{complex}]$$

$$k_{obs} = k_1 + k_2\,[\text{nucleophile}]$$

where k_{obs} is the pseudo first-order rate constant for loss of the complex. By determining k_{obs} at a variety of different nucleophile concentrations, a plot of k_{obs} vs [nucleophile] will yield slope = k_2 and y-intercept = k_1. In these experiments, the concentrations of DDP, *trans*-DDP, CHIP and CBDCA ranged from 0.05 to 1 mM; $[Pt(GSH)_2 \cdot 3H_2O]_n$, 8 to 40 μM; DDTC, 3 to 400 mM; GSH, 5 to 200 mM; Met, 20 to 70 mM; Cys, 10 to 50 mM; and thiosulfate, 2 to 200 mM. Reactions of GSH, Cys, Met, and thiosulfate were performed in phosphate buffer (50 mM, pH 7.4, 23° or 37°). Wherever possible, ionic strength was maintained at 300 mM by the addition of $NaClO_4$. In the reaction of DDTC with $[Pt(GSH)_2 \cdot 3H_2O]_n$, CBDCA, and DDP, 0.5 ml of the reaction mixture was vortexed with 0.5 ml of $CHCl_3$ at various times to extract $Pt(DDTC)_2$ which was quantitated by reversed-phase HPLC as previously described [16]. Platinum complexes in the other reaction mixtures were quantitated by HPLC analysis as described above. The nucleophile was always greater than 10-fold in excess of the platinum complex.

Synthesis of $[Pt(GSH)_2 \cdot 3H_2O]_n$. DDP (600 mg, 2 mmol) and GSH (1.23 g, 4 mmol) were dissolved in 500 ml of N_2-purged H_2O or aqueous NaCl (150 mM) by sonication and heating to 37°. These solutions were incubated under N_2 and in the dark at 37° for 2 days and at 23° for 1 week. Copious precipitate appeared after several days at 23°. The precipitate was collected, washed with water at 4°, and lyophilized. This material was found to be insoluble in acidic or unbuffered aqueous solution but was readily dissolved under slightly alkaline conditions. IR (nujol): 3000, 2960, 1650, 1640, 1540, 1520, 1510, 1460 cm^{-1}. Proton NMR showed two broad, very low-intensity signals at 2.2 and 3.8 ppm. Elemental analysis: C, 27.74%; H, 4.14%; N, 9.90%; S, 7.65%; and Pt, 22.71%; calculated for $Pt(GSH)_2 \cdot 3H_2O$ ($C_{20}H_{32}N_6O_{12}PtS_2 \cdot 3H_2O$): C, 27.87%; H, 4.44%; N, 8.75%, S, 7.44%; and Pt, 22.63%. No free GSH was detectable by the method of Hissin and Hilf [17].

^{1}H-NMR analysis of the reaction of platinum complexes with GSH and thiosulfate. The reaction of GSH (2 mM) and DDP (2 or 10 mM) in phosphate-buffered D_2O [75 mM, pD (pH in D_2O) = 7.4, 23°] was monitored over 14 hr by proton NMR. In another experiment, solutions containing CBDCA or *trans*-DDP (3 mM) with GSH (6 mM) were prepared in H_2O and allowed to react at 23° in the dark and under N_2 for 2–3 weeks. The CBDCA/GSH solution changed from clear and colorless to dark yellow and slightly cloudy. The *trans*-DDP/GSH solution remained pale yellow and clear. Samples of the solution were lyophilized, resuspended in phosphate-buffered D_2O, (75 mM, pD = 7.4, 23°), and analyzed by ^{1}H-NMR as described above. The reaction of thiosulfate (100 mM) and $[Pt(GSH)_2 \cdot 3H_2O]_n$ (1 mM) in D_2O (23°) was followed by ^{1}H-NMR using the previously described parameters.

Detection of polymer in a solution of $[Pt(GSH)_2 \cdot 3H_2O]_n$ *and in solutions of CBDCA or* trans*-DDP with GSH.* The production of large molecular weight platinum-GSH species was assessed in 2- to 3-week-old solutions containing platinum complexes and GSH (3 and 6 mM respectively) and in a solution of $[Pt(GSH)_2 \cdot 3H_2O]_n$ (3 mM). Aliquots (2 ml) of the reaction mixtures were placed in Centricon 10 and 30 Microconcentrators with molecular weight cut-offs of 10,000 and 30,000 daltons respectively (cellulose-based membranes). These solutions were centrifuged at 1000 *g* until 1 ml of ultrafiltrate had formed. The retained solution and ultrafiltrate were analyzed for platinum using an Hitachi

Zeeman-Effect flameless atomic absorption spectrophotometer. Adsorption of the platinum species to the filter membrane did not occur when the solutions were passed through 0.2 μm filters of the same composition.

Effect of platinum complexes on enzyme activity in vitro. The brush border enzymes GGTP and leucine aminopeptidase (LAP) and basolateral-membrane Na^+/K^+- and Mg^{2+}-ATPases were isolated from the kidneys of male Fisher 344 rats by previously described methods [2, 19]. For the GGTP and LAP experiments, brush border membranes (1 mg/ml) were incubated at 37° in Tris buffer (20 mM, pH 7.4, 37°) containing DDP, DDP + 100 mM NaCl, or *cis*-diammine diaquoplatinum(II) (aquated DDP), prepared by treating DDP with slightly less than 2 equivalents of $AgClO_4$ overnight and filtering away the AgCl precipitate. Similar experiments were performed with CHIP and CBDCA ± 100 mM NaCl in MOPS buffer (40 mM, pH 7.4, 37°.) At various times, GGTP was assayed as described previously [2], and LAP was assayed under the following conditions: 5 mM leucine-*p*-nitroanilide, 10 mM Mg^{2+}, and phosphate buffer (50 mM, pH 7.4, 23°) in 1.0 ml with 25 μg protein. The release of *p*-nitroaniline was monitored spectroscopically at 410 nm.

ATPase experiments were performed as follows. Basolateral membranes (0.3 mg/ml protein) were diluted 10-fold with HEPES buffer (40 mM, pH 7.4, 37°) containing DDP, CHIP, or CBDCA and incubated at 37° for 2 hr. The effect of chloride ion was assessed by adding 100 mM choline chloride to platinum-containing solutions and 200 mM sucrose to control solutions. Na^+/K^+-ATPase activity was calculated as the difference between total ATPase activity and Mg^{2+}-ATPase (ouabain-inhibitable) activity, assayed as described previously [19]. Protein was determined by the method of Lowry *et al.* [20].

The effect of thiourea, thiosulfate, and DDTC on platinum-inhibited enzymes was examined by methods previously described [2]. Membranes exposed to DDP or CBDCA and CHIP were resuspended in Tris buffer (100 mM, pH 7.4, 37°) or MOPS buffer (150 mM, pH 7.4, 37°), respectively, containing one of the three nucleophiles.

Effect of DDP on rat kidney enzymes in vivo. DDP (7.5 mg/kg) was administered by lateral tail vein injections to male Fisher 344 rats (200–250 g), and the animals were killed 4 and 24 hr later. The kidneys were removed, and tissue from the corticomedullary junction were isolated by dissection. Basolateral membrane ATPases and brush border GGTP and LAP were isolated and assayed as described above. Alkaline phosphatase (AP) activity in the brush border was assayed spectroscopically (410 nm) by monitoring the release of *p*-nitrophenol under the following conditions: 10 mM *p*-nitrophenylphosphate and 0.5 mM Mg^{2+} in $NaHCO_3$ buffer (100 mM, pH 10, 23°).

Effect of GSH on the binding of DDP to DNA. DNA from salmon testes was dissolved in 50 ml of 20 mM $NaClO_4$ by gentle stirring overnight at 23°, and the concentration of bases was adjusted to 2.77 mM with 20 mM $NaClO_4$, assuming an extinction coefficient of 6600 $M^{-1}\,cm^{-1}$ at 260 nm. HEPES was added to a concentration of 10 mM, and the pH was adjusted to 7.0 (37°) with 5 N NaOH. This stock solution was purged with N_2 for 10 min and divided into several 15-ml aliquots, to which GSH was added to a final concentration of 0.5 or 5 mM. The pH was again adjusted to 7.0 (37°) with 5 N NaOH. Equal volumes of water were added to control solutions to account for dilution. Finally, all the solutions were preheated to 37°, and aliquots of 2 mM DDP in 20 mM $NaClO_4$ were added to the solutions to achieve the following conditions: 2.7 mM DNA bases, 27 μM DDP, 20 mM $NaClO_4$, 10 mM HEPES, pH 7.0, and ±0.5 or 5 mM GSH. The solutions were maintained under N_2 to prevent GSH oxidation. At various times, 1-ml aliquots were removed and diluted with 1 ml of 0.9% NaCl, placed in a Centricon 30 Microconcentrator, and centrifuged for 10 min at 1000 *g*. The filtrate was diluted 1:1 with 0.9% NaCl and assayed for platinum by flameless AAS.

RESULTS

Rates of substitution reactions of platinum complexes. The second-order rate constants for the reactions of several sulfur-containing nucleophiles with DDP, *trans*-DDP, CHIP and CBDCA at 37° are presented in Table 2, and representative plots of k_{obs} vs [nucleophile] are shown in Fig. 1. It is apparent from Table 2 that Met and Cys share similar reactivities with DDP and that the reactivity of the cysteinyl sulfur dropped 2-fold when it was incorporated into GSH. S-Methylation of GSH increased its reactivity with DDP. The platinum complexes studied here differed widely in their reactivities to GSH; the order of reactivity was 1:1.5:22:6500 for CHIP:CBDCA:DDP:*trans*-DDP. The reaction half-lives for *trans*-DDP, DDP, CBDCA and CHIP with 5 mM GSH at 37° were 0.5, 180, 2500, and 3800 min respectively.

A similar range of reactivities with the chemoprotectors DDTC and thiosulfate is seen that parallels the GSH data. The rate constant for the reaction of DDP with thiosulfate agrees well with the value determined by Riley *et al.* [21] when allowance is made for the temperature difference: $358 \times 10^{-4}\,M^{-1}\,sec^{-1}$ at 30° and $570 \times 10^{-4}\,M^{-1}\,sec^{-1}$ at 37°.

Despite the apparent polymeric nature of the $[Pt(GSH)_2 \cdot 3H_2O]_n$ complex isolated in these experiments, DDTC was able to displace GSH and form $Pt(DDTC)_2$ at a rate similar to that for DDP (Table 2). Thiosulfate did not appear to react with $[Pt(GSH)_2 \cdot 3H_2O]_n$ as indicated by the HPLC, ^1H-NMR and GSH assay experiments. It appears that reaction of DDTC with DDP or CBDCA occurs via direct substitution, since the rate constants determined by measuring loss of parent complex or appearance of $Pt(DDTC)_2$ are essentially identical and faster than the loss of chloride ion.

The second-order rate constant ($[H_2O] = 55.5$ M) at 23° for the hydrolysis of chloride ligands from DDP determined here, $4.3 \pm 0.2 \times 10^{-7}\,M^{-1}\,sec^{-1}$, agrees well with the value of $4.5 \times 10^{-7}\,M^{-1}\,sec^{-1}$ reported by Reishus and Martin [22] at 25°. Chloride ligands were released from CHIP 34-fold more slowly than from DDP, with a second-order rate constant of $1.3 \pm 0.4 \times 10^{-8}\,M^{-1}\,sec^{-1}$. Phosphate buffer

Table 2. Second-order rate constants ($k_2 \times 10^4 M^{-1} sec^{-1}$) for substitution reactions of platinum complexes at 37°*

	DDTC	GSH	Thiosulfate
trans-DDP	255,000 ± 21,000	39,100 ± 1,700 (9271 ± 464)†	
DDP	614 ± 26 (660 ± 22)‡	132 ± 8 (42.0 ± 1.8)†	570 ± 27
CBDCA	76.2 ± 4.5 (101 ± 7.6)‡	9.15 ± 0.30 (2.39 ± 0.18)†	85.1 ± 7.2
CHIP	6.55 ± 0.58	6.0§	
$[Pt(GSH)_2 \cdot 3H_2O]_n$	499 ± 190‡		<0.1
	S-methyl GSH	Met	Cys
DDP	219 ± 9	396 ± 7 (182 ± 5)†	386 ± 44 (151 ± 12)†

* Unless noted, all constants are mean ± SD for 4–7 nucleophile concentrations.

† Second-order rate constants at 23°.

‡ Second-order rate constant for the appearance of $Pt(DDTC)_2$; pseudo first-order rate constants were determined from a plot of $\ln(H_\infty - H_t)$ vs time, where H_∞ and H_t are the HPLC peak heights for $Pt(DDTC)_2$ at $t = \infty$ and at various times during the reaction respectively. See Ref. 16 for details of HPLC procedure.

§ Three concentrations of nucleophile were employed.

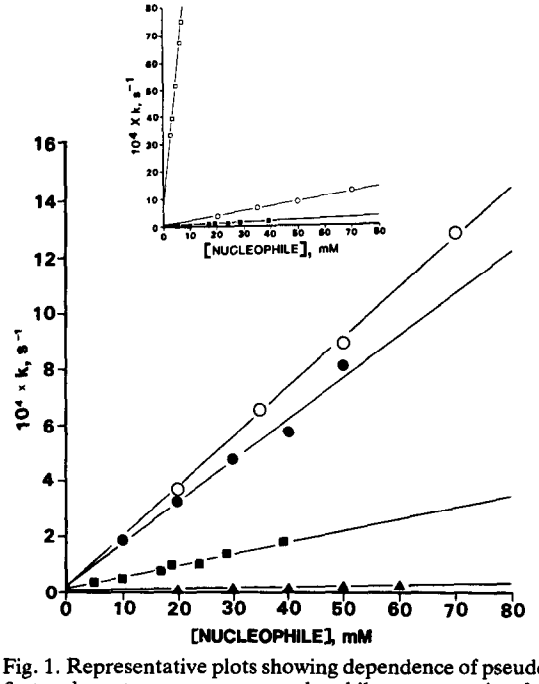

Fig. 1. Representative plots showing dependence of pseudo first-order rate constants on nucleophile concentration for the following reactions at 23°: (○) Met and DDP; (●) Cys and DDP; (■) GSH and DDP; (▲) GSH and CBDCA; and (□) GSH and *trans*-DDP. Inset scaled to include *trans*-DDP/GSH reaction.

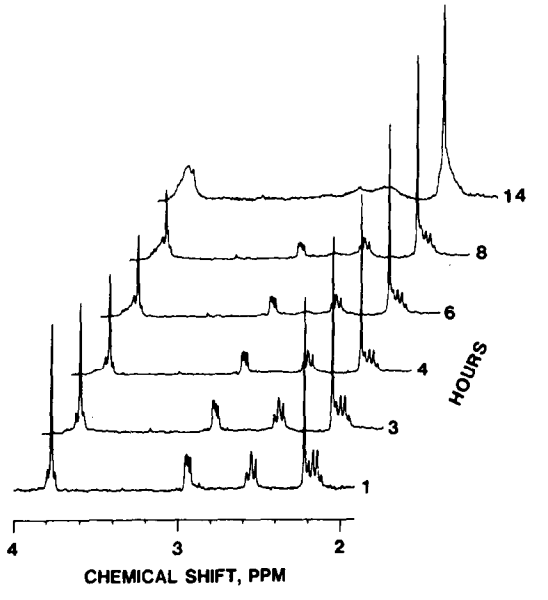

Fig. 2. Reaction of DDP and GSH (2 mM each) at 23° monitored by ^{1}H-NMR. Chemical shift assignments: 2.15 ppm (quartet), β-methylene Glu; 2.25 ppm (singlet), acetone; 2.55 ppm (triplet), γ-methylene Glu; 2.95 ppm (multiplet), β-methylene Cys; 3.80 ppm (singlet), α-methylene Gly; and 3.80 ppm (triplet), α-methine Glu. The α-methine Cys signal was lost in gated decoupling of HOD signal.

concentration was constant in all experiments in which it was employed and thus affects only k_1, the rate constant for the solvent-mediated pathway. Perchlorate ion is inert in substitution reactions of square planar complexes [23].

Reaction of GSH and DDP monitored by ^{1}H-NMR. It is apparent from Fig. 2 that there is a progressive loss of GSH resonance intensity as a function of time, presumably due to line broadening of the signal. The acetone resonance at 2.15 ppm was not broadened, indicating that the GSH line broadening was not an experimental artifact. In the experiment with 2 mM DDP and 10 mM GSH, the signal intensities decreased by a maximum of 40%

and thereafter remained unchanged (data not shown). This is consistent with the formation of a high molecular weight 2:1 complex of GSH and platinum.

Products of the reaction of GSH with DDP, CBDCA and trans-*DDP.* The precipitate that formed in the reaction of 6 mM GSH and 3 mM DDP represents a bis adduct of GSH with platinum and is similar to the product isolated by Odenheimer and Wolf [24]. The structure they suggest is a symmetrical bis-bidentate adduct with both the sulfur and the amide nitrogens of the Cys residues *trans* to each other. It is clear from our experiments, however, that a high molecular weight species had formed. In a 3 mM solution of $[Pt(GSH)_2 \cdot 3H_2O]_n$, > 97% of the platinum was retained by both the 10,000 and 30,000 dalton cut-off filters. Large platinum–GSH complexes were also present in the CBDCA/GSH mixture, in which 85% of the products were retained by the 10,000 dalton filter. None of the *trans*-DDP GSH products was retained by either filter. A large molecular weight reaction product obtained from 3 mM CBDCA and 6 mM GSH is also suggested by the presence of greatly broadened NMR signals of the GSH protons (data not shown) similar to that seen in the 14-hr spectrum of DDP and GSH (Fig. 2). The 1,1-cyclobutanedicarboxylate methylene proton resonances occurred at 1.8 and 2.35 ppm, indicating that this group is no longer bound to platinum; when this group is bound to platinum, the protons at 2.35 ppm are shifted to 2.9 ppm.

The NMR spectrum of the reaction products from 3 mM *trans*-DDP and 6 mM GSH showed a 0.2 ppm upfield shift of the Cys β-carbon protons, which are normally located at 2.95 ppm (Fig. 2), and a change in the environment of these protons as indicated by the altered coupling pattern. These findings indicate bonding of the GSH sulfhydryl to *trans*-DDP and are similar to those reported by Rabenstein [25] for the bonding of methylmercury to GSH. A small amount of free GSH still remained as indicated by HPLC analysis and residual signal at 3.1 ppm, while the presence of two sets of signals centered at 3.2 ppm indicated small amounts of oxidized GSH. The signals from the other hydrogen atoms of GSH were not appreciably affected by bound platinum, and resolution of individual signals remained high, indicating an absence of the line broadening that occurred with CBDCA and GSH.

Inhibition of enzymes by platinum complexes in vitro *and restoration of activity by sulfur-containing nucleophiles.* The activities of LAP and GGTP in rat renal brush border preparations have been shown previously to be inhibited by DDP [2], and two other groups of investigators have demonstrated DDP inhibition of ATPases [5, 26]. The effects of several platinum complexes on the activities of LAP and GGTP are shown in Fig. 3 and on the activity of ATPases in Table 3. The extent of inhibition of these enzymes parallelled the reactivity of these complexes with other nucleophiles: DDP ≫ CBDCA > CHIP.

It is apparent from Fig. 3 that the aquation of DDP is an important factor in its reactivity with renal brush border LAP and GGTP. The initial rate of GGTP inhibition by DDP was first-order, and in the presence of 100 mM chloride the rate constant was

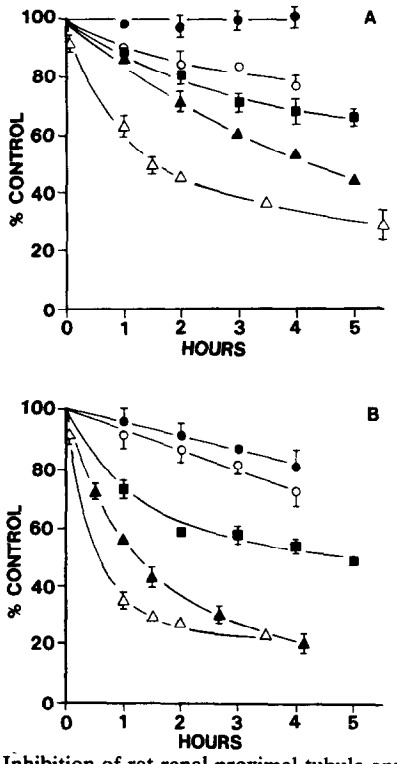

Fig. 3. Inhibition of rat renal proximal tubule enzymes by platinum complexes. (A) GGTP; (B) LAP. Key: (●) 15 mM CHIP; (○) 15 mM CBDCA; (■) 2 mM DDP + 100 mM NaCl; (▲) 2 mM DDP; and (△) 2 mM $Pt(NH_3)_2(H_2O)(OH)^+$. GGTP activity as percent of control at 2-hr incubation: CHIP, 100.9 ± 3.0; CBDCA, 78.6 ± 3.0; DDP + NaCl, 80.3 ± 3.0; DDP, 71.4 ± 3.4; $Pt(NH_3)_2(H_2O)(OH)^+$, 45.0 ± 2.0. LAP activity as percent of control at 2-hr incubation: CHIP, 73.8 ± 0.3; CBDCA, 79.6 ± 5.4; DDP + NaCl, 27.5 ± 2.0. For both enzymes, the DDP, DDP + NaCl, and $Pt(NH_3)(H_2O)(OH)^+$ values are significantly different from each other by Student's *t*-test, $P < 0.05$; $\phi = 4$. Values represent mean ± SD for N = 3. See Materials and Methods for experimental details. See Table 4 for absolute values of control enzyme activities.

reduced by 1.7-fold from $4.7 \times 10^{-5}\,sec^{-1}$ to $2.8 \times 10^{-5}\,sec^{-1}$. A similar effect was seen with LAP. The effect of chloride on the inhibition of LAP and GGTP by CBDCA and CHIP was only significant for CBDCA and LAP; a 2-hr incubation with 50 mM CBDCA and 50 mM CBDCA + 100 mM NaCl resulted in enzyme activity that was 79.6 ± 5.4 and 96.6 ± 2.2% of control activity respectively. These values are significantly different by Student's *t*-test, $P < 0.05$ and $\phi = 4$. The data in Table 3 indicate that chloride had a much smaller impact on ATPase inhibition by platinum complexes, which is in agreement with the findings of Daley-Yates and McBrien [5].

The rate of inhibition of GGTP by 50 mM CBDCA was similar to that for 2 mM DDP (Fig. 4). As we previously demonstrated for DDP-inhibited GGTP [2], DDTC was capable of restoring activity to the CBDCA-inhibited enzyme as indicated in Fig. 4. The kinetics of restoration of activity in both cases was first-order with a rate constant of $2 \times 10^{-4}\,sec^{-1}$ at 10 mM DDTC. This corresponds to a second-order rate constant of $2 \times 10^{-2}\,M^{-1}\,sec^{-1}$ which is

Table 3. *In vitro* ATPase activity following exposure to platinum complexes

	Activity as % of control*	
	Na^+/K^+-ATPase	Mg^{2+}-ATPase
2 mM DDP	8.3 ± 1.2	45.8 ± 5.5†
2 mM DDP + 100 mM NaCl	10.3 ± 4.9	57.3 ± 2.2†
2 mM CBDCA	88.0 ± 8.8	93.5 ± 3.2
15 mM CBDCA	44.5 ± 5.2	69.7 ± 4.8
15 mM CBDCA + 100 mM NaCl	38.1 ± 7.7	78.8 ± 4.0
2 mM CHIP	80.3 ± 14.4	88.0 ± 0.5
15 mM CHIP	42.6 ± 9.2	71.8 ± 5.0

* Values are mean ± SD (N = 3) for 2-hr exposure to the complexes as described in Materials and Methods. Values for DDP are for 1-hr exposure. See Table 4 for absolute values of control enzyme activity.

† Values are significantly different by Student's *t*-test, $P < 0.05$; $\phi = 4$.

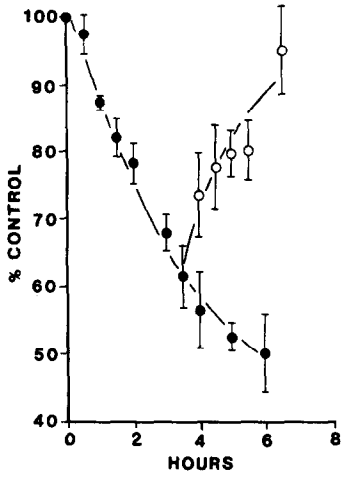

Fig. 4. Inhibition of rat renal proximal tubule GGTP by 50 mM CBDCA (●) and restoration of activity by 10 mM DDTC (○) added after 3.5 hr of exposure to CBDCA. Values represent mean ± SD for N = 3. See Materials and Methods for experimental details. See Table 4 for absolute values of control enzyme activities.

similar to the value of $5 \times 10^{-2} M^{-1} sec^{-1}$ for the reaction of DDTC with $[Pt(GSH)_2 \cdot 3H_2O]_n$. The results of exposure of DDP-treated brush border microvilli to thiourea and thiosulfate are shown in Fig. 5. Thiourea restored LAP activity from 40 to 80% of control over 4 hr, whereas thiosulfate restored activity to only 55% of control value. Similar results are apparent for GGTP, but the thiosulfate rescue was not statistically significant, as indicated in Fig. 5B.

Effect of DDP on rat renal enzymes in vivo. The activities of five renal proximal tubule membrane-bound enzymes were measured in Fisher 344 rats at 4 hr and 18 or 24 hr after DDP administration. The results are shown in Table 4. Four hours after intravenous injection of DDP none of the enzymes was reduced in activity, whereas at 24 hr only AP and LAP activities were reduced significantly. At 18 hr, neither ATPase activity was affected by DDP. Administration of DDTC 2 hr after DDP did not prevent loss of enzyme activity (data not shown). Uozumi and Litterst [26] also demonstrated that DDP has no effect on ATPase activity *in vivo*.

Effect of GSH on the binding of DDP to DNA. This experiment was designed to assess the effect of GSH on the binding of DDP to DNA. GSH significantly reduced the amount of platinum bound to DNA, as assessed by loss of platinum from the filtrate. GSH at concentrations of 0.5 and 5 mM reduced the rate constant of platinum–DNA binding from $7.40 \times 10^{-5} sec^{-1}$ (SD = 0.17×10^{-5} sec^{-1}, N = 6) to $5.88 \times 10^{-5} sec^{-1}$ (SD = $0.52 \times 10^{-5} sec^{-1}$, N = 3) and $1.68 \times 10^{-5} sec^{-1}$ (SD = $0.13 \times 10^{-5} sec^{-1}$, N = 3) respectively. Values determined in the presence of GSH are significantly different from control by Student's *t*-test, $P < 0.05$ and $\phi = 7$. The rate constant of $7.4 \times 10^{-5} sec^{-1}$ found here for the binding of DDP to DNA was slower than that reported previously [2] and probably results from the chelation of aquated platinum species by the HEPES buffer.

Table 4. Effect of cisplatin on rat renal enzymes *in vivo*

	AP	GGTP	LAP	Mg^{+2}-ATPase	Na^+/K^+-ATPase
Control*	3.1 ± 0.1	14.4 ± 2.2	0.69 ± 0.01	0.81 ± 0.03	0.45 ± 0.06
Cisplatin* (7.5 mg/kg)	1.9 ± 0.2	13.1 ± 1.8	0.62 ± 0.03	0.77	0.45
% Control	61†	91	90†	95	100
Time after cisplatin (hr)	24	24	24	18	18

* Values represent μmoles/min/mg protein ± SD for N = 6 except for the ATPases where N = 2.

† Significant difference between treatment and control activities by Student's *t*-test, $P < 0.001$; $\phi = 10$.

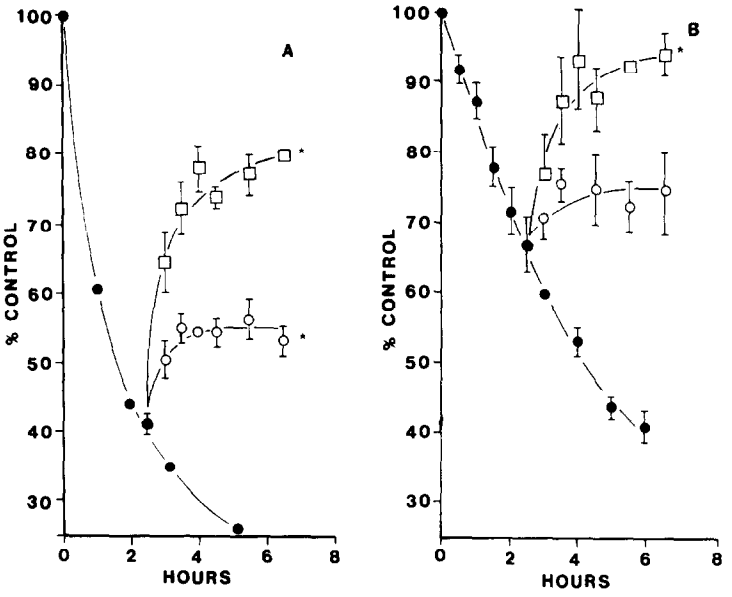

Fig. 5. Inhibition of rat renal proximal tubule enzymes by 2 mM DDP (●) and restoration of activity by 10 mM thiourea (□) and 10 mM thiosulfate (○) after a 2.5-hr exposure to DDP. (A) LAP; (B) GGTP. Values represent mean ± SD for N = 3 except for LAP inhibition data (N = 2). See Materials and Methods for experimental details. Key: * Values are significantly different from t = 2.5-hr enzyme inhibition values by Student's *t*-test, $P < 0.05$; $\phi = 4$. See Table 4 for absolute values of control enzyme activities.

DISCUSSION

Pt(II) complexes are relatively unreactive compared to their Pd(II) and Ni(II) counterparts by factors of 10^5 and 10^7 respectively [18]. Despite this apparent low reactivity, nucleophilic substitution reactions govern the biologic activity of platinum complexes in terms of antitumor activity, toxicity and biotransformation. In this work, we have characterized the reactions of several important platinum complexes with sulfur-containing amino acids, peptides, proteins and nonbiologic molecules that have been implicated as affecting the biologic activity of these complexes.

DDP is cleared rapidly from the plasma following its administration, with a $T_{\frac{1}{2}} \simeq 30$ min [27]. Direct reactions of DDP with free Met, Cys, and GSH in the plasma would occur with a $T_{\frac{1}{2}} > 1000$ min given the low concentrations of these species: 50, 49, and 30 μM respectively [28]. Even serum albumin with its plasma concentration of 700 μM and single reactive sulfhydryl group [8] would react directly with a $T_{\frac{1}{2}} \simeq$ 1300 min assuming a rate constant similar to that of GSH. The loss of DDP from plasma by reaction with plasma components, however, appears to occur at a rate similar to the rate of aquation of DDP [29, 30] despite the presence of 100 mM chloride in the plasma. While high concentrations of chloride will increase the rate of formation of DDP from $Pt(NH_3)_2(H_2O)(Cl)^+$ (the annation reaction), the hydrolysis of the chloride ligands of DDP proceeds at the same rate regardless of the chloride concentration. Nucleophiles more reactive than chloride ion, such as the sulfhydryl group of Cys, will trap the aquated form before the annation reaction can occur. Thus, despite nucleophile concentrations too low to allow significant *direct* reaction with DDP, reactive moieties of plasma proteins and other nucleophiles can bind aquated DDP in the presence of 100 mM chloride.

Once inside the cell, the low chloride concentration shifts the equilibrium toward aquated forms of DDP. GSH is the most prevalent intracellular sulfur nucleophile, ranging in concentration from 0.5 to 10 mM in most cells [31]. The results presented here show that 5 mM GSH can inhibit the rate of binding of DDP to DNA, presumably by interception of reactive platinum complexes. Evidence for the role of GSH as an interceptor of platinum complexes comes from the work of Hamilton *et al.* [11], who have demonstrated that buthionine sulfoximine (BSO)-mediated reduction of intracellular GSH by 3- to 9-fold in A2780 human ovarian carcinoma cells results in a 3- to 4-fold increase in the sensitivity of the cells to DDP.

To characterize more thoroughly the products formed in reactions of GSH with platinum complexes, we isolated an adduct of GSH. The Cys sulfhydryl group is bound to platinum as indicated by the absence of the characteristic S-H band at 2515 cm^{-1} in the IR spectrum of the compound. In the *trans*-DDP/GSH product, the presence of a Pt—S bond is suggested by the altered coupling pattern and upfield shift of the Cys β-methylene hydrogens in the NMR spectrum of a GSH/*trans*-DDP reaction mixture. It is apparent that, under conditions of high platinum concentration, GSH and *cis*-complexes of platinum form high molecular weight species but *trans*-DDP does not. It should be emphasized that there is no evidence to suggest that these large molecules form at the low concentrations of platinum achieved *in vivo*.

The decreased reactivities of CBDCA and CHIP towards nucleophilic substitution demonstrated here

may account for their different biologic activities compared to DDP. Optimal antitumor activity of these drugs in mice occurs at doses 10-fold higher than for DDP [32, 33]. Micetich *et al.* [34] have shown that CBDCA is 45-fold less potent than DDP against L1210 cells and that peak DNA cross-linking occurs 12 hr later for CBDCA. According to Knox *et al.* [4], the carboxylate ligands of CBDCA hydrolyze 100-fold more slowly than the chloride ligands of DDP, and it is this reduced rate of aquation that accounts for the reduced rate of initial binding of CBDCA to DNA. The stability of the 1,1-cyclobutanedicarboxylate ligand also accounts for the decreased reactivity of CBDCA toward the sulfur-based nucleophiles examined here. We have shown that CHIP chloride ligands are hydrolyzed thirty-four times more slowly than those of DDP. Whether the biologic activity of CHIP occurs via direct substitution reactions, reduction to Pt(II) complexes, or by substitution reactions catalyzed by Pt(II) remains uncertain [35].

All of the enzymes studied here may be regarded as nucleophiles with respect to platinum complexes. Each has a sulfhydryl group(s) essential for activity [36–38], and it is generally assumed that enzymes must possess a Cys or Met for reaction with and inhibition by platinum complexes [39]. The relative order of enzyme inhibition by DDP, CBDCA, and CHIP *in vitro* parallels their reactivities with other nucleophiles. Caution must be used in interpreting the *in vitro* enzyme inhibition experiments since steric, charge, and lipophilicity factors as well as the number and nature of nucleophilic sites all play a role in the interaction of the platinum complexes with these enzymes. Furthermore, several hours of exposure to the platinum complexes are required to significantly inhibit most of the enzymes. The different effects of chloride on the enzyme inhibition by DDP, CHIP and CBDCA underscores the complex nature of the inhibition. The suppressed inhibition of LAP and GGTP in the presence of 100 mM chloride suggests that aquated DDP species are important in the inhibition of these enzymes. Inhibition of ATPase activity was much less affected by chloride, however, which suggests a rapid direct reaction with DDP. The rat renal proximal tubule cell enzymes, GGTP, LAP and Na^+/K^+- and Mg^{+2}-ATPase provide an excellent model to study the binding of platinum complexes to proteins and to study the platinum inhibition of an important biochemical process. These results, however, do not implicate these enzymes as primary targets for DDP toxicity as indicated by the *in vivo* findings.

The three sulfur-containing compounds, DDTC, thiosulfate, and thiourea, have all been shown to protect against platinum complex nephrotoxicity [13, 14]. The experimental results presented here suggest differing mechanisms of chemoprotection for these agents. The reversal of enzyme inhibition by thiourea and DDTC represents a ligand exchange reaction between the presumed Pt—S bond of the enzyme and the nucleophile. The structural differences between, and known reactivities of, DDTC and thiourea indicate that enzyme activity can be restored by the chelation and removal of platinum as opposed to disulfide interactions or other direct effects on the enzymes. DDTC undergoes facile exchange with GSH in $[Pt(GSH)_2 \cdot 3H_2O]_n$, and we have shown previously that DDTC restores activity in a concentration-dependent manner to GGTP inhibited by DDP [2]. From Fig. 4, it is apparent that DDTC also restores activity to GGTP inhibited by CBDCA, with the same kinetics as with DDP. Thiourea was nearly as effective in restoration of enzyme activity as DDTC. Thiosulfate, however, was ineffective at restoring activity to DDP-inhibited GGTP or LAP (Fig. 5, A and B), and did not displace GSH from $[Pt(GSH)_2 \cdot 3H_2O]_n$. While DDTC, thiosulfate and thiourea share similar reactivities with DDP (the rate constant for the reaction of thiourea with DDP is $519 \times 10^{-4}\,M^{-1}\,sec^{-1}$ at 30° [21]), these agents differ in their abilities to remove platinum bound to other ligands.

These mechanistic data are consistent with biologic observations on the chemoprotection afforded by thiosulfate and DDTC. Chemoprotection with thiosulfate is optimum when it is administered concomitantly with DDP which, together with the demonstrated high reactivity for DDP, suggests that it exerts its chemoprotective effects by directly chelating DDP and/or other reactive platinum species in the kidney nephron. Uozumi and Litterst [26] have shown that thiosulfate does not affect the level of platinum bound to kidney tissue, consistent with our findings that thiosulfate did not remove platinum bound to proteins. DDTC has been shown to cause a reduction in renal platinum content that correlates with a reduction in nephrotoxicity [14]. DDTC is unique in that it affords protection from DDP toxicity when administered after DDP at a time when most of the reactive platinum species have been taken up by cells or excreted in the urine. Even at a maximal plasma DDTC concentration of 1 mM, the reaction between DDTC and DDP would occur with a $T_{\frac{1}{2}} \simeq$ 190 min, precluding significant reaction given the rapid clearance of DDTC from the plasma ($T_{\frac{1}{2}} \simeq$ 10 min) [2]. Thus, while both DDTC and thiosulfate are effective chemoprotectors, their mechanisms of action may differ by virtue of their differing chemical reactivities with bound platinum.

In summary, the data we have presented quantify the varying reactivities of antitumor platinum complexes with various nucleophiles. The kinetics of reactions of *trans*-DDP, DDP, CBDCA and CHIP with biologic nucleophiles suggest that differences in chemical reactivity account for their differing antitumor and toxic properties. Similarly, the mechanisms of action of the chemoprotective agents thiourea, thiosulfate, and DDTC appear to differ as indicated by their reactivities with platinum bound to macromolecules.

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