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ORIGINAL ARTICLE

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Effect of glutathione depletion on the cytotoxicity of cisplatin and iproplatin in a human melanoma cell line

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Abstract Previous studies from our laboratory have indicated that glutathione (GSH) may affect the cytotoxicity of iproplatin to a greater extent than four other platinum agents tested including cisplatin. Therefore we studied the effect of GSH depletion by buthionine sulfoximine (BSO) on the cytotoxicity of iproplatin and cisplatin in a human melanoma cell line SK-MEL-2. Depletion of GSH was dependent on the concentration and time of incubation with BSO. BSO (100 μM) depleted GSH by 85% at 24 h and by 91% at 48 h. BSO (10 to 100 μM) by itself was not cytotoxic to SK-MEL-2 cells. At 85% depletion of GSH, cytotoxicity of iproplatin was increased by a factor of >7 and that of cisplatin by <2. These results confirm the previous finding that GSH interferes with the cytotoxicity of iproplatin to a significantly greater extent than that of cisplatin. Equitoxic IC_{65} and IC_{90} values of cisplatin (2 μM and 5 μM) or iproplatin (25 μM and 50 μM) had no effect on the intracellular GSH levels in SK-MEL-2 cells. Also, depletion of GSH by BSO had no effect on the accumulation of platinum from either cisplatin or iproplatin in this cell line. Our results suggest that the effect of GSH on the cytotoxicity of cisplatin and iproplatin in this cell line was not a consequence either of differences in GSH–Pt conjugate formation, or of differences in platinum accumulation induced by GSH depletion. GSH may have modulated the cytotoxicity of these platinum complexes by other means such as effects on DNA repair, apoptosis, free radical scavenging or through other yet unidentified mechanisms.

Key words Glutathione · Cytotoxicity · Cisplatin · Iproplatin

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Introduction

Glutathione (GSH) is a predominant nonprotein thiol found in cells and plays an important role in the defense mechanism of the cell by acting as an antioxidant or by reacting with electrophiles [8]. GSH can react with many toxic agents to form conjugates which are eliminated from the cell [8, 26]. While this mechanism confers protection against many types of toxins, it can also counteract the effects of many chemotherapeutic agents including platinum drugs.

Elevation of GSH in cellular resistance to platinum agents has been widely demonstrated [2–4, 15, 34]. Inactivation of platinum complexes through reactions with GSH is thought to be one of the mechanisms by which cells protect themselves from the cytotoxic action of these agents [9]. In vitro and in vivo conjugation reactions of GSH and platinum agents have been described [6, 17]. Active elimination of the GSH–Pt conjugates has been demonstrated in murine L1210 cells and human promyelocytic leukemia HL60 cells [17, 18]. The elimination of GSH-Pt conjugates from cells has been suggested as an important mechanism that reduces the intracellular accumulation of platinum complexes [17]. GSH may also affect the efficacy of platinum drugs through other mechanisms, such as enhancement of the repair of DNA damage [1, 7, 19, 33].

A correlation between intracellular GSH levels and resistance to a number of platinum agents has been reported in several human and murine tumor cell lines [13, 14, 16, 25, 27]. Our own studies using ten human tumor cell lines and five platinum complexes (cisplatin, carboplatin, oxaliplatin, tetraplatin and iproplatin), have shown that the correlation between cellular GSH and the IC_{50} values was strongest for iproplatin (*cis*-dichloro-*trans*-dihydroxy-bis-isopropylamine Pt-IV) [32]. We compared the effects of depletion of GSH with buthionine sulfoximine (BSO) on the cytotoxicity of iproplatin and cisplatin in a melanoma cell line

SK-MEL-2. This cell line was chosen because it is least sensitive to the platinum complexes tested among the ten tumor cell lines used in the previous study [32]. GSH interfered with the cytotoxicity of iproplatin to a significantly greater extent than with the cytotoxicity of cisplatin. In order to understand this phenomenon further, the effect of cisplatin and iproplatin on the cellular GSH levels, and the effect of cellular GSH on the accumulation of platinum from these platinum complexes was also studied.

Materials and methods

Drugs and chemicals

Iproplatin was a gift from Bristol-Myers Co. Cisplatin and 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/BRL (Grand Island, N.Y.). Thiolyte (monobromobimane) was purchased from Calbiochem (San Diego, Calif.).

Cell line

The melanoma cell line (SK-MEL-2) was obtained from the American Type Culture Collection (Rockville, Md.). The cells were maintained in Eagle's minimal essential medium with sodium pyruvate and 10% fetal bovine serum at 37 °C, under an atmosphere containing 5% CO_2 at 95% relative humidity.

Cytotoxicity assays

Cytotoxicity was measured by the sulforhodamine-B assay (SRB) using 96-well plates [36]. The survival fractions shown for the dose response curves are the ratios of the absorbance values obtained for the drug-treated cells over the controls.

In experiments in which the effect of BSO was tested on the cytotoxicity of SK-MEL-2 cells, the cells were plated on day 0 and allowed to attach overnight. The medium was removed and replaced with fresh medium containing 0, 10, 25, 50 or 100 μM BSO. The plates were allowed to incubate for up to 72 h. At 24, 48 and 72 h duplicate plates containing the four concentrations of BSO and the control were subjected to the SRB.

In experiments in which the effect of BSO on the cytotoxicity of cisplatin and iproplatin were tested, the cells were plated on day 0, and after an overnight incubation, were treated for 24 h with 0, 10, 25, 50 or 100 μM BSO. The cells were then exposed to the platinum agents in the continued presence of BSO or vehicle, and incubation was continued for a further 24 h. The cells were washed to remove the drug(s) and fresh medium added. After an additional 96-h incubation, the SRB was carried out.

GSH measurements

GSH in the cells was measured using a specific and sensitive HPLC assay as reported previously [10, 32]. GSH in 5% sulfosalicylic acid extract of cells was derivatized with monobromobimane (thiolyte) and separated on a C18 column with a gradient consisting of 15% methanol (in 0.25% acetic acid) to 100% methanol. Detection was by fluorescence with excitation set at 385 nm and emission at 490 nm. Protein was measured using the assay described by Lowry et al. [22].

Effect of cisplatin and iproplatin on cellular GSH levels

For these experiments, 2×10^6 cells were plated and allowed to adhere to the flask overnight, then exposed to either cisplatin (2 or 5 μM) and iproplatin (25 or 50 μM) for 3 or 6 h. These concentrations produced approximately 65% and 90% growth inhibition, respectively, from the cytotoxicity experiments described above. At the end of each treatment, the drug was removed, and the cells were washed three times with phosphate-buffered saline (PBS), trypsinized and pelleted. GSH was measured by HPLC as described above.

Effect of GSH depletion by BSO on the accumulation of platinum from cisplatin and iproplatin

Total cellular platinum was measured following a 2-h exposure to equimolar concentrations (30 μM) of cisplatin or iproplatin at 37 °C, after depletion of GSH with 25 μM or 50 μM BSO for 24 h. Cells treated identically with the vehicle but not BSO were the controls. At the end of the drug exposure, cells were washed repeatedly with PBS, harvested by trypsinization, counted, and pelleted by centrifugation. Cell pellets were digested in concentrated HNO_3 and boiled with 30% H_2O_2 prior to analysis by atomic absorption spectrophotometry [24]. Platinum in cell digests was quantitated with a Perkin-Elmer 4100ZL atomic absorption spectrophotometer with Zeeman background correction, relative to elemental platinum standards.

Data analysis

The dose modification factor (DMF) calculated as the ratio of the inhibitory concentration (IC) in the absence of BSO pretreatment to that after BSO pretreatment (i.e. IC_{50} with no BSO/IC_{50} after BSO pretreatment). DMF values were determined at the IC_{50} to IC_{90} for each of the drugs at each concentration of the BSO pretreatment, because of the observed cytotoxicity profile for iproplatin.

Results

Effect of BSO on SK-MEL-2 cell survival and GSH levels

BSO at concentrations up to 100 μM was not cytotoxic to SK-MEL-2 cells even after a 72-h continuous incubation (Fig. 1). BSO depleted the levels of GSH in the SK-MEL-2 cells in a dose-dependent manner (Fig. 2). Untreated controls had a cellular GSH concentration of 11.0 ± 0.3 nmol/10^6 cells at 24 h and 11.5 ± 1.8 nmol/10^6 cells at 48 h. BSO (50 μM) had depleted GSH by 77% at 24 h and by 85% at 48 h. BSO (100 μM) had depleted GSH by 85% at 24 h and by 91% at 48 h. Thus by 24 h a significant depletion of cellular GSH had occurred at the higher concentrations of BSO. A further 24-h incubation decreased the GSH levels further, although to a small extent. Therefore, based on these results, a 24-h pretreatment with BSO was chosen to study the effect of GSH depletion on the cytotoxicity

of the platinum agents. The appropriate BSO concentration was maintained during the platinum drug exposure for the continued maintenance of lower GSH levels.

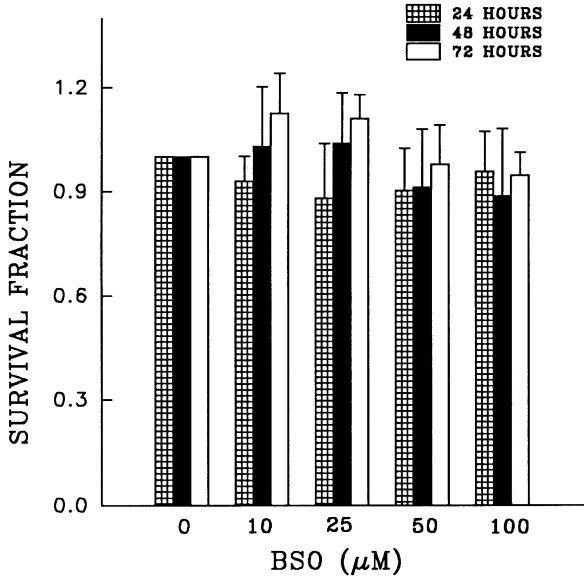

Fig. 1 The effect of BSO on the survival of SK-MEL-2 cells as measured by the SRB assay. The values presented are the ratio of survival of cells after BSO treatment to that of control cells (no BSO treatment) after 24, 48 and 72 h continuous exposure to BSO at the concentrations (10 to 100 μ*M*) specified. The results presented are averages from three different experiments

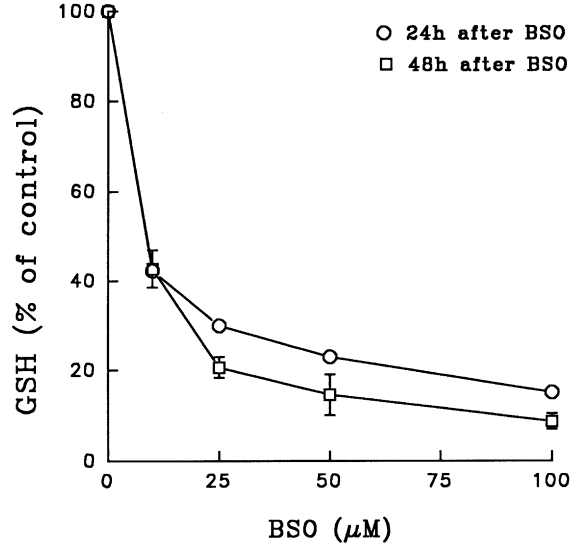

Fig. 2 Effect of BSO (10, 25, 50 and 100 μ*M*) on cellular GSH levels after 24 and 48 h of continuous exposure. GSH was measured by HPLC and the results are expressed in relation to the BSO-untreated control cells. The results are averages from three separate experiments

Effect of BSO on the cytotoxicity of cisplatin and iproplatin

As seen in Figs. 3 and 4, the depletion of cellular GSH with BSO potentiated the cytotoxicity of both cisplatin

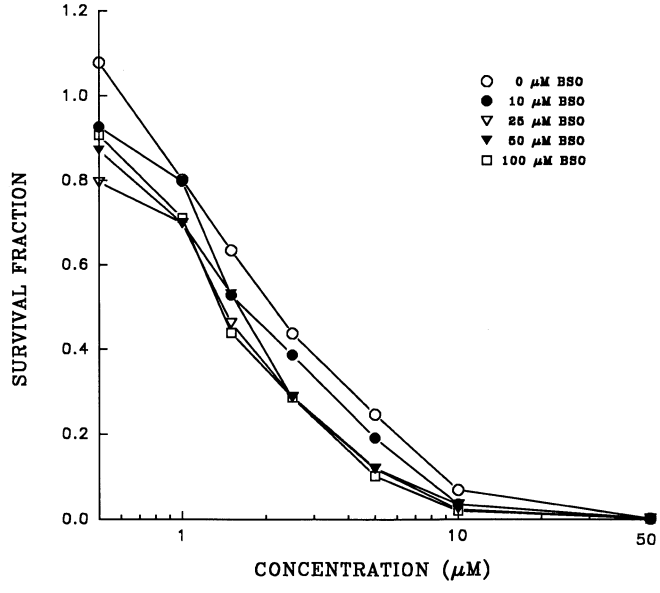

Fig. 3 The effect of a 24-h BSO pretreatment on the cytotoxicity of cisplatin in the SK-MEL-2 cell line. BSO concentrations (10 to 100 μ*M*) used are as specified. Data shown are from a representative experiment. Data from three such experiments were used to calculate the mean DMF (±SD) values at IC_{50} to IC_{90} concentrations shown in Fig. 5

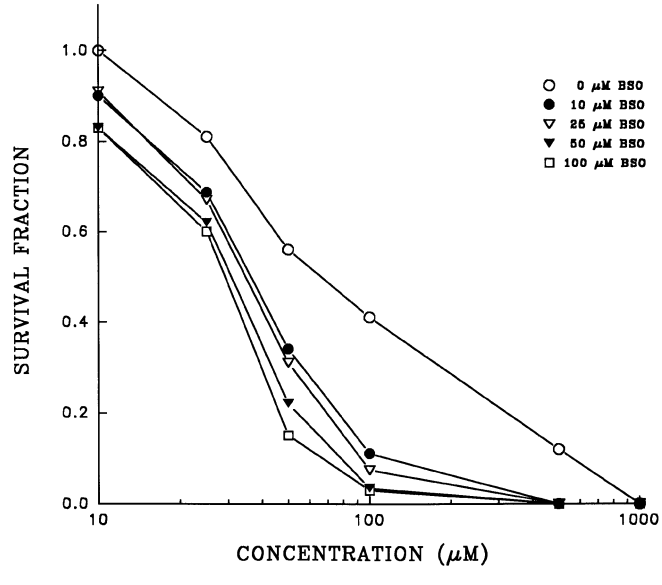

Fig. 4 The effect of a 24-h BSO pretreatment on the cytotoxicity of iproplatin in the SK-MEL-2 cell line. BSO concentrations (10 to 100 μ*M*) used are as specified. Data shown are from a representative experiment. Data from four such experiments were used to calculate the mean DMF (±SD) values at IC_{50} to IC_{90} concentrations shown in Fig. 6

and iproplatin. The degree of potentiation for cisplatin was relatively small and not strictly BSO dose-dependent. The potentiation of cytotoxicity for iproplatin was much greater than that for cisplatin and increased with increasing concentrations of BSO (Fig. 4). In order to better interpret the cytotoxicity curves, a mean DMF resulting from BSO pretreatment was calculated from the data from several experiments for both cisplatin and iproplatin at the IC_{50} to IC_{90} (Figs. 5 and 6). As is apparent from the data, the BSO-mediated depletion of GSH had a differential effect on cisplatin and iproplatin cytotoxicity. An increase in the DMF was achieved for iproplatin with increasing concentrations of BSO (Fig. 6). The DMF achieved for iproplatin at the IC_{90} with 100 μM BSO pretreatment was >7. In contrast, the DMF for cisplatin was <2 and essentially unchanged with the higher concentrations of BSO (Fig. 5). As seen in Fig. 6, at any given BSO concentration, the DMF achieved for iproplatin increased with the iproplatin concentration from IC_{50} to IC_{90}, whereas for cisplatin the DMF was unchanged at different cisplatin concentrations (Fig. 5).

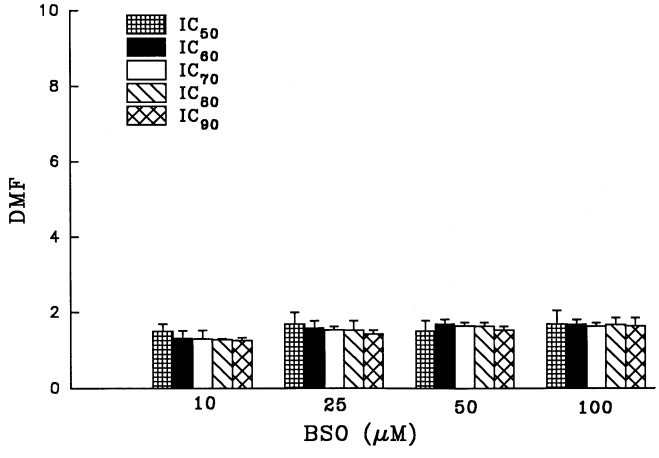

Fig. 5 The dose modification achieved with BSO depletion of GSH for cisplatin in the SK-MEL-2 cell line. Results shown are averages of three experiments

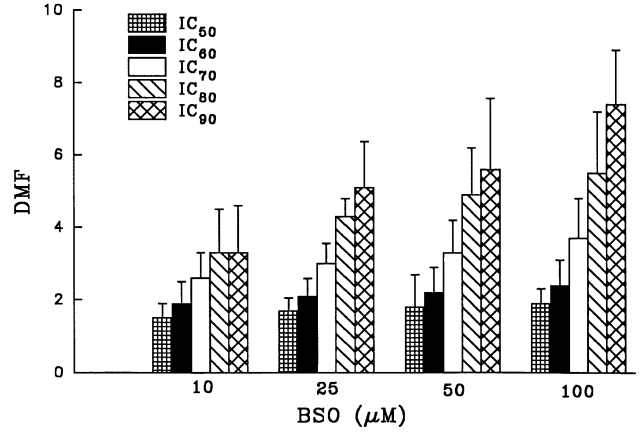

Fig. 6 The dose modification achieved with BSO depletion of GSH for iproplatin in the SK-MEL-2 cell line. Results shown are averages of four experiments

Effect of cisplatin and iproplatin on cellular GSH levels

The effects of equitoxic (IC_{65} and IC_{90}) concentrations of cisplatin and iproplatin on GSH levels in SK-MEL-2 cells are shown in Table 1. The GSH levels in both controls and platinum-treated cells declined by approximately 10% to 15% from baseline values at 3 and 6 h. However, no platinum-induced depletion of GSH was evident in either cisplatin- or iproplatin-treated cells.

Effect of GSH depletion by BSO on the accumulation of platinum from cisplatin and iproplatin

The effects of GSH depletion on cellular accumulation of cisplatin and iproplatin were quantitated following pretreatment with 25 μM or 50 μM BSO for 24 h, concentrations which depleted GSH by approximately

Table 2 Accumulation of platinum by GSH-depleted SK-MEL-2 cells following a 2-h exposure to cisplatin or iproplatin. The values shown are the means and standard deviation of four replicates from two separate experiments

Pretreatment with BSO (μM)	Total cellular platinum (ng/10^6 cells) after exposure to	
	Cisplatin (30 μM)	Iproplatin (30 μM)
0	18.75 ± 4.07	10.27 ± 1.34
25	18.23 ± 2.26	12.87 ± 3.04
50	19.17 ± 4.15	11.07 ± 1.19

Table 1 Cellular GSH after treatment with equitoxic concentrations of cisplatin and iproplatin. The results presented are the means ± SD from two separate experiments with two to four replicates per treatment. The time 'zero' controls are the same for all the treatments

Time of treatment (h)	Cellular GSH (nmol/mg protein)				
	No drug	Cisplatin (μM)		Iproplatin (μM)	
		2	5	25	50
0	90.80 ± 6.10	–	–	–	–
3	76.98 ± 8.98	75.07 ± 7.99	75.93 ± 5.96	82.56 ± 5.90	80.9 ± 14.0
6	76.07 ± 6.90	74.73 ± 3.08	74.07 ± 2.84	77.41 ± 5.85	82.82 ± 3.89

70% and 80% (Table 2). At equimolar concentrations of the platinum agents (30 μ*M*), the accumulation of platinum from iproplatin was about 40% less than that from cisplatin. However, no effect of GSH depletion was noted on platinum accumulation for either of the platinum complexes.

Discussion

The results presented here confirm our previous observation [32] that GSH affects the cytotoxicity of iproplatin to a greater extent than that of cisplatin. Depletion of GSH with BSO potentiated the cytotoxicity of iproplatin severalfold more than the cytotoxicity of cisplatin. The DMF achieved for iproplatin (at the IC_{90}) was >7, but for cisplatin was <2 at all BSO concentrations. These results are consistent with two previous reports indicating that BSO has a greater impact on the cytotoxicity of iproplatin than that of cisplatin [27, 37].

Our previous in vitro studies have shown that iproplatin is quite nonreactive. It does not bind to proteins and DNA, whereas its divalent metabolite *cis*-dichloro bis-isopropylamine (CIP) readily binds [30, 31]. The DNA binding of CIP in vitro is inhibited by GSH [31]. Iproplatin exists in the cells predominantly in the platinum (II) form [29, 30]. Thus our previous work with iproplatin suggests that the GSH-reactive species in the case of iproplatin is its active metabolite CIP, most likely in its aquated form [31]. The reported reaction half-time for iproplatin and GSH in vitro is approximately 63 h and that for cisplatin 2.8 h [6]. Neither the reaction rates between CIP and GSH nor those beween cisplatin metabolites and GSH are known.

In order to determine whether the observed differential effects of GSH depletion on cytotoxicities of cisplatin and iproplatin were a consequence of the differences in the ability of these drugs to undergo intracellular conjugation reactions with GSH, the effects of these agents on the cellular GSH levels were quantitated. It is well established that exposure of cells to agents that react with the thiol groups of GSH [such as diethylmaleate or phorone (diisopropylidine acetone)] results in cellular depletion of GSH [26]. Similar depletion of cellular GSH has been reported for ifosphamide metabolites [20]. If there is greater reactivity between GSH and iproplatin metabolites in the cells than between GSH and cisplatin, one would expect greater depletion of intracellular GSH after iproplatin exposure than after cisplatin exposure. However, neither compound was found to cause significant lowering of cellular GSH. The small decline (10–15%) in GSH in both treated and control cells most probably reflected the normal GSH efflux that has been described for many cells [26].

Depletion of GSH has been reported to affect the accumulation of cisplatin and carboplatin, but not tetraplatin in some cell lines [28]. Formation of GSH–Pt conjugates followed by their export from cells has been suggested as a possible basis for decreased accumulation of platinum in other studies [17]. Our experiments, carried out to evaluate whether BSO depletion of GSH has an affect on the accumulation of cisplatin and iproplatin, indicated no differences for these two platinum complexes in the SK-MEL-2 cells.

Our results suggest that the observed differential effect of GSH on the cytotoxicity of cisplatin and iproplatin was not a consequence of the differences in the conjugation reactions between each of the platinum agents and GSH, but possibly resulted from other factors. Depletion of GSH has been shown to be associated with a marked increase in DNA interstrand crosslink formation in a human carcinoma cell line [35]. Similar increases in DNA interstrand crosslinks have also been seen in vivo in cisplatin-resistant rat ovarian tumors depleted of GSH by BSO treatment [7]. Depletion of GSH by BSO inhibited DNA repair as measured by unscheduled DNA synthesis in an ovarian carcinoma cell line [19]. Replenishment of GSH in BSO-treated cells with GSH monoethyl ester resulted in a complete recovery of DNA repair activity [19]. GSH may affect the activities of DNA repair enzymes [1, 33]. Depletion of GSH with BSO resulted in decreased activities for DNA polymerases and ligases in a human astrocytoma cell line [1, 33]. GSH may also have a role in modulating the mode of cell death following toxic injury [11]. The observation that GSH-depleted leukemia cells undergo necrosis when exposed to melphalan, while non-GSH-depleted cells undergo apoptosis supports this hypothesis [11]. In addition, GSH modulates the activity of the transcription factor NFkB [38, 39] and modulates Fos/Jun induction [5]. Finally GSH is an antioxidant and a scavenger of free radicals [8]. Whether any or all of these mechanisms contribute to the observed differences in the cytotoxicities of iproplatin and cisplatin remains to be determined.

It is apparent from the resuls presented here that clear differences exist between the platinum complexes in their response to cellular GSH concentrations. Although iproplatin, a clinically active agent [12, 21, 23], is no longer undergoing development, it was used as a model in the present studies to explore the differences in the effect of GSH depletion, because in our previous studies cellular GSH showed the best correlation with the IC_{50} of iproplatin [32]. An assessment of the influence of GSH on the activities of other platinum complexes in clinical development is warranted, to better define the complexes whose activity may be potentiated through appropriate modulations of GSH.

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