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Intracellular glutathione and cytotoxicity of platinum complexes

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Abstract Although there have been a number of reports correlating cellular GSH levels with cytotoxicity of platinum agents, none has examined the relationship between GSH concentrations and cytotoxicity. In this study, using a highly specific HPLC method for measuring GSH and expressing GSH as concentration and also per cell number, we evaluated the correlation between GSH levels and the cytotoxicity to five agents in ten human tumor cell lines. The five platinum agents included the platinum(II) complexes cisplatin, carboplatin and oxaliplatin and platinum(IV) complexes iproplatin and tetraplatin. The correlation between intracellular GSH concentration and cytotoxicity was highly significant only for iproplatin ($P = 0.002$) followed by tetraplatin, which demonstrated a trend toward statistical significance ($P = 0.06$). Cytotoxicity of the other platinum complexes showed no relation to GSH concentration, cisplatin itself showing a P-value of 0.09. In contrast, the GSH levels normalized to cell number showed a statistically significant correlation with the cytotoxicity of four of the five platinum agents, the exception being carboplatin; the strongest correlation observed was that for iproplatin and tetraplatin. Glutathione-*S*-transferase (GST) activity in these cell lines showed no correlation with cytotoxicity of any of the platinum complexes. Our results, from the analyses of both GSH concentration as well as GSH per cell number, suggest a significantly higher interaction between GSH and iproplatin compared with the other platinum agents. Moreover, our data suggest that relationships between cytotoxicity and GSH levels on a per-cell basis may not persist when differences in cell volume are taken into account.

Key words Glutathione · Cytotoxicity · Platinum

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Introduction

Glutathione (γ-glutamyl-cysteinyl-glycine, GSH), the predominant intracellular non-protein thiol, is a major defense against exogenous toxins and oxidants in cells. GSH may spontaneously (non-enzymatically) conjugate and inactivate many electrophiles, including drugs used in cancer chemotherapy [13]. In other cases, as for some alkylating agents, this reaction is catalyzed by glutathione-*S*-transferase [51]. Published data suggest that platinum complexes can react directly with GSH without the aid of GST [9, 31].

A number of studies have demonstrated increased GSH levels as one mechanism of cellular resistance to platinum complexes [4, 6, 7, 29, 44]. Recent studies indicate a linear correlation between intracellular GSH levels and resistance to platinum agents in tumor cell lines of both human and murine origin [23, 28, 30, 34, 35]. One study extended this observation to several non-platinum drugs [26]. The possibility that cellular GSH can influence the cytotoxicity of certain platinum(IV) complexes to a significantly greater degree than that of the platinum(II) complexes has been demonstrated in GSH modulation studies [35, 47]. Thus, in these studies depletion of GSH by buthionine sulfoximine enhanced the cytotoxicity of iproplatin and tetraplatin to a greater extent than cisplatin [35, 47].

GSH can potentially antagonize the cytotoxicity of platinum agents by several mechanisms [1, 11, 14, 32, 43]. For example, GSH can complex directly to the agent and inactivate it. Alternatively, GSH can conjugate with monofunctional platinum-DNA adducts, preventing the formation of the bifunctional adducts [14]. GSH may also affect DNA repair processes as suggested by the observation that depletion of GSH reduces the activities of several ligases and polymerases in a human astrocytoma cell line resistant to BCNU and cisplatin [1, 43]. Depletion of GSH also increases the cisplatin-induced DNA interstrand crosslink

formation in a rat ovarian tumor [11]. There is evidence that GSH may facilitate DNA repair through mechanisms that are not yet completely understood. Thus, depletion of GSH produces a decrease in the unscheduled DNA synthesis after DNA damage with cisplatin in a cisplatin-resistant ovarian carcinoma cell line [32].

In the present investigation, we explored the relationship between cellular GSH concentration (as well as GSH expressed as nanomoles per million cells) and the cytotoxicity of five platinum complexes with a variety of leaving and non-leaving (carrier) ligands in a panel of ten human tumor cell lines derived from five different tumor types with a highly specific HPLC assay. The platinum complexes used included three platinum(II) complexes (cisplatin, carboplatin and oxaliplatin) and two platinum(IV) complexes (tetraplatin and iproplatin). Although the latter two drugs are platinum(IV) complexes, both are reduced either intra [40] or extracellularly [22] and exist in the cell predominantly in the active platinum(II) form [10, 40]. We present the results of this study which suggest that cellular GSH interactions with iproplatin are stronger than with the other platinum complexes.

Materials and methods

Drugs and chemicals

Cisplatin was purchased from Sigma Chemical Co. Carboplatin and iproplatin were gifts from Bristol-Myers Co. Tetraplatin was a gift from UpJohn Co. Oxaliplatin was a gift from Debiopharm, Lausanne, Switzerland. Thiolyte (monobromobimane) was purchased from Calbiochem (San Diego, Calif.). All the other chemicals and reagents used in this study were purchased from Sigma Chemical Co (St. Louis, Mo.). All cell culture media were purchased from GIBCO (Grand Island, N.Y.).

Cell lines

All tumor cell lines were of human origin. Two bladder carcinoma cell lines (RT4 and TCCSUP), two ovarian carcinoma cell lines (OVCAR3 and A2780 and a cisplatin-resistant variant of A2780, A2780/CP), a colon carcinoma cell line (HT-29), two glioblastoma cell lines (U-373MG and U-87MG) and two melanoma cell lines (SK-MEL-2 and HT-144) were used in the study. All cell lines except A2780 and A2780/CP were obtained from American Type Culture Collection (Rockville, Md.). The A2780 and A2780/CP were a gift from Dr. R. Ozols of Fox Chase Cancer Center, Philadelphia, Pa.

The origin and other pertinent information on the cell lines used in this study are given in Table 1. The majority of cell lines used in the study are from tumors that are normally non-responsive to cisplatin/carboplatin therapy (e.g. melanoma, glioblastoma and colon). With the exception of A2780/CP (selected for resistance from in vitro cisplatin exposure), none of the other cell lines was specifically selected for platinum resistance in vitro. The OVCAR3 cell line was derived from a patient with ovarian carcinoma refractory to cisplatin-based therapy [27].

Table 1 Human tumor cell lines used in this study

Cell line	Origin	Reference
U-87MG	Glioblastoma/astrocytoma	42
U373-MG	Glioblastoma/astrocytoma	42
HT-144	Malignant melanoma	19–21
SKMEL-2	Malignant melanoma	20
RT-4	Transitional cell papillary tumor of bladder	45
TCCSUP	Transitional cell carcinoma of the bladder	37
HT-29	Adenocarcinoma of the colon	20
OVCAR3	Adenocarcinoma of the ovary	27
A2780	Adenocarcinoma of the ovary	17
A2780/CP	In vitro-derived resistant variant of A2780	7

Cytotoxicity assays

The cytotoxicity of the five platinum complexes was determined using the sulforhodamine-B (SRB) assay. The assay protocol and procedure were as described by Rubinstein et al. [46], with a continuous drug exposure of 48 h as described previously [38].

GSH measurements

GSH in the cells was measured using the HPLC assay described by Fahey and Newton [18] with some minor modifications. Cell extracts were prepared according to the method of Anderson [2] by sonicating 1×10^6 cells into 0.5 ml 5% sulfosalicylic acid (SSA) at 4°C, followed by the removal of cell debris by centrifugation. The GSH in the supernatant was derivatized with monobromobimane (thiolyte; 40 µl of a 7 mg/ml solution to 220 µl of extract) in the dark at pH 8.0 for 20 min. The reaction was stopped with 50% SSA and, after another centrifugation, a 10-µl aliquot was injected on to the HPLC column.

The HPLC system consisted of a Waters Associates M6000A pump, a model 710B refrigerated automatic injector WISP, a model 470 scanning fluorescence detector, with an Autochrom 1 pump gradient maker. The HPLC system was interfaced with a Hewlett Packard computer equipped with Nelson Analytical chromatography software. The HPLC separation of GSH from other thiols and the hydrolysis products of thiolyte was carried out on an Altech C18 column with mobile phase consisting of 15% methanol (in 0.25% acetic acid, pH 3.2) run isocratically for 5 min followed by a gradient to 100% methanol in 45 min at a flow rate of 1.0 ml/min. The detection was with a fluorescence detector with excitation at 385 nm and emission at 490 nm. Quantitations were based on identically prepared analytical standards.

Thiolyte derivatizes the free GSH and other thiols with free sulfhydryls. The total glutathione (free + mixed disulfides including protein disulfides) in the cells was measured after reduction to the free form with dithiothreitol (2.5 mM). Thus both the free GSH and the total glutathione were measured in these cell lines.

GSH concentrations were expressed as nanomoles per million cells and/or millimolar concentrations based on cell volume measurements made with a Coulter Counter (Model ZM). All GSH and GST (see below) measurements were obtained in the mid-log phase for all cell lines (48–72 h after passage).

GST measurements

The activity of GST was measured spectrophotometrically with the 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to

the method of Habig and Jakoby [25], since CDNB is a substrate for the different isozymes and gives an estimate of total GST activity [52]. In this assay, the change in the absorbance (at 340 nm) of the substrate when it is conjugated with GSH was measured at 25°C. The enzyme activity is expressed as nanomoles of product formed per minute per milligram protein. Protein was measured by the 'Bradford' assay with bovine serum albumin as the standard.

Data analysis

Correlations between GSH level and IC_{50} of each of the platinum complexes and that between GST activity and the IC_{50} were determined by linear regression analysis using the computer program 'Epistat'.

Results

Cell lines and cytotoxicity to the platinum agents

The cell lines used in the study differed in their in vitro sensitivity to the five platinum complexes (Fig. 1). The melanoma line SK-MEL2 was the most resistant to all the platinum complexes except carboplatin and the ovarian A2780 was the most sensitive. Some cell lines like OVCAR3 and TCCSUP were relatively cisplatin-sensitive, but were relatively resistant to both the diaminocyclohexane platinum-containing complexes tetraplatin and oxaliplatin.

GSH levels and GST activity

The measured free GSH levels and GST activity are shown in Table 2. The free GSH levels ranged from 1.07 ± 0.73 to 13.14 ± 0.05 nmoles/10^6 cells in the ten cell lines, the lowest being in the A2780 cell line and the highest in the SK-Mel2 cell line. The GSH concentration ranged from 1.25 mM (HTB-14) to 4.72 mM (SK-Mel 2). Free GSH constituted $> 85\%$ of the total in each of these cell lines. The specific activity of GST (nmoles product formed/min per mg protein) ranged from 2.63 ± 0.25 (RT-4) to 164.05 ± 12.45 (OVCAR-3).

Relationship between GSH, GST and cytotoxicity

A number of studies have shown correlations between GSH levels (per cell number or per milligram protein) and cytotoxicity to platinum complexes, but there are no studies that have attempted to relate GSH concentration to cytotoxicity. Since cell lines may show a great deal of variation in size we examined the affect of using GSH concentration, in addition to GSH per cell number, for evaluating these correlations. Thus, GSH was calculated both as concentration and per cell number for these evaluations.

A plot of GSH concentration (mM) against the IC_{50} in these cell lines is shown in Fig. 2. A highly

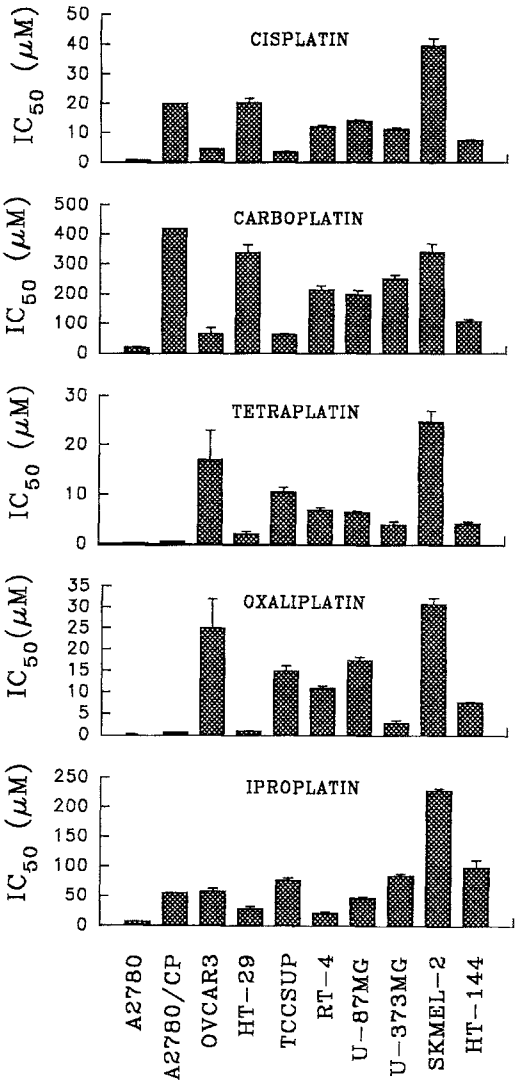

Fig. 1 Sensitivity of the ten human tumor cell lines used in this study to the five platinum complexes

Table 2 GSH levels and GST activity in the cell lines. The numbers shown are mean ± SD of at least four sample extracts

Cell line	GSH (nmoles/10^6cells)	GSH (mM)	GST (nmoles product/min/mg protein)
U-87MG	3.73 ± 0.15	1.91 ± 0.08	43.68 ± 3.1
U373-MG	6.19 ± 0.11	3.05 ± 0.05	69.69 ± 6.3
HT-144	5.61 ± 0.25	3.05 ± 0.14	63.80 ± 5.8
SKMEL-2	13.14 ± 0.05	4.72 ± 0.02	29.30 ± 1.6
RT4	6.60 ± 3.7	2.57 ± 1.44	2.63 ± 0.25
TCCSUP	5.48 ± 0.62	3.91 ± 0.44	63.66 ± 8.5
HT-29	4.09 ± 2.0	2.25 ± 1.12	124.60 ± 9.6
OVCAR3	5.9 ± 0.2	1.82 ± 0.06	164.10 ± 12.5
A2780	1.07 ± 0.73	1.25 ± 0.85	54.40 ± 2.9
A2780/CP	3.10 ± 0.07	2.37 ± 0.05	81.60 ± 2.83

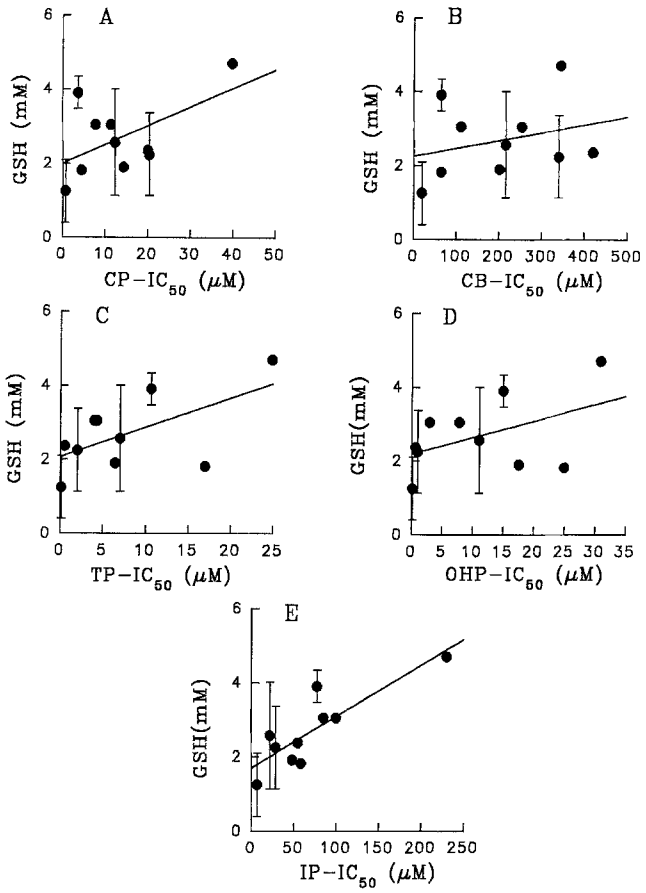

Fig. 2A–E Correlation between cellular free GSH concentration (mM) and IC_{50} values for the five platinum agents. **A** Cisplatin, **B** carboplatin., **C** tetraplatin, **D** oxaliplatin, **E** iproplatin. The line shown is a regression line

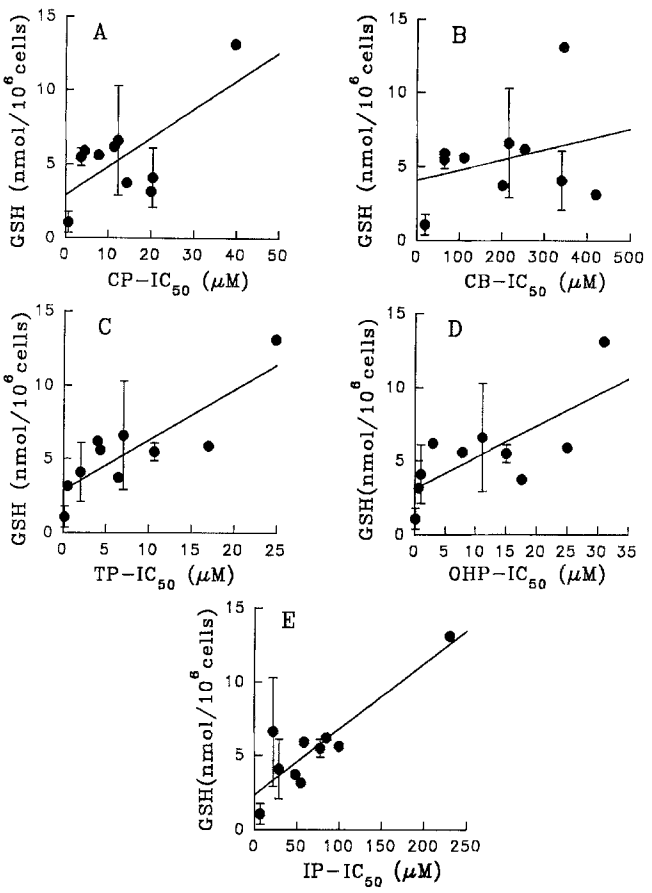

Fig. 3A–E Correlation between free GSH (nmoles/10^6 cells) and IC_{50} values for the five platinum agents. **A** Cisplatin, **B** carboplatin, **C** tetraplatin, **D** oxaliplatin, **E** iproplatin. The line shown is a regression line

statistically significant correlation was apparent only between the GSH and the IC_{50} for iproplatin (Table 3). The correlation for tetraplatin showed a trend toward statistical significance ($p = 0.06$). No correlation was found between GSH concentration and the IC_{50} for cisplatin ($P = 0.09$), oxaliplatin ($P = 0.169$) or carbo-platin ($P = 0.43$). The correlations between total glutathione (mM) and the IC_{50} values showed exactly the same pattern as that for the free GSH (data not shown).

The intracellular levels of GSH (nanomoles per million cells) plotted against the IC_{50} values for the five platinum complexes are shown in Fig. 3. The total glutathione showed a nearly identical profile (data not shown). A statistically significant correlation was observed between cellular GSH levels and the IC_{50} values for cisplatin, tetraplatin, oxaliplatin and iproplatin (Table 3). The strongest correlations observed were for the two platinum(IV) complexes iproplatin and tetraplatin. No correlation was apparent between GSH and

Table 3 The observed correlations between GSH and IC_{50}, and GST and IC_{50} for the five platinum complexes tested in all the cell lines

Platinum complex	GSH(nmoles/10^6) cells		GSH(mM)		GST(nmoles product/min/mg protein)	
	r	*P*	*r*	*P*	*r*	*P*
Cisplatin	0.69	0.027	0.56	0.093	−0.24	0.510
Carboplatin	0.30	0.399	0.28	0.435	−0.09	0.799
Tetraplatin	0.85	0.002	0.61	0.062	−0.01	0.988
Oxaliplatin	0.73	0.015	0.47	0.169	−0.04	0.910
Iproplatin	0.89	<0.001	0.85	0.0019	−0.21	0.563

Table 4 Correlations between GSH and IC_{50}, for the five platinum complexes evaluated in nine cell lines (without the SKMEL2)

Platinum complex	GSH(nmoles/10^6 cells)		GSH (mM)	
	r	*P*	*r*	*P*
Cisplatin	0.005	0.989	0.007	0.987
Carboplatin	−0.008	0.983	0.05	0.906
Tetraplatin	0.60	0.08	0.17	0.66
Oxaliplatin	0.47	0.200	0.05	0.89
Iproplatin	0.55	0.125	0.71	0.03

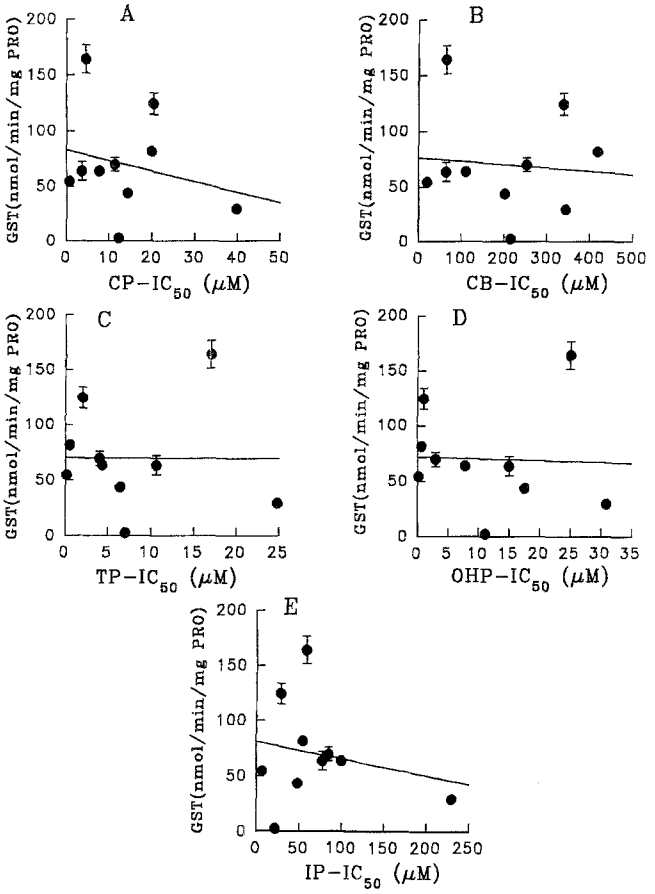

Fig. 4A–E Correlation between GST activity (nmoles/min/mg protein) and IC_{50} values for the five platinum agents. **A** Cisplatin, **B** carboplatin, **C** tetraplatin, **D** oxaliplatin, **E** iproplatin. The line shown is a regression line.

carboplatin cytotoxicity in these cell lines. The correlations between total glutathione (per million cells) and the IC_{50} values showed an identical pattern to that of the free GSH (data not shown).

For some drugs, correlations may have been substantially affected by the data for the SKMEL-2 cell line (Figs. 1 and 3). Therefore, we recalculated the correlation coefficients excluding the data point for this cell line (Table 4). The correlation coefficients recalculated for the nine cell lines showed a statistically significant correlation only for GSH concentration and iproplatin cytotoxicity ($r = 0.71$, $P = 0.03$). Since cell lines of different origin vary a great deal in size, GSH concentration would be expected to be an important parameter. In our experiments GSH concentration correlated consistently with iproplatin cytotoxicity. There is a suggestion that tetraplatin also may react readily with GSH, but only when all the cell lines are taken into consideration (Table 3). It should also be noted that, in the panel of cell lines used in this study, the cell line with the highest GSH was the least sensitive and the one with the lowest GSH was the most sensitive to four out of five platinum agents.

No correlation was found between the GST activity and the IC_{50} for any of the platinum complexes (Fig. 4, Table 3).

Discussion

Cellular resistance to platinum complexes may involve one or more biochemical mechanisms including decreased drug accumulation, increased intracellular inactivation of the drug and increased repair of the platinum-DNA adducts [3, 33, 41, 44, 49]. GSH and metallothionein are two sulfhydryl molecules, that are implicated in the intracellular inactivation of platinum agents [41]. GSH elevation in acquired resistance to platinum agents and alkylating drugs has been reported widely [5, 15, 34, 50]. A linear correlation between GSH and cytotoxicity to cisplatin has been reported in ovarian carcinoma cell lines with varying degrees of acquired resistance (all derived from a common parental line) [24], in unrelated lines derived from multiple ovarian cancer patients [35], and in lines representing several different tumor types [28]. Some of these studies also showed correlations to other platinum complexes [30, 34, 35]. In one study [35], significant correlations were found between GSH and the cytotoxicity of cisplatin, carboplatin and iproplatin but not tetraplatin. In another [30], which used L1210 cells of varying degrees of platinum resistance, correlations between GSH and cisplatin, GSH and iproplatin, but not GSH and tetraplatin, were observed. One other study showed significant correlations between GSH and the cytotoxicity of seven platinum complexes that included cisplatin, carboplatin, iproplatin and tetraplatin among others [34].

In each of the studies that showed a correlation between GSH and the cytotoxicity to platinum agents, the measured GSH was either normalized to cell number [23, 30, 35], or to cellular protein content [28, 30, 34, 35]. In our study when the GSH was normalized to cell number, a statistically significant correlation was found for four out of the five platinum complexes tested, carboplatin being the exception; the strongest correlation found was that for iproplatin. When GSH concentration, instead of GSH per cell number, was

taken into account, the correlation to the cytotoxicity of iproplatin remained strong, that for tetraplatin was close to attaining statistical significance, while that for the others no longer existed.

The significance of the differences found when GSH was normalized to cell number as opposed to cell volume is unclear at this time. However, since cell lines of different origin and those with acquired resistance often show a great deal of variation in cell size, the GSH concentration rather than amount per number of cells may be the parameter that should be of significance for evaluating correlations. Our data, independent of how the GSH levels were expressed, indicate a definite interaction between GSH and iproplatin. Since the distribution of the data points for cisplatin and iproplatin is such that the SK-MEL2 data point may have affected the significance of the conclusion. Reanalysis of the data without the SK-MEL2 data point showed a statistically significant correlation only between GSH concentration and iproplatin cytotoxicity ($r = 0.71$, $P = 0.03$). This result further supports the observation that GSH may interact quite readily with iproplatin compared to the other platinum agents. The stronger influence of GSH on iproplatin compared to cisplatin and other agents has been also demonstrated in two other studies in which cell lines subjected to buthionine depletion of GSH showed a significantly greater potentiation of iproplatin cytotoxicity than cisplatin cytotoxicity [35, 47].

The reported reaction half-time for iproplatin and GSH in vitro is approximately 63 h, longer than that for carboplatin which has been reported to be about 42 h [9]. However, we have previously demonstrated that iproplatin exists in the cells predominantly in the platinum(II) form [40], and that in vitro the binding of CIP (*cis*-dichloro-bisisopropylamine, the reduced form of iproplatin) to DNA is inhibited by GSH [39]. Thus, it is possible that GSH may interact with and inactivate the bisisopropylamine platinum(II) species much more readily than it does the other complexes. There is some suggestion from our results that GSH may also react readily with tetraplatin. Again, tetraplatin is a platinum(IV) complex that readily gets reduced to platinum(II) species in culture media [22] and intracellularly [10]. Thus, the unique reactivity of iproplatin and tetraplatin could be due to platinum(II)-assisted platinum(II) ligand exchange reactions following the reduction of iproplatin and tetraplatin [10]. A greater proportion of tetraplatin–GSH conjugate has been found in an ovarian cell line compared with the cisplatin–GSH conjugate [36]. If intracellular levels of GSH affect the activity of individual platinum complexes differently, this finding may have a significant implication for future platinum drug development.

The SK-Mel2 cell line with the highest GSH level showed the most resistance for four out of five platinum complexes. The A2780 cell line with the lowest GSH was the most sensitive to all the platinum complexes. While the possibility that at very high intracellular GSH concentrations all the platinum complexes may be affected to a certain degree remains, it is apparent that the intracellular interactions between GSH and the platinum agents with differing carrier and leaving ligands need to be carefully evaluated.

GSH is measured most commonly with an indirect, reductase recycling enzymatic procedure using dithiobisnitrobenzoic acid, a thiol reagent, commonly called Ellman's reagent [16]. This procedure can overestimate the GSH content of the cells because Ellman's reagent reacts with all cellular thiols [16]. In this study, we used a highly specific HPLC assay which separates the various thiols and gives a measure of free GSH. It is possible that GSH by itself may have a selective impact on the activity of platinum complexes while the total thiol content of cells may have a greater role in the inactivation of all platinum complexes. This total thiol may include not only the non-protein sulfhydryls, but also the protein sulfhydryl such as that contributed by metallothionein and other sulfhydryl-rich proteins.

In our study, we found no correlations between the activity of GST and the cytotoxicity to any of the platinum complexes tested. The lowest GST activity was found in a bladder carcinoma cell line (RT-4) which is relatively resistant to all the platinum complexes and the highest in OVCAR-3, an ovarian carcinoma cell line which is relatively cisplatin-sensitive, but relatively highly resistant to DACH-platinum complexes. This lack of correlation between GST and the cytotoxicity of platinum agents is consistent with the observations of other investigators and with the hypothesis that the effect of GSH on platinum cytotoxicity may not be mediated by GST [23, 28, 30, 34, 35]. However, the possibility remains that a specific isozyme of GST which is not picked up in this general assay may be involved in the catalysis of the GSH conjugation to platinum agents. Specific isozymes of GST are known to catalyze the GSH conjugation reaction with specific alkylating agents [8, 12, 48].

All the studies that show a relationship between GSH and platinum resistance including ours, however, are in vitro studies, and there is little information on the relevance of these findings to the clinical situation. Our HPLC assay for GSH allows quantitation of GSH in tumor biopsies of approximately 10 mg (Pendyala et al., unpublished data). Using this assay we hope to define the role of GSH in clinical resistance to platinum agents.

The studies presented in this paper call into question whether GSH plays a general role in resistance to platinum complexes and suggest that the extent to which GSH interacts with platinum complexes is dictated by the structure of the complex.

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