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GLUTATHIONE-MEDIATED MODULATION OF TETRAPLATIN ACTIVITY AGAINST SENSITIVE AND RESISTANT TUMOR CELLS

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Abstract—Tetraplatin (Ormaplatin) has good antitumor activity against some cisplatin-resistant cells and is currently being studied in clinical trials. We have studied the effect of extracellular reduced glutathione (GSH) on the cytotoxicity and biochemical pharmacology of tetraplatin in L1210 leukemia cells. Parent L1210/0 cells were exposed to tetraplatin for 2 hr with or without GSH in Hanks' balanced salt solution (HBSS), and cytotoxicity was assessed by a soft agar clonogenic assay. GSH (10 or 100 μM) increased tetraplatin (10 μM)-induced cell kill by about 2 logs; concentrations of the thiol 10-fold below or above these levels increased cell kill to a lesser degree. GSH-mediated increases in the cytotoxicity of tetraplatin were also observed against cisplatin-resistant L1210/DDP and tetraplatin-resistant L1210/DACH cells. An equimolar concentration of 1,2-diaminocyclohexane-platinum(II) dichloride [DACH-Pt(II)Cl_2] alone was as cytotoxic as the combination of tetraplatin and GSH. Intracellular accumulations of tetraplatin in both L1210/0 and L1210/DDP cells were increased by GSH, whereas in L1210/DACH cells platinum uptake decreased in the presence of the thiol. Reactions between tetraplatin and salmon sperm DNA in the presence or absence of GSH (1 or 100 μM), performed at 37° in HBSS, revealed that levels of total and interstrand DNA-platinum adducts were minimal in the absence of GSH, whereas in the presence of GSH DNA adducts of tetraplatin were substantial and similar to those seen with DACH-Pt(II)Cl_2. Tetraplatin (10 μM) incubated at 37° in HBSS with GSH (10 μM–1 mM) was reduced chemically to the DACH-Pt(II) species within 5 min; a 200-μM tetraplatin solution required a GSH concentration of at least 100 μM for substantial reduction to occur. This chemical reduction of tetraplatin appears to be a prerequisite for its biological activity. Thus, extracellular GSH can modulate the biological activity of tetraplatin, and the combination may prove useful in specific clinical applications, such as intracavitary platinum therapy.

Key words: tetraplatin; glutathione; chemical reduction; cytotoxicity; intracellular uptake; DNA adducts

The discovery of cisplatin† by Rosenberg *et al.* [1] has had a significant impact in the treatment of testicular, ovarian and bladder cancers [2, 3]. However, the therapeutic efficacy of cisplatin is limited because of its significant side-effects, which include nephrotoxicity, nausea and vomiting, myelosuppression and peripheral neuropathy [4]. Development of drug resistance [5, 6] is another major factor that has also impeded cisplatin use. Several platinum complexes have been developed and tested in an effort to overcome these problems [7]. One such complex is tetraplatin (Ormaplatin), which entered clinical trials in 1990 [8, 9] as a result of its activity against cisplatin-resistant murine [10, 11] and human [12, 13] cell lines. It has equal or greater therapeutic effectiveness [10, 11, 14] and is less nephrotoxic than cisplatin [15, 16].

In light of the fact that substitution reactions with platinum(IV) complexes, relative to platinum(II), are slow, it has been suggested that tetravalent compounds such as iproplatin express their antitumor activities via chemical reduction to biologically active platinum(II) species [17, 18]. Recently, Chaney and his colleagues investigated the biotransformation of tetraplatin *in vitro* [19–22] and *in vivo* [23] by using a two-column HPLC separation system; they demonstrated that tetraplatin was reduced rapidly to DACH-dichloroplatinum(II) [DACH-Pt(II)Cl_2], with a half-life of 5–15 min in RPMI 1640 tissue culture medium containing 15% FBS and 3 sec in undiluted rat plasma. Their studies indicated that protein sulfhydryl was the major extracellular reducing agent for tetraplatin *in vitro* and *in vivo*.

GSH is a tripeptide thiol that is a major constituent of the intracellular nonprotein sulfhydryl pool of most cell types and participates in many important cellular functions, including detoxication of xenobiotics, amino acid transport, and DNA repair mechanisms [24]. Moreover, increases in cellular GSH levels have been associated with resistance to cisplatin in certain murine and human cancer cell lines [5]. Many studies have been conducted in which the activity of cisplatin was potentiated by depletion

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† Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); tetraplatin (Ormaplatin), *trans-R,R-S,S*-1,2-diaminocyclohexane tetrachloroplatinum(IV); DACH, *trans-R,R-S,S*-1,2-diaminocyclohexane; GSH, reduced glutathione; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); ISC, interstrand cross-link; TSH, total sulfhydryl; and NPSH, non-protein sulfhydryl.

Tetraplatin
[TP]

trans-DACH-Pt(II)Cl_2
[DACH-Pt(II)]

Fig. 1. Structures of tetraplatin and DACH-Pt(II)Cl_2.

of the intracellular GSH level using buthionine-*SR*-sulfoximine, an inhibitor of glutathione synthesis [25–30]. Eastman [31], on the other hand, demonstrated the importance of GSH in the reduction/activation of the inert tetraplatin before its interaction with purified DNA. This suggests that extracellular concentrations of GSH will play a significant role in the reduction/activation process (Fig. 1) and may also modulate the biochemical pharmacology of the platinum(IV) and/or the resulting platinum(II) species. However, the few studies that have been reported on the subject are not clear on the role of GSH in the cytotoxicity of tetraplatin [19–23, 31], and no information is available at all with resistant cell lines. The experiments described here were designed to assess the influence of extracellular GSH on cytotoxicity and intracellular accumulation of tetraplatin in platinum-sensitive and -resistant L1210 cells and on total DNA adduct and interstrand cross-link formation with salmon sperm DNA.

MATERIALS AND METHODS

Drugs and chemicals. The two drugs used in this study, DACH-Pt(II)Cl_2 and tetraplatin (Fig. 1), were prepared as described previously [10]. The compounds were dissolved in saline immediately before use. HBSS, RPMI 1640 tissue culture medium and FBS were purchased from Whittaker M.A. Bioproducts Inc. (Walkersville, MD); GSH, DTNB and salmon sperm DNA were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell lines. Platinum-sensitive and -resistant murine leukemia L1210 cell lines were provided by Dr. Alan Eastman (Department of Pharmacology, Dartmouth Medical School, Hanover, NH) and have been well characterized [29, 32, 33]. The extent of resistance of L1210/DDP to cisplatin and of L1210/DACH to tetraplatin depends on the assay used; in a proliferative cell-count assay, we have reported these cells to be 50- and 36-fold resistant, respectively, while in a clonogenic assay the L1210/DACH cells were found to be 123-fold resistant to tetraplatin [34]. All cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin and 0.3 mg/mL L-glutamine. Cells were grown at 37° in a humidified atmosphere of 5% CO_2 in air. The original cell lines were stored at −70° and fresh cells were removed and cultured from original stock every 3–4 months.

Clonogenic assays. Exponentially growing cells were exposed for 2 hr to the drug (0.1 to 200 μM) with or without GSH (0.1 μM to 10 mM) in HBSS at 37°, and then washed twice with PBS. Between 10^2 and 10^5 cells were suspended in 3 mL of complete medium containing 0.1% Noble agar in closed tubes (in triplicate), as described previously [34], and colonies were counted after 10–14 days of incubation. The IC_{50} and IC_{90} values, defined as the concentrations (μM) needed to reduce cell survival by 50 and 90%, respectively, compared with control cells, were determined from sigmoidal plots of surviving fraction versus log drug concentration, using a computer program (GraphPAD Software, San Diego, CA).

Drug uptake studies. Exponentially growing cells were resuspended to 10^6 cells/mL in HBSS and were exposed for 2 hr to the platinum drug (0–100 μM) at 37° with or without GSH (0–1000 μM). After 2 hr, duplicate 1.5-mL portions were transferred to microcentrifuge tubes for assessment of drug accumulation. The cells were washed twice with ice-cold PBS, pelleted by centrifugation (12,500 *g*, 2 min) and stored at −20° until analyzed for platinum by flameless atomic absorption spectrophotometry (model AA300/GTA-96; Varian, Victoria, Australia) using conditions, described previously [35], that gave a detection limit of 100 pg platinum.

Sulfhydryl measurements. Non-protein (NPSH) and total sulfhydryl (TSH) contents were determined using a modification of the Ellman method [36, 37]. Briefly, 10^8 cells were disrupted in a sonicator in 2 mL of 0.02 M EDTA. For determination of NPSH, the cell lysate (500 μL) was treated with 10% trichloroacetic acid (500 μL) and centrifuged (12,500 *g*, 2 min), and the supernatant (800 μL) was adjusted to pH 8.9 with 0.4 M Tris buffer (1.6 mL) before the addition of Ellman's reagent (10 mM DTNB in methanol, 25 μL). TSH was determined by adding the cell lysate (100 μL) to 0.2 M Tris buffer (1 mL, pH 8.2) and 0.02 M EDTA (0.9 mL) followed by 20 μL of the Ellman's reagent. All samples were allowed to develop for 30 min at room temperature, and absorbance was then read at 412 nm. Protein sulfhydryl content was determined from the difference between the TSH and NPSH contents. The protein concentration of cell lysate was determined by the method of Lowry *et al.* [38].

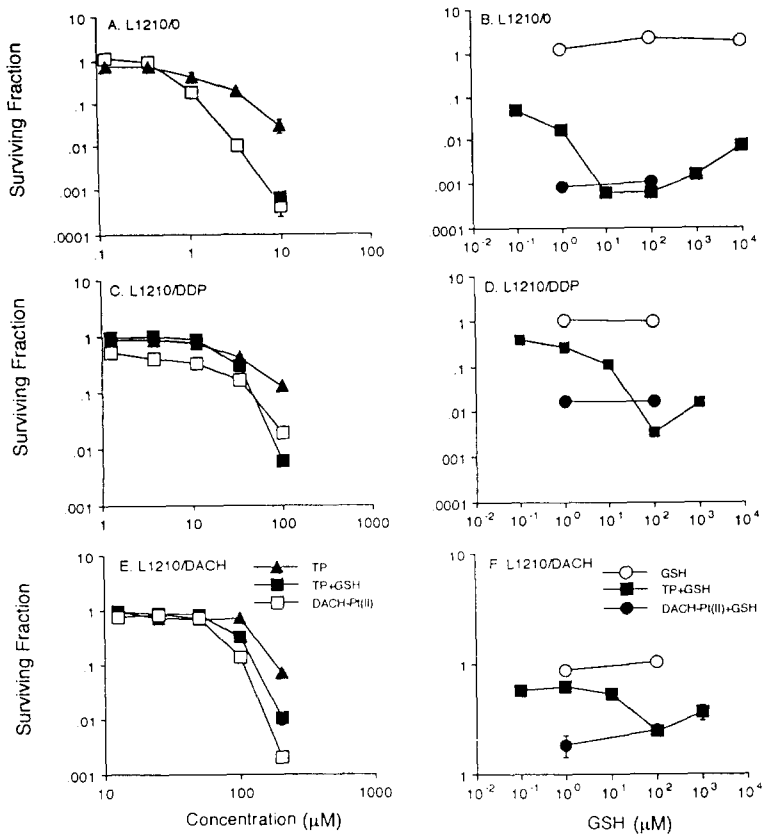

Fig. 2. Cytotoxicity of DACH-Pt(II)Cl_2 and tetraplatin (TP) in the presence and absence of GSH. Left panels show survival of L1210/0 (A), L1210/DDP (C) and L1210/DACH (E) cells exposed for 2 hr to a range of concentrations of tetraplatin, tetraplatin with GSH (10 μM in A, 100 μM in C and E), or DACH-Pt(II)Cl_2 in HBSS. Right panels: survival of L1210/0 (B), L1210/DDP (D) and L1210/DACH (F) exposed to tetraplatin or DACH-Pt(II)Cl_2 with various concentrations of GSH. Platinum drug concentrations used were 10 μM against L1210/0 and 100 μM against L1210/DDP and L1210/DACH cells. Data are means ± SD (shown if larger than the size of the symbols); N = 3.

In vitro *reduction of tetraplatin with GSH.* Tetraplatin (10 and 200 μM) was incubated with GSH (0.1 μM to 1 mM) at 37° in HBSS, aliquots were removed at predetermined times, and unchanged tetraplatin was determined by HPLC. The HPLC system consisted of a Waters 600 multisolvent delivery system (Waters Associates, Milford, MA) with a 250 × 4.6 mm Hypersil ODS (C18) 5 μm column (Alltech Associates, Inc., Deerfield, IL), a model 481 UV detector, and a peak-integrating data module. The mobile phase was water flowing at 1.0 mL/min, and the analytical wavelength was 197.5 nm. Under these conditions, tetraplatin and DACH-Pt(II)Cl_2 have retention times of 6.2 ± 0.1 and 7.8 ± 0.1 min (mean ± SD, N = 10), respectively. Concentration × peak area curves for tetraplatin and DACH-Pt(II)Cl_2 were linear by regression analysis ($r > 0.999$) in the range 0–8 nmol.

Binding of platinum complexes to DNA. Multiple 500-μg aliquots of salmon sperm DNA in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) were incubated at 37° with platinum complexes (5–20 μM, approximately 1 platinum : 75 nucleotides at 20 μM) with or without GSH (1 or 100 μM). After 24 hr, the reaction was quenched by the addition of 200 μL of 10 M ammonium acetate and precipitation of DNA with 2.5 mL of absolute ethanol. The precipitated DNA was washed twice with 70% ethanol and then dissolved in 250 μL of water at room temperature overnight. The DNA content was assessed by absorption at 260 nm, and the amount of platinum in the sample was determined by flameless atomic absorption spectrophotometry.

DNA interstrand cross-links (ISC). Salmon sperm DNA (100 μg/mL in TE buffer, pH 8.0) was treated with platinum complexes (0–10 μM) with or without GSH (1 or 100 μM) at 37° for 24 hr. DNA was precipitated, as described above, and dissolved in TE buffer. ISC were detected using an ethidium bromide fluorescence assay as described before [39, 40]. Briefly, two identical sets of triplicate 100-μL aliquots containing 10 μg of DNA were transferred to borosilicate glass tubes, and 400 μL of an assay buffer (20 mM potassium phosphate buffer containing 2 mM EDTA, pH 12.0) was added. The DNA in one set was denatured by heating to 100°

Table 1. Cytotoxicity of tetraplatin, tetraplatin with GSH, and DACH-Pt(II)Cl_2 in L1210 cells

Treatment	L1210/0		L1210/DDP		L1210/DACH	
	IC_{50}* (μM)	IC_{90}* (μM)	IC_{50}* (μM)	IC_{90}* (μM)	IC_{50}* (μM)	IC_{90}* (μM)
TP	1.2	5.8	34.0	100	144.3	190
	(1)†	(1)	(28)	(17)	(120)	(33)
TP + GSH‡	0.75	1.5	24.9	54.6	87.8	140
	(1)	(1)	(33)	(36)	(117)	(93)
DACH-Pt(II)	0.61	1.4	32.8	53.3	73.9	100
	(1)	(1)	(54)	(38)	(121)	(71)

* The concentration of tetraplatin (TP) or DACH-Pt(II)Cl_2 (DACH-Pt(II)) that, after a 2-hr exposure in HBSS, reduced cell survival to 50% (IC_{50}) or 90% (IC_{90}) of control values, as measured by a clonogenic assay.

† Values in parentheses indicate resistance factors relative to L1210/0.

‡ GSH concentration: 10 μM, L1210/0; 100 μM, L1210/DDP and L1210/DACH.

for 10 min in a heating block, and then cooled immediately to 15°. One milliliter of the assay buffer was added to each tube, followed by 1.5 mL of a 2 μg/mL ethidium bromide (EB) solution in the assay buffer to yield a final EB concentration of 1 μg/mL in a volume of 3 mL. The fluorescence was measured in an LS5B digital spectrofluorometer (Perkin–Elmer, Norwalk, CT) with wavelengths set at 305 nm for excitation and 590 nm for emission. The cross-linking index (CLI) was calculated using the following equation that was derived from the report of Brent [39]:

$$CLI = [(-\ln X_{drug\text{-}treated}) - (-\ln X_{control})]$$

where X = (FNH − FH)/FNH, FNH = fluorescence without heating, and FH = fluorescence after the heating/cooling cycle.

Statistical analysis. Statistical significances were determined by Student's *t*-test; values of $P < 0.05$ were considered significant.

RESULTS

Cytotoxicity evaluation. Cytotoxicities against the three L1210 lines of tetraplatin alone, tetraplatin with GSH and DACH-Pt(II)Cl_2 alone are presented in Fig. 2 (panels A, C and E); the data are summarized as IC_{50} and IC_{90} values in Table 1. Against all three cell lines, DACH-Pt(II)Cl_2 was more potent than tetraplatin alone. Simultaneous exposure of cells to tetraplatin and GSH, however, resulted in an increase in potency that approached the level of cell kill observed with DACH-Pt(II)Cl_2 at an equivalent concentration. The greatest thiol-mediated increases in tetraplatin cytotoxicity (by 0.8 to 1.7 log cell kill; $P < 0.05$ vs tetraplatin alone) were observed at the highest drug concentration examined. Figure 2 (panels B, D and F) also shows the effects of extracellular GSH levels on the cytotoxicity of a 10 (in L1210 cells) or 100 μM (in L1210/DDP and L1210/DACH cells) concentration of the platinum complex. In all cell lines, GSH alone displayed no cytotoxicity and had no gross effect on the cytotoxicity of the platinum(II) compound. GSH, however, had a substantial effect on the cytotoxicity of tetraplatin: increasing GSH concentration above 0.1 μM increased tetraplatin-induced cell kill until a maximal increase in cytotoxicity ($P < 0.05$ vs tetraplatin alone) was seen at 10 and/or 100 μM thiol, with higher concentrations of GSH (≥ 1 mM) having a lesser effect.

Intracellular accumulation. Figure 3 (panels A, C and E) shows intracellular levels of platinum following a 2-hr incubation of the platinum complex (6.25 to 100 μM) with or without GSH (1 or 100 μM). There were no gross differences in platinum accumulation between tetraplatin and tetraplatin + 1 μM GSH or between DACH-Pt(II)Cl_2 and tetraplatin + 100 μM GSH. However, in L1210/0 and L1210/DDP cells, DACH-Pt(II)Cl_2 and tetraplatin + GSH (100 μM), in general, produced greater intracellular platinum levels than those seen with tetraplatin alone or tetraplatin + GSH (1 μM), with a 2-fold difference ($P < 0.05$) being observed between these groups at 100 μM platinum drug concentration. In contrast, accumulation of DACH-Pt(II)Cl_2 and tetraplatin + 100 μM GSH in L1210/DACH cells was significantly less ($P < 0.05$) than accumulation of tetraplatin or tetraplatin + 1 μM GSH at all platinum drug concentrations examined. Figure 3 (panels B, D and F) also demonstrates the effect of extracellular GSH concentration on intracellular platinum accumulation in L1210 cells exposed to 100 μM tetraplatin or DACH-Pt(II)Cl_2. Intracellular accumulations of tetraplatin in both L1210/0 and L1210/DDP cells increased progressively with an increase in GSH concentration, and at 100 μM GSH the accumulation was significantly greater ($P < 0.05$) than in cells exposed to tetraplatin alone or tetraplatin + 1 μM GSH; a higher GSH level (1 mM) caused a subsequent decline in cellular platinum content. In L1210/DACH cells, uptake of tetraplatin decreased significantly with increasing GSH concentration above 10 μM ($P < 0.05$ vs tetraplatin alone). Accumulation of DACH-Pt(II)Cl_2 also decreased significantly in the presence of 1 mM GSH level ($P < 0.05$ vs drug alone) in all three cell lines.

Reduction of tetraplatin with GSH. Incubation at 37° of tetraplatin with GSH in HBSS resulted in a reduction of the platinum(IV) complex and a concomitant appearance of DACH-Pt(II)Cl_2, as monitored by HPLC. Typical HPLC chromatograms for standards and for the reaction between tetraplatin and GSH are shown in Fig. 4. No interfering peaks were seen at the retention times of interest, and this facilitated peak integration with high accuracy for quantitative purposes. Results monitoring the disappearance of tetraplatin are summarized in Table 2. About 25–40% drop in 10 μM tetraplatin was seen by 2 hr in the absence or presence of low concentrations of GSH (0.1 or 1.0 μM). Higher levels of GSH (≥ 10 μM), on the other hand, completely reduced this concentration of tetraplatin to DACH-Pt(II)Cl_2 within 5 min. The higher concentration of tetraplatin (200 μM) underwent negligible spontaneous transformation and required a GSH level of 100 μM or higher for the reduction

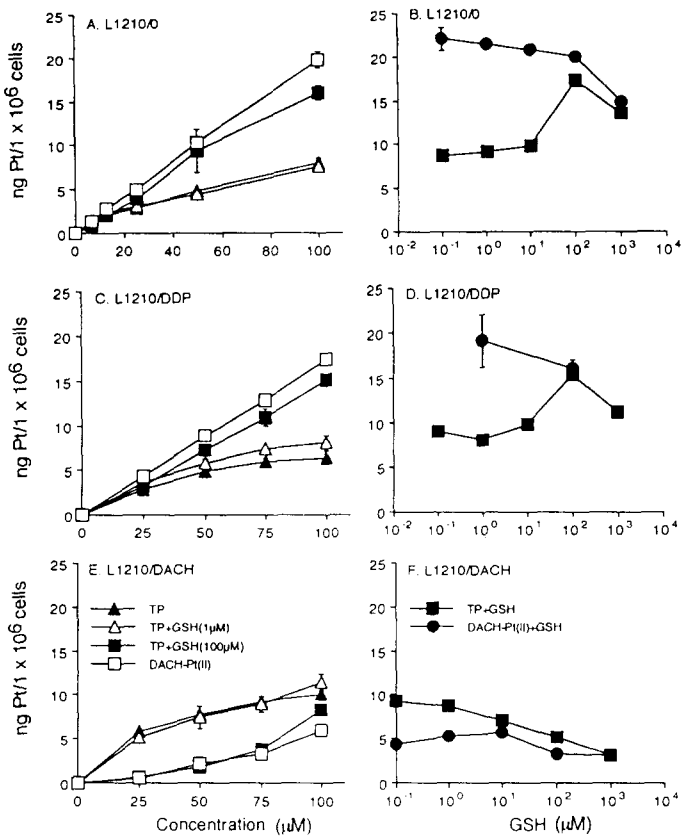

Fig. 3. Accumulation of DACH-Pt(II)Cl_2 and tetraplatin (TP) in the presence and absence of GSH. Left panels show uptake of tetraplatin, tetraplatin with GSH (1 or 100 μM), and DACH-Pt(II)Cl_2 by L1210/0 (A), L1210/DDP (C) and L1210/DACH (E) cells exposed for 2 hr to a range of platinum drug concentrations. Right panels: uptake of 100 μM tetraplatin or DACH-Pt(II)Cl_2 by L1210/0 (B), L1210/DDP (D) and L1210/DACH (F) cells in 2 hr in the presence of various concentrations of GSH. Data are means ± SD (shown if larger than the size of the symbols); N = 3.

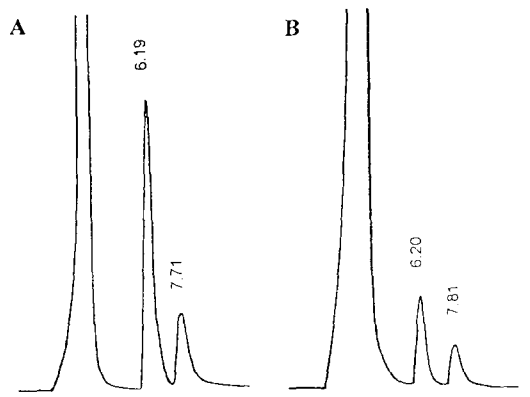

Fig. 4. HPLC chromatograms of tetraplatin and DACH-Pt(II)Cl_2. Tetraplatin and the platinum(II) complexes eluted at retention times of 6.2 and 7.7 to 7.8 min, respectively. The large peak is the solvent front eluting at about 3.3 min. The chromatograms show (A) the separation of the two compounds (2 nmol each) as a standard mixture, or (B) the extent of conversion of tetraplatin to DACH-Pt(II)Cl_2 5 min after the reaction between tetraplatin (200 μM) and GSH (100 μM). Each of the two standards, when examined individually, gave a single peak after the solvent front at the indicated elution times (not shown).

Table 2. Reduction of tetraplatin with GSH*

	% Tetraplatin remaining									
	TP (10 μM)				TP (200 μM)					
	GSH (μM)				GSH (μM)					
Time (min)	0	0.1	1	10	0	0.1	1	10	100	1000
5	100†	86.1	80.7	0	100	100	100	90.7	34.4	0
120	72.4	75.5	60.9	—‡	99.9	100	100	83.4	7.6	—
240	—	—	—	—	—	—	—	62.4	3.8	—
360	—	—	—	—	84.5	89.6	81.9	—	—	—

* Reactions were conducted in HBSS, and disappearance of tetraplatin (TP) was monitored by HPLC.
† Results are presented as means of two experiments.
‡ Not determined.

reaction to approach or reach completion within the 4- to 6-hr time span of the study.

Reaction of platinum complexes with salmon sperm DNA. Reactions between tetraplatin, tetraplatin + GSH (1 or 100 μM) or DACH-Pt(II)Cl_2

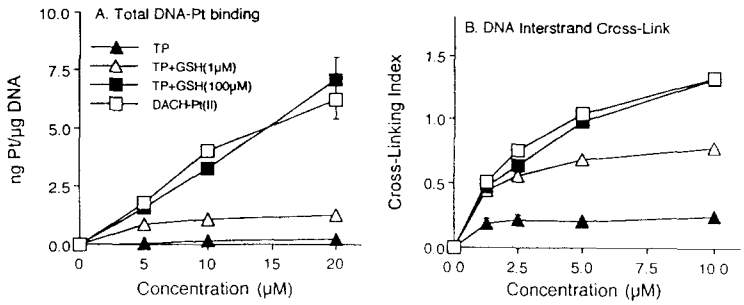

Fig. 5. Total DNA-platinum binding (A) and DNA-platinum interstrand cross-links (B). DNA was incubated with tetraplatin (TP) or DACH-Pt(II)Cl_2 with or without GSH (1 or 100 μM), and adduct levels were measured 24 hr later. Data are means ± SD (shown if larger than the size of the symbols); N = 3.

Table 3. Total (TSH) and non-protein (NPSH) sulfhydryl contents in L1210 cells

Cell line	TSH (nmol/mg protein)	NPSH (nmol/mg protein)	Protein (μg/l × 10^6 cells)
L1210/0	72.3 ± 1.9*	18.1 ± 1.6	131.3 ± 14.5
L1210/DDP	91.2 ± 6.9†	32.6 ± 4.0†	190.1 ± 24.9†
L1210/DACH	77.8 ± 5.8	20.3 ± 3.8	155.3 ± 18.5

* Mean ± SD (derived from four independent experiments).
† $P < 0.05$ vs L1210/0.

and DNA were investigated at 37° in HBSS at several platinum drug concentrations, and both total DNA-platinum adducts (Fig. 5A) and DNA interstrand cross-links (Fig. 5B) were measured 24 hr later. Tetraplatin without GSH showed minimal reaction toward DNA, whereas with GSH (100 μM) the platinum(IV) complex produced substantial total and interstrand adduct levels, which were similar to those seen with DACH-Pt(II)Cl_2. Tetraplatin + GSH (1 μM) reacted with DNA to a lesser degree and lower levels of total DNA-platinum binding and cross-links were observed.

TSH and NPSH contents in L1210 cells. The thiol levels were determined to assess the potential of cells to reduce tetraplatin intracellularly. Levels of TSH in the three cell lines were 3- to 4-fold greater than NPSH (Table 3). Some differences in thiol levels were also apparent between the cell lines. Relative to the parent L1210/0 line, TSH, NPSH (primarily GSH) and protein contents were significantly higher by 30–80% in L1210/DDP cells ($P < 0.05$), but were similar in L1210/DACH cells.

DISCUSSION

Tetraplatin is a third-generation platinum complex in which the central platinum is in the +4 oxidation state with an octahedral configuration (Fig. 1). This complex has shown antitumor activity comparable with or superior to that of cisplatin against a number of murine and human tumor lines both *in vitro* and *in vivo*, and has been shown to maintain that activity against cisplatin-resistant cells [10–14]. Its mechanism of action is likely to be through its interaction with DNA to form interstrand and intrastrand cross-links (or adducts), as has been demonstrated for cisplatin [7]. Its activity, however, depends on its rapid chemical reduction to the +2 oxidation state as a first step, with thiols playing a major reductive role [19–23, 31]. Although intracellular protein and non-protein thiol concentrations can be high in tumor cells (Table 3), the rapid *in vivo* chemical reduction of tetraplatin ($T_{1/2} = 3$ sec) in the thiol-rich plasma will predominate [20, 23], and tissue distribution of the intact platinum(IV) complex *per se* is likely to be low. Thus, the cytotoxicity of tetraplatin will be dependent on accessibility of tumor to the reduction products. *In vitro*, reduction in tissue culture systems ($T_{1/2} = 5$–15 min) appears to be relatively slower [19], and the status of thiol levels in the medium on cytotoxicity of tetraplatin becomes critical.

In this investigation, we have demonstrated that, in the absence of extracellular GSH, DACH-Pt(II)Cl_2 has greater cytotoxicity than tetraplatin. Furthermore, it is transported in L1210/0 and L1210/DDP cells to a greater extent and in L1210/DACH cells to a lesser extent than tetraplatin. This observation in L1210/DACH cells is interesting, but the underlying mechanism of the preferential accumulation of tetraplatin over the platinum(II) drug in this cell line is not known. The data, nevertheless, draw the speculation that L1210/DACH cells may have distinctly different membrane properties from the other two L1210 lines. Although comparisons of biological properties indicate superiority of DACH-Pt(II)Cl_2, tetraplatin may still be favored in any chemotherapeutic application from the viewpoint of aqueous solubility, which is very poor for the platinum(II) complex (0.26 mg/mL for the *R,R* component) and relatively higher for the platinum(IV) agent (21.5 mg/mL for the *R,R* complex) [41].

The results presented in this report further indicate that cytotoxicity, intracellular accumulation and formation of DNA adducts with tetraplatin were modulated by GSH, and under optimal conditions were similar to levels seen with DACH-Pt(II)Cl_2 alone. It is very likely, therefore, that in the extracellular fluid GSH accelerates the chemical reduction of tetravalent platinum complex to the platinum(II) compound, which then enters the cell and interacts with DNA to elicit biological activity. That tetraplatin can transform rapidly to the DACH-Pt(II)Cl_2 complex was confirmed with our HPLC system. Complete reduction of tetraplatin can occur with less than equimolar concentration of the thiol, which suggests that other mechanisms may be at play in the process, such as platinum(II)-assisted chemical reduction that has been reported recently [21]. Higher GSH levels reduced the uptake and cytotoxicity of tetraplatin. Eastman [31] has observed a similar decrease in the reaction between tetraplatin and purified DNA with increasing GSH, and proposed that this may be due to a direct chemical reaction between the platinum and the thiol, leading to the formation of a relative inert platinum-GSH product.

Reduction of tetraplatin inside the cell to cytotoxic species following its accumulation is highly probable based on substantial thiol levels that are present in L1210 leukemia cells and on the demonstration of cytotoxicity in HBSS, a solution which does not support significant tetraplatin reduction, as has been noted here and elsewhere [21, 31]. Although cisplatin-resistant L1210/DDP cells have higher levels of the thiol than parent L1210/0 or tetraplatin-resistant L1210/DACH cells, intracellular reduction of tetraplatin is likely to be rapid in all cell lines and, therefore, the reductive process can be excluded as a factor responsible for differences in the potencies of this drug among the three lines. Our conclusion supporting intracellular chemical reduction of tetraplatin as an initial cytotoxic process agrees with that of Eastman [31] but contrasts with that of Chaney *et al.* [21], who have suggested that intracellular reduction of tetraplatin is not important for biological activity. Our data, however, do indicate that tetraplatin alone was about 2- to 4-fold less potent (comparing IC_{90} values) than DACH-Pt(II)Cl_2 against wild type and variant L1210 cells, but this was consistent with lower intracellular accumulations of the platinum(IV) complex in L1210/0 and L1210/DDP cells. In L1210/DACH cells, on the other hand, the lower potency of tetraplatin was not consistent with its greater accumulation relative to DACH-Pt(II)Cl_2, and this indirectly supports the finding of Chaney *et al.* [21] that intracellular biotransformation pathways of tetraplatin may be different from those of DACH-Pt(II)Cl_2. These differences in uptake and, thus, cytotoxicity were observed in a non-reducing extracellular environment provided by HBSS, but in the presence of extracellular GSH, which rapidly reduces tetraplatin, differences between the two complexes were greatly diminished. Lack of a difference in biological activity between the drugs in a reducing environment is consistent with the data of Gibbons *et al.* [19], who conducted their *in vitro* investigations with tetraplatin and DACH-Pt(II)Cl_2 in RPMI 1640 medium that could facilitate tetraplatin reduction.

In summary, extracellular GSH can enhance the *in vitro* cytotoxicity of tetraplatin through its chemical reduction to DACH-Pt(II)Cl_2. As platinum(IV) complexes acquire greater interest, it becomes critical to monitor thiol levels in cells and in the extracellular compartments as potential modulators of drug cytotoxicity during preclinical evaluation. Clinically, thiol monitoring in the peritoneal cavity may be particularly important in examining the recently reported potential of tetraplatin for intraperitoneal chemotherapy [42].

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