[CANCER RESEARCH 46, 2745-2750, June 1986]

Effect of Diethyldithiocarbamate on *cis*-Diamminedichloroplatinum(II)-induced Cytotoxicity, DNA Cross-Linking, and γ-Glutamyl Transpeptidase Inhibition[1]

Donald L. Bodenner, Peter C. Dedon, Peter C. Keng, and Richard F. Borch[2]

Departments of Pharmacology [D. L. B., P. C. D., R. F. B.] and Radiation Oncology [P. C. K.] and The Cancer Center, University of Rochester, Rochester, New York 14642

ABSTRACT

Diethyldithiocarbamate (DDTC) has been shown to protect against the toxicity of *cis*-diamminedichloroplatinum(II) (DDP) without inhibition of antitumor effect. We report here that DDTC is unreactive toward DDP complexes in which both chlorides have been replaced by guanine residues but removes platinum from a variety of other ligands, and that this difference in reactivity may provide the basis for the selective protection observed with DDTC. Platinum-DNA complexes were unreactive toward DDTC (10 mM, >4 h) when the platinum:base ratio $r < 0.02$. DDTC did not react with the 1:2 complex of DDP:guanosine but reacted rapidly with the 1:1 complex and with the 1:2 complexes of DDP:adenosine. Reaction of DDP with DDTC was second order with a rate constant $k = 4.4\ M^{-1}\ min^{-1}$ at 37°C, corresponding to a $t_{½} = 150$ min at [DDTC] = 1 mM. Treatment of L1210 cells with DDTC (0.5–1 mM) after exposure to DDP indicated that DDTC had no effect on cell kill if DDTC treatment was delayed for 1 h after DDP. The effect of DDTC on DDP-induced DNA interstrand cross-links was also examined in L1210 cells. Interstrand cross-links were decreased by ~50% when cells were treated with DDTC immediately after DDP; no change in DNA interstrand cross-links was observed when DDTC treatment occurred 3 h after DDP. A modified alkaline elution procedure was used to evaluate the effects of high concentrations of DDTC, thiourea, and cyanide on platinum:DNA cross-links from L1210 DNA. Exposure to DDTC (0.5 M, 4 h) did not alter interstrand cross-links, but both thiourea and cyanide caused extensive reversal of cross-links at concentrations as low as 10 and 1 mM, respectively. Both commercial and rat kidney brush border preparations of γ-glutamyl transpeptidase were inhibited by exposure to 2 mM DDP; exposure of the inhibited enzyme to DDTC (1 or 10 mM) resulted in significant restoration of enzyme activity. These data indicate that DDTC has unique chemical specificity in its reactions with platinum complexes and that this specificity is ideal for application as a chemoprotective drug against *cis*-platinum toxicity.

INTRODUCTION

DDP[3] is believed to generate tumoricidal lesions by formation of bifunctional adducts with DNA (1). In contrast, the mechanistic basis for DDP's toxicity to the kidney and other organ systems is not known. We previously hypothesized that nephrotoxicity and gastrointestinal toxicity may be a consequence of platinum binding and inactivation of thiol-containing enzymes, and that the ability of DDTC to inhibit DDP toxicity results from DDTC's ability to remove platinum from these protein thiol groups (2). Evidence in support of this enzymatic basis for DDP toxicity has appeared (3, 4). A number of sulfur nucleophiles has been studied as inhibitors of DDP-induced nephrotoxicity (5–10); however, selective protection of normal tissue without inhibition of antitumor effect has been difficult to achieve. Thiourea reacts rapidly with platinum complexes and has been shown to reverse platinum:DNA cross-links in solution (11) and inhibit platinum:DNA cross-links in cultured cells (12, 13). We and others (14–21) have shown that DDTC provides protection against renal, gastrointestinal, and bone marrow toxicity induced by DDP without concomitant inhibition of DDP's antitumor effect. We describe here the results of our studies on the reaction of DDTC with a number of platinum complexes, which support the hypothesis that DDTC selectively reverses platinum:thiol complexes without reversal of platinum:DNA cross-links.

MATERIALS AND METHODS

Chemicals. *cis*-Platinum was obtained through the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, or from Bristol Laboratories. Sodium diethyldithiocarbamate, salmon sperm DNA, γ-glutamyl *p*-nitroanilide, guanosine, adenosine, cytidine, Tris, sodium dodecyl sulfate, and γ-glutamyl transpeptidase were obtained from Sigma Chemical Co. HPLC solvents, sodium cyanide, thiourea, and EDTA were obtained from Baker Chemical Company. Noble agar was obtained from Difco Laboratories, and proteinase K and tetrapropyl ammonium hydroxide were obtained from Merck and RSA, Inc., respectively. Radiolabeled thymidine and Aquasol were purchased from New England Nuclear. RPMI Medium 1640 and horse serum were obtained from Gibco.

Hydrolysis of DDP. A chloride ion-specific electrode (Orion, Inc.) was placed in a solution of DDP (1.7 or 3.3 mM in 5 mM sodium nitrate), the electrode was connected to a Corning pH meter set on the millivolt scale, and liberated chloride ion was measured every 2 min for 2 h by comparison of the millivolt readings to those obtained from chloride ion standard curves. Determinations were made in triplicate at both 20°C and 37°C.

Kinetics of the Reaction between DDP and DDTC. A solution of 5 mM DDTC and 50 mM Tris (pH 7.4, 37°C) in distilled water was divided into ten 975-μl portions. To each sample were added 25 μl of a freshly prepared DDP solution (0.60 mg in 1.0 ml of distilled water) to give a final DDP concentration of 50 μM. Samples were removed from the constant temperature bath at intervals over 2 h; $CHCl_3$ (2.0 ml) was added, the sample was agitated for 1 min, and the $CHCl_3$ layer was analyzed for platinum bis(diethyldithiocarbamate) by HPLC (Footnote 4; Ref. 22). The pseudo-first order rate constant was determined from the slope of the plot of $\ln(H_\infty - H_t)$ *versus* t, where H_∞ and H_t are the $Pt(DDTC)_2$ peak heights at 3 h and time t, respectively. The slope was $3.62 \times 10^{-4}\ s^{-1}$ determined by linear regression, $r^2 = 0.995$.

Kinetics of DDP Binding to DNA. A solution of salmon sperm DNA was prepared in 20 mM $NaClO_4$ to give a solution which was 2.5 mM in DNA base, determined by absorbance at 260 nm. A solution of DDP (1 mg/ml in 20 mM $NaClO_4$) was prepared, and aliquots were added to the DNA solution in amounts required to produce platinum:base ratios of 0.01, 0.02, 0.05, 0.08, and 0.1, respectively. Final base concentration was adjusted to 2.2 mM by the addition of 20 mM $NaClO_4$. Aliquots (1 ml) were removed every 15 min for 3 h and every 30 min for an additional 5 h, added to 1 ml of 0.1 M NaCl, and centrifuged for 7 min at 450 × *g* through spin dialysis cones (Amicon; M_r cutoff, 30,000). The platinum concentration in the ultrafiltrate was determined by flameless atomic absorption analysis and by formation, extraction, and HPLC analysis of platinum bis(diethyldithiocarbamate) (Footnote 4; Ref. 22). Excellent agreement was obtained between the two methods.

The effect of phosphate buffer on binding kinetics was examined at a platinum:base ratio of 0.01. The solutions were prepared as described

Received 7/25/85; revised 12/10/85; accepted 1/24/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by Grants CA 22925, CA 34620, and CA 11198. Taken in part from the Ph.D. Thesis of D. L. B., University of Minnesota, December 1983.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: DDP, *cis*-diamminedichloroplatinum(II); DDTC, sodium diethyldithiocarbamate; GGT, γ-glutamyl transpeptidase; HPLC, high-pressure liquid chromatography.

[4] P. H. Lieder and R. F. Borch, unpublished results.

above, except that the appropriate concentrations of sodium phosphate buffer were used in place of the $NaClO_4$ solutions.

Reversal of Platinum:Base and Platinum:DNA Complexes. Platinum:DNA complexes were prepared at platinum:base ratios 0.01–0.1 using the procedures described above and allowing the reactions to go to completion in the dark. The resulting solutions of the platinum:DNA complexes were equilibrated at 37°C for 15 min, and DDTC was added to a final concentration of 10 mM. Aliquots were removed at 30-min intervals and extracted with $CHCl_3$ (1 ml), and the $CHCl_3$ extract was analyzed for $Pt(DDTC)_2$ by HPLC as described previously (Footnote 4; Ref. 22).

The platinum:bis-nucleoside complexes were prepared by reacting 80 μmol of adenosine, guanosine, or cytidine and 20 μmol of DDP in 20 ml of water. The samples were heated to 50°C in the dark for 48 h and then allowed to go to completion at room temperature; the extent of reaction was monitored by HPLC as described below. The platinum:guanosine monoadduct was prepared by reaction of guanosine with excess DDP; the reaction was monitored by HPLC and terminated when monoadduct formation was maximal. Adducts were isolated by preparative HPLC and characterized by UV, nuclear magnetic resonance, and platinum atomic absorption spectroscopy, and by comparison of HPLC retention times using conditions identical to those reported by Eastman (23). The bisadenosine complexes were obtained as an inseparable mixture of two isomers, and subsequent reactions were carried out on the isomeric mixture. The kinetics of platinum:base reversal was measured by adding DDTC to these solutions to a final concentration of 10 mM at 37°C. Aliquots were removed, and the platinum:base complex was quantified by HPLC.

Cell Treatments. Exponentially growing L1210 cells in RPMI Medium 1640 containing 10% horse serum were labeled for 24–48 h with [2-^{14}C]thymidine (0.01 μCi/ml). The cells were washed twice with RPMI Medium 1640 and then resuspended in this medium to give a final density of 5–8 × 10^5 cells/ml after addition of drug solution. The cells were equilibrated at 37°C for 15–30 min and then treated with DDP for 1 h. Cells were then washed 3 times with supplemented medium, resuspended in the same medium, and treated with DDTC for 1 h at the concentrations and times indicated. Again cells were washed 3 times in supplemented medium and assayed for viability by the method of Chu (24). Between 10^2 and 10^5 cells were suspended in 3 ml of medium containing 0.01% Noble agar, and colonies were counted after 12–15 days of incubation.

DNA Interstrand Cross-Linking. The alkaline elution assay was performed as previously described (25). L1210 cells were labeled and exposed to DDP with or without DDTC as described above. Experimental cells were subjected to 300 rads of X-ray at 4°C, and ^{3}H-labeled internal standard L1210 cells received 100 rads of X-ray at 4°C. Equal numbers (2 × 10^5) of treated and control cells were mixed, deposited on polyvinyl chloride filters, and lysed with a solution of 2% sodium dodecyl sulfate, 0.3% glycine, 20 mM EDTA, and proteinase K (0.5 mg/ml). The filters were then eluted with a solution of 0.1 M tetrapropylammonium hydroxide and 0.02 M EDTA at pH 12.1 with an elution rate of 2 ml/h. A total of ten 3-ml fractions was collected, and the radioactivity of the eluted fractions and filter was determined by scintillation counting in Aquasol. Cross-linking was expressed in rad equivalents with the 35% internal standard DNA retention as the end point as described previously (13).

A modified alkaline elution procedure was developed to evaluate platinum:DNA reversal by toxic levels of various drugs. Cells were labeled and exposed to DDP as described above and then incubated in RPMI Medium 1640 containing 10% horse serum for 8–12 h at 37°C to maximize cross-linking. The cell suspensions were then irradiated and lysed on polyvinyl chloride filters as described above. The filters were washed twice with EDTA (1.5 ml, 20 mM, pH 10.0). Solutions of sodium DDTC, thiourea, and sodium cyanide at the appropriate concentrations containing 20 mM EDTA were placed on the filter and eluted at the rate of 2 ml/h for the specified times. The filters were then washed with EDTA, and the DNA was eluted as described above. Reversal was determined from the difference in rad-equivalent cross-links between control and filter-treated DNA and is expressed as a percentage of the control value.

Effect of DDP with or without DDTC on GGT Activity. Bovine kidney GGT (Sigma) (30 units/ml) was incubated in Tris buffer (50 mM, pH 7.4, 37°C) in the presence or absence of DDP (1 or 2 mM) which had been incubated in distilled water overnight. A sample of the solution was removed at appropriate intervals, diluted 10-fold with Tris buffer, and assayed by measuring the liberation of *p*-nitroaniline from γ-glutamyl-*p*-nitroanilide (26). Enzyme activity was constant for > 4 min during the assay, and < 10% of activity was lost over 8 h at 37°C in control experiments. After incubating for 6 h, the mixture was dialyzed for 1 h against the incubation buffer to remove excess DDP, and DDTC was added to give a final concentration of 1 or 10 mM. Enzyme activity was measured as described above. Enzyme solution without drug exposure was otherwise treated identically, and enzyme activities were determined as a percentage of the control enzyme activity at that time.

GGT activity was also measured in brush border preparations isolated as described by Booth and Kenny (27) from Fischer 344 rats. The membranes were isolated and stored frozen in a solution of 10 mM mannitol and 2 mM Tris (pH 7.0) at a protein concentration of 1.0 mg/ml. Neither freezing nor prolonged incubation at 37°C affected enzyme activity. Aliquots were thawed and centrifuged at 15,000 × *g* for 15 min, and the pellets were resuspended in an identical volume of Tris (20 mM, pH 7.4, 37°C). The resulting membrane suspensions were incubated with 2 mM platinum solutions consisting of DDP, DDP plus 100 mM NaCl, or DDP treated with 2 equivalents of $AgClO_4$. Enzyme activity was assayed as described above. The incubation mixtures were then centrifuged (15,000 × *g*, 15 min), the supernatant was discarded, and the pelleted membranes were resuspended in Tris (100 mM, pH 7.4, 37°C) containing 0–10 mM DDTC. Enzyme activity was measured at 30- and 60-min intervals. Addition of DDTC to control enzyme preparations had no effect on activity.

RESULTS

DDP Reaction Kinetics. Rate constants for the hydrolysis of DDP in 5 mM aqueous $NaNO_3$ were 1.7 ± 0.3 × 10^{-3} min^{-1} and 6.4 ± 0.8 × 10^{-3} min^{-1} at 20°C and 37°C, respectively, determined by measuring chloride release from the platinum complex. This corresponds to a half-life of almost 2 h at 37°C for the replacement of the first chloride ligand by a water molecule. These rate constants are in reasonable agreement with reported values (28) and were determined to provide a base-line value for the experiments to be described. The second-order rate constant for the reaction of DDP with DDTC was 4.4 M^{-1} min^{-1} at 37°C, over 4 orders of magnitude greater than the rate constant for reaction of DDP with water. When [DDTC] = 1 mM, however, the reaction rates between DDP and water or DDTC are comparable ($t_{½}$, 110 and 150 min, respectively, at 37°C). Thus, DDTC is essentially unreactive toward DDP at maximal plasma concentrations of approximately 1 mM observed after a protective dose *in vivo*, suggesting that the mechanism of DDTC protection does not involve direct interception and inactivation of the parent drug.

The rate constant (6.2–6.9 × 10^{-3} min^{-1}) for reaction of DDP with salmon sperm DNA (pH 6.8, 37°C) was essentially identical to the rate of DDP hydrolysis and was independent of the platinum:base ratio over the range 0.01–0.1. Reaction of salmon sperm DNA with the diaquocomplex of DDP was complete in minutes. These results are consistent with DDP hydrolysis as the rate-determining step in DNA binding as reported previously (29).

The presence of phosphate buffer decreases the rate of platinum binding to DNA in a concentration-dependent manner (Table 1); 100 mM phosphate decreases the rate of binding approximately 3.5-fold. The rate of binding at a constant phosphate buffer concentration is also pH dependent, with a slower rate at increased pH above 6.8. These data are consistent with the reactive platinum species having the structure

Table 1 *Effect of phosphate concentration and pH on the rate of cis-platinum binding to salmon sperm DNA (platinum:base ratio r = 0.01) at 37°C*

PO_4 (mM)	pH	$10^3 \times k$ (min^{-1})[a]
5	6.8	5.2
20	6.8	4.3
50	6.8	3.3
100	6.8	1.9
50	6.5	3.5
50	7.0	2.4
50	7.5	1.4

[a] Pseudo-first-order rate constant k determined from the slope of ln (DDT_t/DDP_0) *versus* t derived from linear regression; $r^2 > 0.98$ for all experiments.

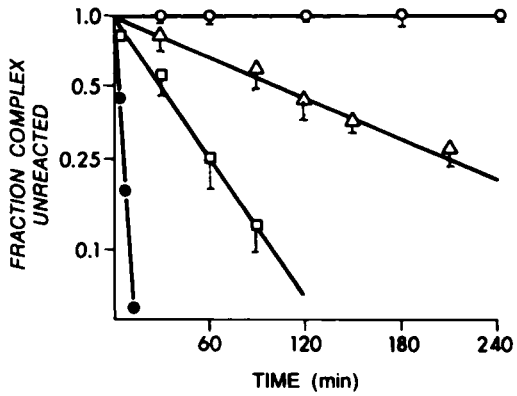

Fig. 1. Reaction of DDP:guanosine and DDP:adenosine complexes with DDTC (10 mM, 37°C). Unreacted complex was quantitated by HPLC analysis and expressed as a fraction of that present at time zero. ●, $Pt(NH_3)_2$ (guanosine)$(OH_2)^{2+}$; ○, $Pt(NH_3)_2$ (guanosine)$_2^{2+}$; △, □, two isomeric complexes of $Pt(NH_3)_2$ (adenosine)$_2^{2+}$. n = 5; *bars*, ± SD. (●, SD < 0.03, bars omitted for clarity).

$Pt(NH_3)_2(\underline{H_2O})(OH)^+$; the pK_a of the underlined hydrogen is 7.2, and as this proton dissociates, the easily replaced water molecule becomes a far less reactive hydroxide ligand. Phosphate presumably competes with DNA for the reactive platinum species to form a stabilized phosphate complex.

Effect of DDTC on Platinum:DNA and Platinum:Purine Complexes. Platinum:DNA complexes were prepared by reaction of salmon sperm DNA with DDP at different platinum:base ratios. These complexes were then reacted with DDTC (10 mM, 37°C), and the platinum was removed and quantified as the DDTC complex by HPLC analysis. For platinum:base ratios < 0.05, < 1% of the platinum bound to DNA was removed after 2 h of DDTC treatment (data not shown). This suggests that DDTC should not reverse the cytotoxic lesion in tumor cells, where the platinum:base ratio is ≪ 0.01 (27). Reaction of DDTC with the DDP:guanosine mono- and bisadducts and with a mixture of the DDP:adenosine bisadducts was also examined (Fig. 1). The bisadduct with guanosine was stable to DDTC (10 mM, 37°C), showing no evidence of reaction after 4 h. In contrast, the DDP:guanosine monoadduct and the two DDP:adenosine bisadducts reacted rapidly with DDTC, showing pseudo-first-order kinetics with half-times of approximately 2, 30, and 100 min, respectively. These data suggest that replacement of both chloride ligands by guanosine residues confers unique stability to DDTC for the platinum complex; the absence of significant platinum:DNA reversal at low platinum:base ratios is consistent with the observations of Eastman that guanine is the initial platinum coordination site in DNA (23).

Effect of DDP with or without DDTC on L1210 Cell Survival. In order to assure that DDTC was used at concentrations which had no direct effect on cell survival, L1210 cells were exposed for 1 h to DDTC alone at a concentration ranging from 0.5–5 mM, and cell survival was evaluated by the soft agar cloning assay. Cell survival was >90% at DDTC concentrations ≤ 2 mM, so DDTC was used at a concentration of 0.5–1 mM in subsequent experiments.

The effect of DDP on L1210 survival was assessed using a 1-h exposure at DDP concentrations of 1–20 μM; this concentration range gave dose-response curves with reproducible D_0 values (the concentration of DDP that reduces survival to 37% as derived from the exponential portion of the survival curve). The effect of a 1-h DDTC exposure beginning 1.5, 2, and 4 h after the start of a 1-h DDP exposure is summarized in Table 2; these times corresponding to an interval of ½, 1, and 3 h, respectively, between the end of DDP treatment and initial DDTC exposure demonstrate that DDTC has no effect on DDP cytotoxicity under these conditions. These data indicate that a delay of at least 1 h between DDP and DDTC administration *in vitro* should be sufficient to prevent the inhibition of DDP-induced cytotoxicity.

Effect of DDP with or without DDTC on DNA Interstrand Cross-Links. The effect of DDTC on DDP-induced DNA interstrand cross-links was evaluated by the alkaline elution technique; all cells received 300 R to introduce single-strand breaks (25). L1210 cells were treated with DDP (20 μM, 1 h) with or without DDTC (1 mM, 1 h) 3 h later. The effect of DDTC alone was also evaluated in cells receiving no DDP. Alkaline elution was carried out 6 h after DDP treatment. DDTC alone had no effect on the DNA elution profile; similarly, DDTC treatment 3 h after DDP had no effect on the extent of DDP-induced DNA interstrand cross-linking. The effect of DDTC immediately and 3 h after DDP treatment and washing of cells was evaluated by alkaline elution at different times. The results are expressed in rad-equivalent cross-links (Fig. 2). Although exposure to DDTC 3 h after DDP had no effect on interstrand cross-linking, a significant reduction in cross-links was observed when cells were exposed to DDTC immediately after DDP treatment.

The alkaline elution technique was modified in order to examine the effect of toxic concentrations of DDTC and other agents on platinum:DNA interstrand cross-links formed after an 8–12 h incubation period following cell treatment with DDP. Rad-equivalent cross-links were determined as described for the normal alkaline elution procedure; reversal is expressed in percentage and determined from the decrease in cross-links resulting from exposure of DNA to drug on the filter compared with controls (Table 3). DDTC was unable to reverse interstrand cross-links even after exposure of the DNA to 0.5 M DDTC for 4 h. In contrast, exposure of DNA to thiourea caused significant removal of platinum interstrand cross-links;

Table 2 *Effect of 1-h DDTC treatment on L1210 cell survival after cis-platinum exposure (1–20 μM, 1 h)*

DDTC (mM)	Interval (h)[a]	D_0 (μM)[b]
Control		3.6 ± 0.7[c]
0.5	−2.0	8.4 ± 0.4[d]
0.5	1.5	4.4 ± 0.5
1.0	1.5	5.1 ± 0.3
0.5	2.0	3.4 ± 0.3
0.5	4.0	4.1 ± 0.8

[a] Time between the beginning of DDP and DDTC exposure. The interval between the end of DDP and the beginning of DDTC treatment is 1 h less than this value.

[b] D_0 is dose of DDP which decreases survival to 37% of control as derived from the log-linear portion of the survival curve.

[c] Mean ± SD.

[d] Significantly different from control ($P < 0.01$, the Student t test); other values not significantly different ($P > 0.1$).

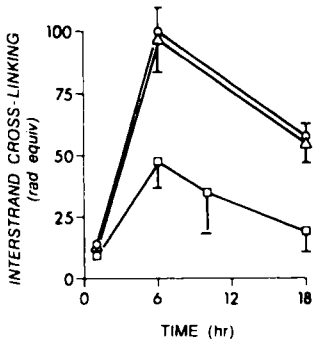

Fig. 2. Effect of DDTC on DNA interstrand cross-linking following 1-h treatment of L1210 cells with 20 μM DDP. Cells were washed and then received no further treatment (○) or were incubated with DDTC (1 mM, 1 h) immediately (□) or 3 h (△) after washing. Alkaline elution was carried out after incubating for various times, as described for Fig. 3. *Bars*, ± SD; $n = 3$.

Table 3 *Effect of DDTC, thiourea, and cyanide on interstrand cross-links after reaction with DNA isolated from L1210 cells previously treated with cis-platinum (20 μM, 1 h)*

Agent	Concentration (mM)	Exposure time (h)	Cross-link reversal (%)[a]
DDTC	10	1	0 ± 5
DDTC	100	4	0 ± 5
DDTC	250	4	0 ± 5
DDTC	500	4	0 ± 5
Thiourea	2	1	15 ± 8
Thiourea	10	4	23 ± 7
Thiourea	500	4	54 ± 10
Cyanide	2	1	27 ± 5
Cyanide	10	2	40 ± 7
Cyanide	40	4	75 ± 9

[a] Percentage of reversal = 100 × (1 − cross-links treated/cross-links control) ± SD; cross-links calculated in rad equivalents using a modified alkaline elution procedure ($n = 3$). See text for details.

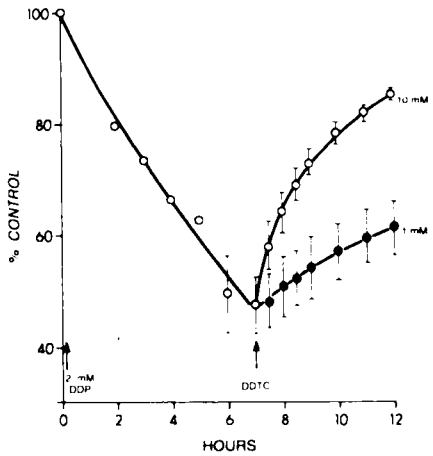

Fig. 3. Effect of DDP and DDTC on bovine γ-glutamyl transpeptidase. GGT (Sigma) (30 units/ml) was incubated with 2 mM DDP and periodically assayed for enzyme activity. After 7 h, excess DDP was removed by dialysis, and the enzyme was treated with 1 mM (●) or 10 mM (○) DDTC. Enzyme activity is expressed as percentage of control enzyme incubated in the absence of drug; $n = 5$. *Bars*, SD.

this property of thiourea has been reported previously (11). Cyanide is a ligand with high affinity for platinum, and its ability to reverse DNA interstrand cross-links was used to demonstrate the feasibility of the DNA filter method.

Effect of DDP with or without DDTC on γ-Glutamyl Transpeptidase Activity. GGT was incubated with 2 mM DDP at 37°C, and enzyme activity was assayed at various times (Fig. 3). DDP clearly inhibits enzyme activity in a time-dependent fashion. DDTC restores activity to the platinum-inhibited enzyme, with the higher DDTC concentration providing more rapid and complete recovery. GGT activity was also measured in F344 rat kidney brush border membranes. Incubation with DDP (2 mM) shows first-order loss of enzyme activity (rate constant $k = 4.2 \times 10^{-3}$ min^{-1}). Addition of chloride ion decreases the concentration of aquated platinum species and retards the rate of enzyme inhibition. In contrast, exposure of enzyme to aquated DDP accelerates the initial rate of enzyme inhibition.

Membrane suspensions were incubated with DDP (2 mM) for 3 h and then incubated with 0–10 mM DDTC (Fig. 4). Restoration of enzyme activity is observed after treatment with 1 or 10 mM DDTC; recovery is more rapid at the higher DDTC concentration. Enzyme activity after treatment with 0.1 mM DDTC is not significantly different from that observed when the enzyme was resuspended in drug-free medium. These results demonstrate that DDP inhibition of a model renal enzyme can be reversed by pharmacological concentrations of DDTC which provide chemoprotection *in vivo* (14).

DISCUSSION

DDP is a relatively unreactive species chemically; even with a powerful nucleophile like DDTC, the reaction half-life is approximately 15 min and 2.5 h at DDTC concentrations of 10 and 1 mM, respectively (37°C). The rate of platinum binding to DNA which we and others (29) have observed, the rate of DDP-induced inactivation of GGT, and the ability of chloride ion to interfere with DNA binding and enzyme inhibition are consistent with DDP hydrolysis as the rate-limiting step in these reactions. In contrast, the reaction of DDP with DDTC is 40,000-fold faster than with water, confirming that DDTC reacts with DDP by direct substitution rather than by initial rate-limiting hydrolysis. It is important to note, however, that the concentration differences between water (55 M) and pharmacological DDTC (1 mM) *in vivo* result in nearly equivalent

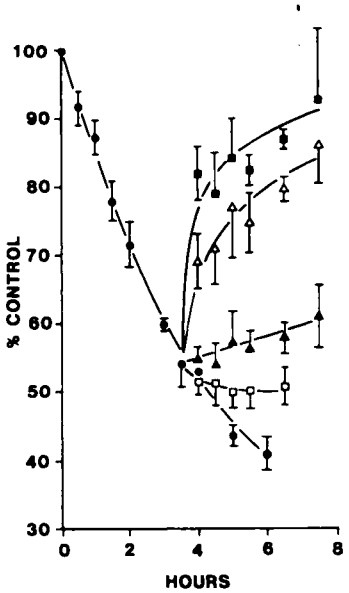

Fig. 4. Effect of DDP and DDTC on γ-glutamyl transpeptidase activity in rat kidney brush border preparations. Brush border membranes were isolated from F344 rats and incubated with 2 mM DDP in Tris buffer. Enzyme activity was assayed at various times for 6 h (●). Identically treated membranes were pelleted after 3 h and incubated with DDTC. Enzyme activity was monitored for an additional 4 h. Activity is expressed as percentage of a control preparation incubated in the absence of drug. *Bars*, SD; $n = 3$. DDTC concentration: ■, 10 mM; △, 1 mM; ▲, 0.1 mM; □, 0 mM.

half-lives under these conditions.

The reaction of DDTC with platinum:nucleoside and platinum:DNA complexes was examined to determine whether DDTC's selective chemoprotection might be based upon a fundamental difference in reactivity among different platinum complexes. Both the initial (30) and predominant (31) cytotoxic platinum:DNA lesions involve replacement of chloride by guanine bases in the same (32) or opposite strand bound at the N-7 position. Our observation that DDTC is unreactive toward platinum:bisguanine complexes prepared from both the nucleoside and from DNA may explain its failure to inhibit DDP's antitumor response.

Thiourea is another ligand with high affinity for platinum complexes. It is effective as a chemoprotector against DDP nephrotoxicity in animal models, but it also inhibits tumor response to DDP *in vivo* (5) and enhances survival of DDP-treated tumor cells *in vitro* (>1 log unit even after a 2-h interval between DDP and thiourea treatment) (13). In contrast, DDTC had no effect on L1210 cell survival when an interval of at least 1 h between DDP and DDTC treatment was used. The effects of thiourea and DDTC on platinum:DNA interstrand cross-linking in L1210 cells are also quite different. Thiourea does not reverse preformed cross-links in cells; it presumably enhances survival by inhibiting subsequent cross-linking of platinum:DNA monoadducts when cells are treated with thiourea after DDP exposure (13). Interstrand cross-links continue to accumulate after DDTC treatment, however, and the extent of cross-linking at later times is unaffected if DDTC treatment is sufficiently delayed. Thiourea (1 M) has been shown to reverse lethal platinum:DNA cross-links in a noncellular system (11). Using a modification of the alkaline elution assay, we observe that thiourea at concentrations as low as 10 mM reverses interstrand cross-links, whereas 500 mM DDTC has no effect on platinum:DNA interstrand cross-links. Thus, in spite of their similarities in affinity for platinum complexes, DDTC and thiourea behave very differently in both their chemical reactions and their effects on DDP-treated cells.

γ-Glutamyl transpeptidase is an enzyme of considerable importance in the renal tubule; it is concentrated in proximal tubule brush border and contains a sulfhydryl group essential for enzymatic activity. This enzyme was chosen as a model to evaluate DDP-induced inhibition and DDTC-induced recovery of enzyme activity. First-order inhibition of the enzyme by DDP was observed at a rate consistent with initial DDP hydrolysis as the rate-limiting step. Enzyme activity was restored to the inhibited complex by treatment with DDTC at concentrations which have been observed *in vivo* after pharmacological doses (14). These results do not implicate GGT as the primary target in DDP-induced nephrotoxicity; however, they do provide a model for DDTC reversal of *cis*-platinum-induced inhibition of an important biochemical process. DDP is known to inhibit other enzymes containing essential sulfhydryl groups (3, 4), and a similar effect of DDTC on DDP-induced cross-linking and inactivation of human α_2-macroglobulin has also been described (33).

The data presented in this paper provide a mechanistic basis for the selectivity of DDTC to inhibit DDP toxicity *in vivo* without inhibition of antitumor effect (14). DDTC provides effective chemoprotection even when given after DDP; this is a unique property of platinum drug chemoprotectors and is consistent with a mechanism involving reversal of toxic platinum lesions. The effect of structural differences on reactivity of platinum complexes can be subtle; DDTC reacts readily with platinum:bisadenine complexes but is essentially unreactive toward the corresponding guanine complexes. The platinum:DDTC complex is thermodynamically stable and chemically and biologically unreactive;[4] thus, platinum removed from sites of potential toxicity is essentially inactivated. We are currently evaluating the potential of DDTC as a DDP chemoprotector in the clinic; the results will be described in a forthcoming publication.

REFERENCES

1. Roberts, J. J., and Thomson, A. J. The mechanism of action of antitumor platinum compounds. Prog. Nucleic Acid Res. Mol. Biol., *22:* 71–133, 1979.
2. Borch, R. F., and Pleasants, M. E. Inhibition of *cis*-platinum nephrotoxicity by diethyldithiocarbamate rescue in a rat model. Proc. Natl. Acad. Sci. USA, *76:* 6611–6614, 1979.
3. Daley-Yates, P. T., and McBrien, D. C. H. The inhibition of renal ATPase by cisplatin and some biotransformation products. Chem.-Biol. Interact., *40:* 325–334, 1982.
4. Guarino, A. M., Miller, D. S., Arnold, S. T., Pritchard, J. B., Davis, R. D., Urbanek, M. A., Miller, T. J., and Litterst, C. L. Platinate toxicity: past, present, and future prospects. Cancer Treat. Rep., *63:* 1475–1483, 1979.
5. Burchenal, J. H., Kalaher, K., Dew, K., Lokys, L., and Gale, G. Studies on cross-resistance, synergistic combination, and blocking activity of platinum derivatives. Biochimie (Paris), *60:* 961–965, 1978.
6. Howell, S. B., and Taetle, R. Effect of sodium thiosulfate on *cis*-dichlorodiammineplatinum(II) toxicity and antitumor activity in L1210 leukemia. Cancer Treat. Rep., *64:* 611–616, 1980.
7. Shrieve, D. C., and Harris, J. W. Protection against *cis*-dichlorodiammine Pt(II) cytotoxicity *in vitro* by cysteamine. Int. J. Radiat. Oncol. Biol. Phys., *8:* 585–588, 1982.
8. Yuhas, J. M., and Culo, F. Selective inhibition of the nephrotoxicity of *cis*-dichlorodiammineplatinum(II) by WR-2721 without altering its antitumor properties. Cancer Treat. Rep., *64:* 57–64, 1980.
9. Graziano, J., Jones, B., and Pisciotto, P. The effect of heavy metal chelators on the renal accumulation of platinum after *cis*-platinum administration to the rat. Br. J. Pharmacol., *73:* 649–654, 1982.
10. Zunino, F., Tofanetti, O., Besati, A., Cavalletti, E., and Savi, G. Protective effect of reduced glutathione against *cis*-dichlorodiammine platinum(II)-induced nephrotoxicity and lethal toxicity. Tumori, *69:* 105–111, 1983.
11. Filipski, J., Kohn, K. W., Prather, R., and Bonner, W. M. Thiourea reverses cross-links and restores biological activity in DNA treated with dichlorodiaminoplatinum(II). Science (Wash. DC), *204:* 181–183, 1979.
12. Micetich, K., Zwelling, L. A., and Kohn, K. W. Quenching of DNA platinum(II) monoadducts as a possible mechanism of resistance to *cis*-diamminedichloroplatinum(II) in L1210 cells. Cancer Res., *43:* 3609–3613, 1983.
13. Zwelling, L. A., Filipski, J., and Kohn, K. W. Effect of thiourea on survival and DNA cross-link formation in cells treated with platinum(II) complexes, L-phenylalanine mustard, and bis(2-chloroethyl)methylamine. Cancer Res., *39:* 4989–4995, 1979.
14. Bodenner, D. L., Dedon, P. C., Keng, P. C., Katz, J. C., and Borch, R. F. Selective protection against *cis*-diamminedichloroplatinum(II)-induced toxicity in kidney, gut, and bone marrow by diethyldithiocarbamate. Cancer Res., *46:* 2751–2755, 1986.
15. Borch, R. F., Bodenner, D. L., and Katz, J. C. Diethyldithiocarbamate and *cis*-platinum toxicity. *In:* M. P. Hacker, E. B. Douple, and I. H. Krakoff (eds.), Platinum Coordination Complexes in Cancer Chemotherapy, pp. 154–164. Boston: Martinus-Nijhoff Publishing, 1984.
16. Borch, R. F., Katz, J. C., Lieder, P. H., and Pleasants, M. E. Effect of diethyldithiocarbamate rescue on tumor response to *cis*-platinum in a rat model. Proc. Natl. Acad. Sci. USA, *77:* 5441–5444, 1980.
17. Borch, R. F., and Katz, J. C. Effect of diethyldithiocarbamate (DDTC) or disulfiram (DSF) rescue on the response of P388 leukemia to *cis*-diamminedichloroplatinum(II) in BDF_1 mice. Proc. Am. Assoc. Cancer Res., *22:* 259, 1981.
18. Evans, R. G., Wheatley, C., Engel, C., Nielsen, J., and Ciborowski, L. J. Modification of the bone marrow toxicity of *cis*-diamminedichloroplatinum(II) in mice by diethyldithiocarbamate. Cancer Res., *44:* 3686–3690, 1984.
19. Gale, G. R., Atkins, L. M., and Walker, E. M. Further evaluation of diethyldithiocarbamate as an antagonist of cisplatin toxicity. Ann. Clin. Lab. Sci., *12:* 345–355, 1982.
20. Gringeri, A., and Borch, R. F. The effect of diethyldithiocarbamate (DDTC) on bone marrow cells treated with *cis*-diamminedichloroplatinum (DDP) and its analogs. Proc. Am. Assoc. Cancer Res., *25:* 371, 1984.
21. Khandekar, J. D. Improved therapeutic index of *cis*-diamminedichloroplatinum by diethyldithiocarbamate in rodents. Res. Commun. Chem. Pathol. Pharmacol., *40:* 55–66, 1983.
22. Borch, R. F., Markovitz, J. H., and Pleasants, M. E. A new method for the HPLC analysis of Pt(II) in urine. Anal. Lett., *12:* 917–922, 1979.
23. Eastman, A. Separation and characterization of products resulting from the reaction of *cis*-diamminedichloroplatinum(II) with deoxyribonucleosides. Biochemistry, *21:* 6732–6736, 1982.

24. Chu, M. Y., and Fisher, G. A. Incorporation of cytosine-^{3}H arabinoside and its effects on the murine leukemia cells (L51784). Biochem. Pharmacol., *17:* 753–767, 1968.
25. Kohn, K. W., Ewig, R. A. G., Erickson, L. C., and Zwelling, L. A. Measurement of strand breaks and cross-links by alkaline elution. *In:* E. C. Friedberg and P. C. Hanawalt (eds.), DNA Repair: A Laboratory Manual of Research Procedures, Vol. 1, pp. 379–401. New York: Marcel Dekker, Inc., 1981.
26. Tate, S. S., and Meister, A. Interaction of γ-glutamyl transpeptidase with amino acids, dipeptides, and derivatives and analogs of glutathione. J. Biol. Chem., *249:* 7593–7602, 1974.
27. Booth, A. G., and Kenny, A. J. A rapid method for the preparation of microvilli from rabbit kidney. Biochem. J., *142:* 575–581, 1974.
28. Reishus, J. W., and Martin, D. S. *cis*-Dichlorodiammineplatinum(II) acid hydrolysis and isotopic exchange of the chloride ligands. J. Am. Chem. Soc., *83:* 2457–2464, 1961.
29. Ushay, H. M., Tullius, T. D., and Lippard, S. J. Inhibition of the *Bam*HI cleavage and unwinding of pBR322 deoxyribonucleic acid by the antitumor drug *cis*-dichlorodiammineplatinum(II). Biochemistry, *20:* 3744–3748, 1981.
30. Eastman, A. Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)platinum(II). Biochemistry, *22:* 3927–3933, 1983.
31. Lippard, S. J. New chemistry of an old molecule: *cis*-$Pt(NH_3)_2Cl_2$. Science (Wash. DC), *218:* 1075–1082, 1982.
32. Pinto, A. L., and Lippard, S. J. Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin) to DNA. Biochim. Biophys. Acta, *780:* 167–180, 1985.
33. Gonias, S. L., Oakley, A. C., Walther, P. J., and Pizzo, S. V. Effects of diethyldithiocarbamate and nine other nucleophiles on the intersubunit protein cross-linking and inactivation of purified human α_2-macroglobulin by *cis*-diamminedichloroplatinum(II). Cancer Res., *44:* 5764–5770, 1984.