

# Modulation of Cisplatin Cytotoxicity and Cisplatin-Induced DNA Cross-Links in HepG2 Cells by Regulation of Glutathione-Related Mechanisms

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## ABSTRACT

Glutathione (GSH), glutathione S-transferase (GST), and glutathione conjugate export pump (GS-X pump) have been shown to participate collectively in the detoxification of many anticancer drugs, including cisplatin. Identification and regulation of the rate-limiting step in the overall system for cisplatin detoxification is of crucial importance for sensitization of human tumor cells to cisplatin. In this study, the GSH content, GST activity, and GS-X pump activity were regulated separately to examine effects of the regulation on cisplatin cytotoxicity and cisplatin-induced DNA interstrand cross-links (ICL) in HepG2 cells. Seventy-percent depletion of GSH by buthionine sulfoximine (BSO) and 50% increase of GSH by monoethyl GSH ester (GSHe) potentiated and decreased cisplatin cytotoxicity, respectively. This was reflected by a significant decrease and

increase of their respective  $IC_{50}$  values by 62 and 107%. Cisplatin-induced ICL was also potentiated by depletion of GSH by BSO and decreased by enrichment of GSH by GSHe, as shown by a 125% increase and a 34% decrease of cross-linked DNA compared with control samples exposed to cisplatin alone ( $p = 0.008$  and  $0.03$ , respectively). On the other hand, inhibition of GST and GS-X pump by ethacrynic acid, quercetin, tannic acid, and indomethacin at concentrations that inhibited activities of GST and GS-X pump by more than 50% had no significant effects on cisplatin cytotoxicity and cisplatin-induced DNA ICL in these cells. The results showed that of the parameters measured, intracellular GSH seems to be the rate-limiting factor, and its regulation would provide a more promising strategy for sensitization of human liver tumor cells to cisplatin.

Cisplatin has been used widely as a chemotherapeutic drug for a variety of malignancies including hepatocellular carcinoma (Go and Adjei, 1999; Leung et al., 1999). However, cellular drug resistance compromised its clinical effectiveness. Potential biochemical and molecular mechanisms of cisplatin resistance have been proposed (Perez, 1998). Glutathione (GSH), glutathione-related enzymes, and the glutathione conjugate export pump (GS-X pump) have been shown to participate in the detoxification of many anticancer drugs, including cisplatin (Tew, 1994; Commandeur et al., 1995; Zhang et al., 1998). GSH could combine with cisplatin to form a less toxic and more water-soluble glutathione conjugate, bis-(glutathionato)-platinum. Export of the GSH conjugate out of tumor cells by GS-X pump, also known as the multidrug-resistance associated protein (MRP) represents the final elimination or "phase III" of the overall detoxification system (Ishikawa, 1992; Ishikawa and Ali-Osman, 1993).

Increased intracellular GSH (Godwin et al., 1992; Iida et al., 1999) and over-expression of GST and GS-X pump (Ishikawa et al., 1994; Bai et al., 1996; Cui et al., 1999) have been correlated closely with cisplatin resistance in tumor cells. Modulation of the GSH content (Meijer et al., 1992; Hansson et al., 1996) and GST activity (Awasthi et al., 1994) has been shown to affect cisplatin cytotoxicity in human tumor cells. Likewise, modulation of the GS-X pump also changed the sensitivity of tumor cells to other anticancer drugs, which are substrates of glutathione-related detoxification mechanisms (Wijnholds et al., 1997; Zhang and Wong, 1997). Because glutathione conjugation of cisplatin and the subsequent export of its conjugate represent a concerted detoxification system, this study attempts to identify the rate-limiting step of this multistep system. Earlier studies to regulate GSH, GST, and GS-X pump with a view of modulating cisplatin sensitivity were made by different groups (Hansson et al., 1991; Meijer et al., 1992; Awasthi et al., 1994; Wijnholds et al., 1997; Zhang and Wong, 1997). A more efficient modulation

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**ABBREVIATIONS:** GSH, glutathione; GS-X pump, glutathione conjugate export pump; MRP, multidrug-resistance associated protein; GST, glutathione S-transferase; ICL, interstrand cross-links; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; BSO, buthionine sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; EB, ethidium bromide; GSHe, monoethyl glutathione ester; DNP-SG, dinitrophenylglutathione; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; AAS, atomic absorption spectrometry.

could conceivably be achieved by targeting the rate-limiting step. In this study, each component of the glutathione-related system, namely the GSH content, GST, and GS-X pump activities, was regulated respectively to examine effects of the regulation on cisplatin cytotoxicity and cisplatin-induced DNA interstrand cross links (ICL) in HepG2 cells. By comparing the effects of regulation of GSH content, GST, and GS-X pump on cisplatin cytotoxicity and cisplatin-induced DNA cross links, it was hoped that the parameters likely to exert a significant influence could be identified. This would provide a strategy for a more efficacious sensitization of cisplatin-resistant tumor cells. In addition, cisplatin accumulation, DNA platination, and cisplatin efflux were also studied.

## Materials and Methods

**Chemicals.** Cisplatin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethacrynic acid, indomethacin, buthionine sulfoximine (BSO), GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and ethidium bromide (EB) were obtained from Sigma Chemical Co (St. Louis, MO). Tannic acid, butein, and quercetin were purchased from Extrasynthesis (Genay, France). GSH colorimetric assay kit and GSH monoethyl ester (GSHe) were obtained from Calbiochem (La Jolla, CA). QIAGEN genomic DNA Maxi kits were obtained from QIAGEN GmbH (Hilden, Germany) and PicoGreen double-stranded DNA quantification reagents were obtained from Molecular Probes (Eugene, OR). Media and sera for cell culture were purchased from Life Technologies (Grand Island, NY). All other chemicals were of analytical grade from standard commercial suppliers.

**Cell Culture.** Human liver tumor cells, HepG2, were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in minimal essential medium containing 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10% fetal calf serum. Cells were maintained in a humidified atmosphere of air/CO<sub>2</sub> (19/1) and were subcultured every 2 to 3 days.

**Assay of Cytotoxicity.** Approximately  $2 \times 10^4$  cells were seeded in each well of 96-well tissue culture plates and incubated in a CO<sub>2</sub> incubator for 24 h. Fresh stock solutions of cisplatin were prepared in dimethyl sulfoxide and aliquots of the stock solutions were added to wells of the plates at desired concentrations. After 18-h incubation with cisplatin, surviving cells in wells were determined by the MTT assay (Hanson et al., 1989). Twenty-five microliters of a 5 mg/ml stock solution of MTT were added to each well, and after 2 h incubation at 37°C, 100  $\mu$ l of the extraction buffer [20% SDS (w/v) in 50% *N,N*-dimethyl formamide (v/v), 2.5% of 80% acetic acid, and 2.5% of 1N HCl, pH 4.7] were added. After an overnight incubation at 37°C, absorbance was measured at 570 nm using a microplate reader (Dynatech Laboratories, Chantilly, VA). For studies of effects of the modulators of glutathione-related factors on cisplatin cytotoxicity, BSO (at a final concentration of 0.5 mM) and GSHe (of a final concentration of 5 mM) were added 20 h and 4 h, respectively, before addition of cisplatin. Ethacrynic acid, quercetin, indomethacin, tannic acid, and butein were each added 1 h before addition of cisplatin; their final concentrations were 10 to 40  $\mu$ M. The cell viability was calculated as the ratio of absorbance in wells containing cisplatin and cisplatin plus modulators compared with control cells. IC<sub>50</sub> values were calculated from a linear regression from dose-dependent curves of at least five points.

**Measurement of Cisplatin-Induced DNA ICL.** DNA cross-links in cisplatin-treated HepG2 cells were measured according to a method reported previously (De Jong et al., 1986). Cells ( $0.5 \times 10^6$ ) were plated in each well of six-well plates and incubated in a CO<sub>2</sub> incubator for 24 h. Cisplatin was added to the wells at a final concentration of 200  $\mu$ M and incubated at 37°C for 6 h. BSO at a final

concentration of 0.5 mM was added 20 h before addition of cisplatin; GSHe (of final concentration of 5 mM) was added 4 h before addition of cisplatin. Ethacrynic acid, quercetin, indomethacin, tannic acid, and butein, each with a final concentration of 40  $\mu$ M, were added 1 h before addition of cisplatin. Cells were then collected by trypsinization and resuspended in 200  $\mu$ l of lysing buffer [4 M NaCl, 50 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, and 0.1% (w/v) Sarkosyl, pH 7.2]. Lysis was carried out at 37°C for 16 h. After lysing, 5  $\mu$ l of heparin (2500 U/ml) was added for 20 min at 37°C. Thereafter, 3 ml of EB solution (10  $\mu$ g/ml EB, 20 mM K<sub>2</sub>HPO<sub>4</sub>, and 0.4 mM EDTA, pH 12.0) were added to the lysates. DNA was then denatured by heating to 100°C for 5 min and cooled rapidly to room temperature. The samples were kept in the dark and intensities of fluorescence were measured by a spectrofluorometer (PerkinElmer LS 50 B). The excitation and emission wavelengths were 525 nm and 580 nm, respectively. The percentage of cross-linked DNA was determined by measuring the difference in fluorescence intensity of denatured cell lysates of controls and drug-treated samples by the following formula: interstrand cross-links (%) =  $(F_t - F_n) / (1 - F_n) \times 100\%$ .  $F_t$  and  $F_n$  are fluorescence fractions after heat denaturation in treated cells and control cells, respectively.  $F_t$  and  $F_n$  were calculated as the ratio of fluorescence in samples after heat denaturation and without heat denaturation.

**Measurement of the Activity of GS-X Pump.** The cells were cultured on six-well plates at a density of  $1.0 \times 10^6$  cells/well/2 ml in a CO<sub>2</sub> incubator for 24 h. They were then incubated with 1 mM CDNB at 10°C for 30 min for loading of dinitrophenylglutathione (DNP-SG). Formation of DNP-SG inside human tumor cells was identified in our previous study (Zhang and Wong, 1996). Cells in the wells were washed three times with 1 ml Hanks' balanced salt solution (HBSS) and then incubated in HBSS at 37°C up to 60 min. DNP-SG exported to extracellular HBSS was quantified at intervals up to 60 min by the HPLC-UV method as described previously (Zhang and Wong, 1996). Extracellular HBSS (0.5 ml) was passed through a 0.45- $\mu$ m filter and 100  $\mu$ l of the filtrate was injected for HPLC-UV analysis using an HP 1100 liquid chromatographic system (Hewlett Packard, Palo Alto, CA) connected to a variable wavelength detector, set at 340 nm. The column (100  $\times$  4.6 mm) was packed with C<sub>18</sub> Hypersil ODS (Hewlett Packard) and elution was carried out with a gradient of 40 to 90% (v/v) methanol in 0.01 M sodium phosphate, pH 6.0, for 6 min, following the solvent system reported previously (Oude Elferink et al., 1993). The flow rate was 1 ml/min. The peak representing DNP-SG at the retention time of 2.1 min was quantified by comparing with DNP-SG standards formed from 1 to 40 nmol of CDNB/ml in the presence of excess GSH and rat liver GSTs purified by *S*-hexylglutathione affinity chromatography (Zhang and Das, 1994). Activity of the GS-X pump, which is a measurement of the rate of efflux of DNP-SG from the human tumor cells, was calculated and expressed as nanomoles per 10<sup>6</sup> cells at 37°C.

**Assay of GST Activity.** HepG2 cells were plated in each well of six-well plates and incubated in a CO<sub>2</sub> incubator for 24 h. The cells were then incubated with ethacrynic acid and quercetin at final concentrations ranging from 5 to 40  $\mu$ M for 1 h. The cells were then harvested, sonicated, and centrifuged at 10,000g for 20 min at 4°C. The supernatants were used for analysis of GST activity according to the method of Habig et al. (1974). The reaction mixture contained 125 mM potassium phosphate buffer, pH 6.5, 1 mM GSH, and 1 mM CDNB. The reaction was started by the addition of GST samples prepared as described above. The rate of formation of the CDNB conjugate with GSH was determined spectrophotometrically by monitoring the absorbance at 340 nm. The GST activities were expressed as nmol of DNP-SG formed/min per mg of protein at 37°C, taking the extinction coefficient as 9.6 mM<sup>-1</sup> cm<sup>-1</sup> (Habig et al., 1974).

**Assay of GSH Content.** The GSH contents of the human tumor cells were determined by a colorimetric GSH assay kit as described previously (Zhang and Wong, 1996). In this assay system, the first step leads to the formation of thioethers between chromogenic re-

agents with all mercaptans; 30% NaOH then specifically transforms the substitution product obtained from GSH into chromophoric thione with a maximal absorbance at 400 nm. After incubation with the modulators (0.5 mM and 1.0 mM BSO for 20 h, 5 mM GSHe for 4 h, and 40  $\mu$ M ethacrynic acid, quercetin, tannic acid, butein, and indomethacin for 1 h), approximately  $2 \times 10^6$  cells were harvested in 0.2 ml of PBS by trypsinization and scraping with a rubber policeman. Cells were then disrupted by a Polytron homogenizer (IKA Labortechnik, Staufen, Germany) and centrifuged at 105,000g for 45 min at 4°C. 100  $\mu$ l of the supernatant fraction was added to 800  $\mu$ l of buffer (200 mM potassium phosphate, pH 7.8, containing 0.2 mM diethylenetriamine pentaacetic acid and 0.025% lubrol) and then 50  $\mu$ l of reagent A (0.012 M chromogenic reagent in 0.2 M HCl) and 50  $\mu$ l of reagent B (30% aqueous sodium hydroxide) were added sequentially. The reaction mixture was incubated at ambient temperature for 10 min and the absorbance was measured at 400 nm. GSH contents of the cells were expressed as  $\mu$ g/ $10^6$  cells.

**Cisplatin Accumulation.** Cell-associated platinum was measured in total cell extracts by atomic absorption spectrometry (AAS). Cells ( $1.0 \times 10^6$ ) were plated in each well of six-well plates and incubated at 37°C overnight. Duplicate wells containing subconfluent, exponentially growing cells were treated with 200  $\mu$ M cisplatin for 4 h at 37°C. The cells were then washed twice with PBS, harvested by trypsinization, and sonicated. Duplicate aliquots were analyzed directly by AAS using a PerkinElmer Zeeman/3030 spectrometer. Platinum absorption at 265.9 nm was monitored using an element lamp of current of 10 mA. Platinum was quantified using a standard curve from elemental platinum. Cellular platinum level was expressed as nanograms of Pt per  $10^6$  cells.

**Total DNA Platination.** Each group of drug-treated and control cells ( $1 \times 10^7$ ) was harvested by trypsinization and used for DNA isolation by a QIAGEN genomic DNA Maxi kit. DNA resuspended in Tris-EDTA buffer was quantified by PicoGreen dsDNA quantification reagents and calculated from a standard curve of Lambda DNA. Platinum content was determined by AAS as described above and expressed as nanograms of Pt per microgram of DNA.

**Measurement of Cisplatin Efflux.** Cells ( $0.8 \times 10^6$ ) were plated in each well of six-well plates and incubated at 37°C overnight. Cisplatin was then added to a final concentration of 200  $\mu$ M and incubation was carried out at 37°C for 2 h. The medium in wells was replaced by HBSS after two washes with PBS. Cells were further incubated in HBSS for 1 h at 37°C. For studies of modulators of glutathione-related mechanisms on cisplatin efflux by HepG2 cells, BSO (0.5 mM), and GSHe (5 mM) were added separately 20 h and 4 h, respectively, before the addition of cisplatin; the final concentrations are given in parentheses. Likewise, ethacrynic acid, quercetin, indomethacin, tannic acid, and butein (final concentration, 40  $\mu$ M) were added 1 h before the addition of cisplatin. The platinum content in the HBSS was determined by the AAS as described above. The efflux of cisplatin was calculated from this measurement and the value was expressed as nanograms of cisplatin per hour per  $10^6$  cells.

**Protein Determination.** The protein contents in samples were measured by the method of Bradford et al. (1976) using the Bio-Rad reagent with bovine serum albumin as a standard.

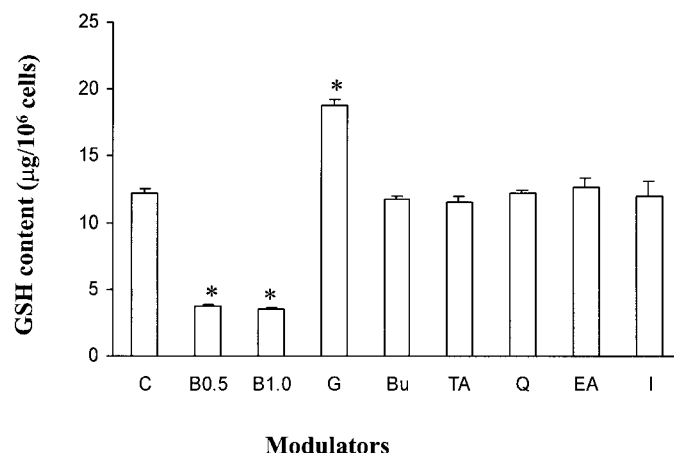
## Results

**Effects of Modulators of GSH Content, GST, and GS-X Pump on Cisplatin Cytotoxicity in Human Liver Tumor Cells.** BSO, which is a specific inhibitor of GSH synthesis (Griffith, 1982), was used to deplete GSH. GSHe, which has been shown to increase effectively intracellular GSH levels (Versantvoort et al., 1995), was used to enrich GSH in HepG2 cells. As shown in Fig. 1, incubation with 0.5 mM BSO for 20 h depleted GSH by 70%. Conversely, incubation with 5 mM GSHe for 4 h increased GSH by 50%. To observe the effect of intracellular GSH content on cisplatin

cytotoxicity, the cells were incubated with BSO and GSHe before addition of cisplatin. As shown in Fig. 2, cisplatin between 12.5 and 150  $\mu$ M showed a dose-dependent cytotoxicity on the cells with an  $IC_{50}$  value of 42.8  $\mu$ M. BSO potentiated the cytotoxicity of cisplatin significantly as reflected in an  $IC_{50}$  value of 16.2  $\mu$ M. On the other hand, GSHe decreased cisplatin cytotoxicity with a significant change in  $IC_{50}$  value to 88.7  $\mu$ M. Measured at 25  $\mu$ M cisplatin, cytotoxicity was potentiated by BSO resulting in a 76% decrease in cell survival, whereas GSHe increased cell viability by 52% compared with controls exposed to only cisplatin. Incubation with BSO or GSHe alone had no significant action on the survival of the cells compared with control cells. These results suggested that the GSH content is a determinant of cisplatin sensitivity in human liver tumor cells.

Ethacrynic acid, a proven GST inhibitor, has been shown to potentiate the cytotoxicity of melphalan and chlorambucil, which are substrates of glutathione conjugation (Tew et al., 1988; Hansson et al., 1991). In our laboratory, quercetin was also demonstrated to be a potent GST inhibitor and it could sensitize human colon tumor cells to chlorambucil (Zhang and Das, 1994; Zhang and Wong, 1997). To examine the effect of ethacrynic acid and quercetin on the GST activity of tumor cells subjected to cisplatin cytotoxicity, the cells were incubated with these compounds for 1 h before exposure to cisplatin. They were then coincubated with cisplatin. As shown in Table 1, the cellular GST activity was inhibited dose dependently by incubation with ethacrynic acid and quercetin with  $IC_{50}$  values of 14.4  $\mu$ M and 17.5  $\mu$ M, respectively. However, incubation of the cells with these inhibitors at the concentrations which inhibit more than 50% of the GST activity had no significant effect on cisplatin cytotoxicity (Table 2). These results suggested that GST is not a rate-limiting factor of the system for cisplatin detoxification.

The phase "III" detoxification of cisplatin by the glutathione-related detoxification system composed of the efflux of the glutathione conjugate of cisplatin by the GS-X pump (Ishikawa, 1992; Ishikawa and Ali-Osman, 1993). Export of the glutathione conjugate of cisplatin may represent an im-



**Fig. 1.** Effects of modulators of GSH-related factors on cellular GSH of human liver tumor cells. Cells were incubated with modulators (C, Control; B0.5, 0.5 mM BSO; B1.0, 1 mM BSO; G, 5 mM GSHe; Bu, 40  $\mu$ M butein; TA, 40  $\mu$ M tannic acid; Q, 40  $\mu$ M quercetin; EA, 40  $\mu$ M ethacrynic acid; I, 40  $\mu$ M indomethacin). Intracellular GSH was measured as described under *Materials and Methods*. Values are means ( $\pm$ S.D.) of two independent experiments carried out in duplicate. \*Compared with control  $P < 0.01$  (Student's  $t$  test)

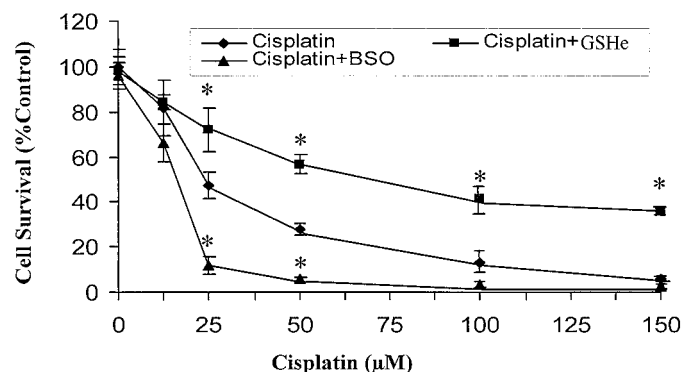


portant mechanism, which reduces the intracellular accumulation of platinum (Ishikawa and Ali-Osman, 1993). Indomethacin has been reported as an inhibitor of GS-X pump and it has been shown to sensitize human MRP overexpressing drug-resistant tumor cells to doxorubicin and vincristine (Draper et al., 1997). Our data also showed that tannic acid and butein, which inhibited the GS-X pump, could sensitize human tumor cells to chlorambucil (Zhang and Wong, 1996,

1997). In the present study, these inhibitors were examined for their effects on the sensitivity of HepG2 cells to cisplatin. As shown in Fig. 3, indomethacin, tannic acid, and butein inhibited the activity of GS-X pump in the human liver tumor cells significantly when measured with dinitrophenylglutathione (DNP-SG), which is a classic substrate of GS-X pump (Ishikawa, 1992). However, incubation of HepG2 cells with indomethacin and tannic acid before and coincubated with cisplatin had no significant effect on cisplatin cytotoxicity (Table 2). These results suggested that in HepG 2 cells, export of the glutathione conjugates of cisplatin may not be a determining or a rate-limiting reaction for the detoxification of cisplatin. Butein may potentiate cisplatin cytotoxicity by a mechanism other than inhibition of GS-X pump.

**Effects of Modulators of GSH Content, GST and GS-X Pump on Cisplatin-Induced DNA Interstrand Cross-Links.** DNA is the most critical target for antitumor action of cisplatin. Among the DNA lesions induced by cisplatin, DNA ICL are likely to have the most severe consequences for the cells. The formation of DNA ICL has been correlated with cisplatin cytotoxicity (Zhen et al., 1992). Therefore, cisplatin-induced DNA ICL was determined in this study to observe the effects of modulators of glutathione-related mechanisms on this DNA lesion. As shown in Fig. 4, cisplatin-induced DNA ICL was potentiated by depletion of GSH by BSO and decreased by enrichment of GSH by GSHe; these were reflected by a 125% increase and a 34% decrease of cross-linked DNA compared with control cells ( $P = 0.008$  and  $0.03$  respectively). On the other hand, inhibitors of GST and GS-X pump (namely ethacrynic acid, quercetin, indomethacin, butein, and tannic acid) had no significant effect on cisplatin-induced DNA ICL ( $P = 0.14$ – $0.96$ ). This result suggested that modulation of cisplatin cytotoxicity by regulation of GSH content was wholly or partially caused by changes in the formation of DNA ICL.

**Accumulation of Cellular Cisplatin, Total DNA Platination and Cisplatin Efflux.** Cisplatin accumulation, DNA platination, and cisplatin efflux were determined to assess whether changes of these parameters were involved in the modulation of cisplatin cytotoxicity by depletion and enrichment of GSH. As shown in Table 3, depletion and enrichment GSH increased and decreased cisplatin accumulation by 40% and 54%, respectively ( $P = 0.01$  and  $0.001$ , respectively). DNA platination was affected by changes of GSH content with a significant 168% increase of DNA platination by BSO and a 64% decrease of DNA platination by GSHe



**Fig. 2.** Effects of BSO and GSHe on cisplatin cytotoxicity on human liver tumor cells. Cells were incubated with 0.5 mM BSO for 20 h and with 5 mM GSHe for 4 h before addition of cisplatin. After incubation with various concentrations of cisplatin for 18 h, cell viability was determined by the MTT method as described under *Materials and Methods*. Values are means ( $\pm$ S.D.) of four experiments. \*Compared with control  $P < 0.01$  (Student's  $t$  test)

**TABLE 1**

Inhibition of GST activity of cultured HepG2 cells by ethacrynic acid and quercetin

HepG2 cells were plated in six well plates ( $7 \times 10^5$  cells/well/3 ml of medium) and incubated at  $37^\circ\text{C}$  overnight. Ethacrynic acid and quercetin were then added to wells at various concentrations for 1 h. After incubation with the inhibitors, cells were collected by trypsinization and homogenized by ultrasonication. The homogenate was centrifuged at  $10,000g$  for 20 min and the supernatants were used for GST activity analysis as described under *Materials and Methods*. GST activity was expressed as nanomoles of dinitrophenylglutathione formed per minute per milligram of cellular protein at  $37^\circ\text{C}$ .

Inhibitor Concentration	GST Activity	
	Ethacrynic Acid	Quercetin
	nmol/min/mg (% inhibition)	
Control	$75.8 \pm 2.8$ (0)	$75.8 \pm 2.8$ (0)
5 $\mu\text{M}$	$62.7 \pm 5.9$ (17.3)	$65.9 \pm 0.9$ (13.1)
10 $\mu\text{M}$	$39.3 \pm 2.5$ (48.2)	$48.2 \pm 5.7$ (36.4)
20 $\mu\text{M}$	$31.4 \pm 0.8$ (59.6)	$36.1 \pm 3.9$ (52.4)
30 $\mu\text{M}$	$28.2 \pm 1.3$ (62.8)	$31.3 \pm 0.9$ (58.8)
40 $\mu\text{M}$	$22.9 \pm 5.0$ (69.8)	$26.4 \pm 3.3$ (65.2)

**TABLE 2**

Effects of inhibitors of GST and GS-X pump on cisplatin cytotoxicity on HepG2 cells

Cells were preincubated with the inhibitors and then coincubated with the inhibitors and cisplatin and cell survival was determined as described under *Materials and Methods*.

Cisplatin	Cell Survival					
	Control	10 $\mu\text{M}$ Bu	20 $\mu\text{M}$ TA	20 $\mu\text{M}$ Q	40 $\mu\text{M}$ EA	40 $\mu\text{M}$ I
	% control					
Control	$100 \pm 7.4$	$96.2 \pm 5.3$	$100.4 \pm 0.5$	$99.7 \pm 3.6$	$100 \pm 5.6$	$97.5 \pm 0.5$
6.25 $\mu\text{M}$	$94.1 \pm 2.7$	$95.1 \pm 5.4$	$98.6 \pm 1.2$	$92.2 \pm 4.3$	$98.1 \pm 1.4$	$99.5 \pm 7.8$
12.5 $\mu\text{M}$	$81.1 \pm 6.4^a$	$82.3 \pm 2.0$	$87.7 \pm 1.7$	$88.6 \pm 2.0$	$87.9 \pm 1.9$	$87.2 \pm 2.9$
25.0 $\mu\text{M}$	$66.0 \pm 2.0^b$	$55.5 \pm 4.1^c$	$67.6 \pm 0.3$	$68.7 \pm 3.2$	$64.0 \pm 1.2$	$68.1 \pm 1.0$
50.0 $\mu\text{M}$	$43.5 \pm 2.2^b$	$33.3 \pm 0.9^d$	$42.0 \pm 1.1$	$42.8 \pm 0.1$	$39.9 \pm 4.4$	$41.2 \pm 2.0$
100.0 $\mu\text{M}$	$24.8 \pm 4.3^b$	$17.8 \pm 2.8^d$	$26.4 \pm 1.8$	$26.2 \pm 1.0$	$27.4 \pm 1.8$	$27.9 \pm 1.4$

Bu, butein; TA, tannic acid; Q, quercetin; EA, ethacrynic acid; I, indomethacin.

<sup>a</sup> Compared with control without cisplatin  $P < 0.05$ , <sup>b</sup> Compared with control without cisplatin  $P < 0.01$  (student's  $t$  test) <sup>c</sup> Compared with control without modulators  $P < 0.05$ , <sup>d</sup> Compared with control without modulators  $P < 0.01$  (student's  $t$  test)

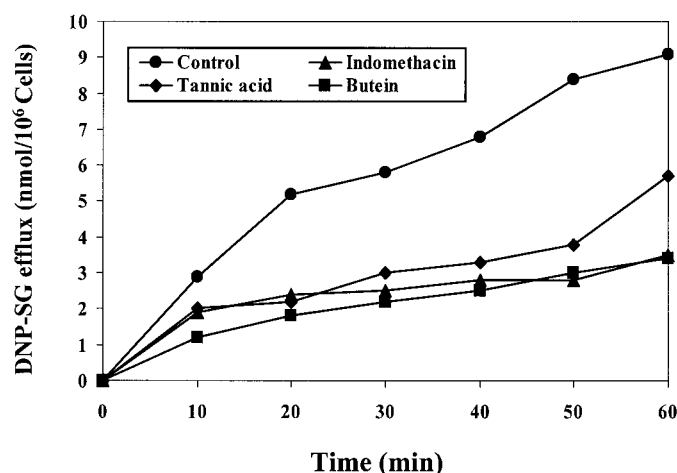
( $P = 0.0001$  and  $0.0008$  respectively). Depletion and enrichment of GSH also affected cisplatin efflux significantly, with a 34% decrease and a 57% increase of cisplatin efflux ( $P = 0.01$  and  $0.03$ ), respectively. This contributed at least partially to changes of cisplatin accumulation and DNA platina-

tion. Cisplatin accumulation, DNA platination, and cisplatin efflux were not affected significantly by inhibitors of GST and GS-X pump.

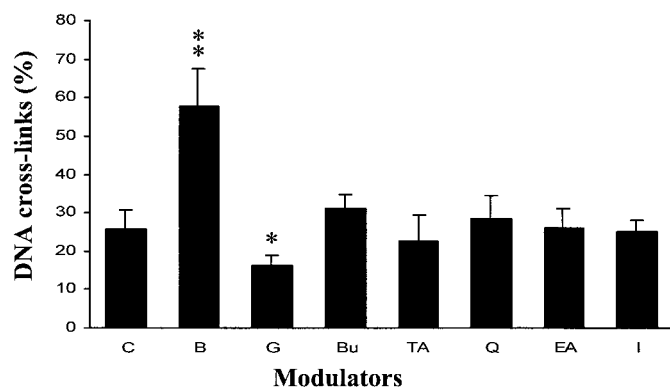
## Discussion

Increased detoxification of cisplatin by glutathione-related reactions is an important biochemical mechanism of cisplatin resistance. Identification of chemosensitizers modulating glutathione-associated cisplatin resistance would be valuable in developing new strategies to overcome drug resistance. This study aimed to identify the rate-limiting step(s) in this GSH-related multistep system. Depletion of GSH by BSO and enrichment of GSH by GSHe could potentiate and decrease cisplatin cytotoxicity, respectively, in human liver tumor cells (Fig. 2). These results concurred with previous findings that intracellular GSH affected the sensitivity of human tumor cells to cisplatin (Meijer et al., 1992) and to other anticancer drugs such as 1,3-bis(2-chloroethyl)-nitrosourea (BCNU) (Ali-Osman et al., 1996) and etoposide (Schneider et al., 1995). On the other hand, inhibitors of GST and GS-X pump had no significant effects.

In our previous study, inhibitors of GST and GS-X pump at low and nontoxic concentrations could sensitize human colon tumor cells to chlorambucil (Zhang and Wong, 1997). Cytotoxicities of a variety of anticancer drugs that are substrates of glutathione conjugation and GS-X pump were potentiated by inhibition of GST and GS-X pump in a number of studies (Tew et al., 1988; Hansson et al., 1991; Wijnholds et al., 1997; Zhang and Wong, 1997; Zhang et al., 1998). It has been well established that cisplatin is detoxified by conjugation with GSH followed by the export of the conjugate by the GS-X pump (Ishikawa and Ali-Osman, 1993). However, in the present study, inhibitors of GST such as ethacrynic acid (Tew et al., 1988) and quercetin (Zhang and Das, 1994; Zhang and Wong, 1997) and inhibitors of the GS-X pump, namely indomethacin (Draper et al., 1997), butein, and tannic acid (Zhang and Wong, 1996), had no significant effects on cisplatin cytotoxicity in human liver tumor cells (Table 2). These compounds were present at concentrations which inhibited GST activity and the GS-X pump by more than 50% (Table 1 and Fig. 3). Transfection of GSH conjugate transport protein (MRP) could induce resistance of HeLa cells and human ovarian cancer cell lines to doxorubicin and vincristine but not to cisplatin (Grant et al., 1994; Sharp et al., 1998). In human lung cancer cell lines, coinubation of a group of nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac and tolmetin could potentiate the cytotoxicity of doxo-



**Fig. 3.** Inhibitory effect of indomethacin, tannic acid, and butein on GS-X pump activity in human liver tumor cells. Cells were preincubated with  $40 \mu\text{M}$  each of the inhibitors for 1 h and GS-X pump activity was measured by an high-performance chromatography method as described under *Materials and Methods*. GS-X pump activity was expressed as efflux of DNP-SG from the cells (nanomoles/ $10^6$  cells).



**Fig. 4.** Effects of modulators on cisplatin-induced DNA ICL in human liver tumor cells. Cells were preincubated with the modulators and then coincubated with the modulators and cisplatin. DNA ICL was measured by a fluorescent method as described under *Materials and Methods*. Modulators: C, control; B,  $0.5 \text{ mM}$  BSO; G,  $5 \text{ mM}$  GSHe; Bu,  $40 \mu\text{M}$  butein; TA,  $40 \mu\text{M}$  tannic acid; Q,  $40 \mu\text{M}$  quercetin; EA,  $40 \mu\text{M}$  ethacrynic acid; I,  $40 \mu\text{M}$  indomethacin. Values are means ( $\pm$ S.D.) of at least three independent experiments. \*Compared with control  $P < 0.05$ ; \*\*Compared with control  $P < 0.01$  (Student's  $t$  test)

TABLE 3

Effects of the modulators on cisplatin accumulation, cisplatin efflux, and DNA platination in human liver tumor cells treated with cisplatin. Cells were preincubated with the  $0.5 \text{ mM}$  BSO for 20 h,  $5 \text{ mM}$  GSHe for 4 h, and  $40 \mu\text{M}$  butein, tannic acid, quercetin, ethacrynic acid, and indomethacin for 1 h before addition of cisplatin. Cisplatin accumulation, DNA platination, and cisplatin efflux were determined as described under *Materials and Methods*.

Modulators	Cisplatin Accumulation	Cisplatin Efflux	DNA Platination
	ng Pt / $10^6$ cells	ng / h / $10^6$ cells	ng Pt / $\mu\text{g}$ DNA
Control	$917.5 \pm 130.1$	$56.1 \pm 7.1$	$0.63 \pm 0.07$
BSO	$1284.0 \pm 119.8^a$	$37.1 \pm 3.2^a$	$1.69 \pm 0.03^a$
GSHe	$421.1 \pm 20.4^b$	$88.1 \pm 16.1^a$	$0.23 \pm 0.02^b$
Butein	$831.0 \pm 69.3$	$54.4 \pm 9.9$	$0.66 \pm 0.02$
Tannic Acid	$902.5 \pm 90.9$	$48.3 \pm 8.9$	$0.65 \pm 0.01$
Quercetin	$936.0 \pm 97.1$	$48.3 \pm 6.5$	$0.64 \pm 0.02$
Ethacrynic Acid	$948.0 \pm 73.5$	$54.4 \pm 15.5$	$0.63 \pm 0.10$
Indomethacin	$863.0 \pm 25.1$	$52.4 \pm 17.2$	$0.60 \pm 0.02$

<sup>a</sup> Compared with control  $P < 0.05$ ; <sup>b</sup> Compared with control  $P < 0.01$  (student's  $t$  test)

rubicin, VP-16, and vincristine but not of cisplatin. This potentiation was shown by inhibition of GST and GS-X pump (Duffy et al., 1998). Combined expression of MRP and GST P1-1 could not affect the sensitivity of MCF7 cells to cisplatin either (Morrow et al., 1998). These results support our findings and suggest that GSH, GST, and GS-X pump may play different roles for detoxification of various substrates. In this study, 70% inhibition of GST activity by EB and 65% inhibition of the GS-X pump activity by indomethacin did not seem to affect the overall detoxification of cisplatin significantly. In the cells, certain thresholds of GST activity and GS-X pump activity may be needed for detoxification of cisplatin. The residual activities of GST and the GS-X pump may be still beyond the thresholds. In contrast, 70% depletion and 50% increase of intracellular GSH affected cisplatin cytotoxicity on the cells significantly (Fig. 2). Therefore, of the three components, namely GSH, GST and GS-X pump, in the detoxification system, the rate-limiting parameter seemed to be the intracellular GSH content. The only exception was butein, an inhibitor of GS-X pump; it potentiated cisplatin cytotoxicity significantly but it did not affect cisplatin induced DNA ICL (Table 2 and Fig. 4). It is conceivable that butein might potentiate cisplatin cytotoxicity by a mechanism other than inhibition of GS-X pump. Butein was shown to be a potent protein tyrosine kinase inhibitor in our laboratory (Yang et al., 1998). It has also been shown that tyrosine kinase activity is required for the chemoresistant phenotype of non-small-cell lung cancer cells and tyrosine kinase inhibitors could sensitize these cells to chemotherapeutic drugs including cisplatin (Zhang and Hung, 1996). Inhibition of protein tyrosine kinase may be the mechanism for potentiation of cisplatin cytotoxicity by butein.

In this study, the effects of modulators of GSH-related mechanisms on the induction of DNA interstrand cross-links by cisplatin was also investigated, to find out whether there is a relationship between increased DNA damage and reduced drug inactivation. Similar effects were observed on cisplatin-induced DNA ICL and cisplatin cytotoxicity when assayed by the MTT method (Table 2, Figs. 2 and 4). Depletion of GSH by BSO and enrichment of GSH by GSHe increased and decreased DNA ICL significantly, while modulators of GST and GS-X pump had no significant effects on cisplatin-induced DNA ICL. The involvement of DNA ICL in cisplatin action was implicated by this correlation between cisplatin-induced DNA ICL and cytotoxicity. In this study, BSO was preincubated and coincubated with cisplatin. The continuous exposure to BSO aimed to prevent resynthesis of GSH. Continuous exposure to BSO has been shown to increase the level of BCNU-induced DNA ICL and BCNU cytotoxicity compared with pre-exposure to BSO alone (Ali-Osman et al., 1996). One possible consequence of depletion of GSH is increase in DNA ICL because of a reduction in its repair. It has been shown that depletion of GSH by BSO decreased DNA repair synthesis in a resistant ovarian carcinoma cell line (Lai et al., 1989). In our study, DNA platination was measured at an early stage of cisplatin action (1 h), whereas effect of GSH depletion on the synthesis of repair patches by DNA polymerase was presumably a later event (Hansson et al., 1991). Therefore, changes of GSH content would probably affect DNA platination by intervention of cisplatin combination with DNA. The actions of modulators on accumulation of cellular cisplatin, DNA platination and

cisplatin efflux were also studied. As shown in Table 3, cisplatin-induced DNA platination increased to 268% by 70% depletion of cellular GSH and decreased to 36% by 45% enrichment of cellular GSH. Accumulation of cellular cisplatin was increased to 140% by depletion of GSH by BSO and decreased to 46% by enrichment of GSH by GSHe. These changes were of a lower magnitude compared with changes of DNA platination (to 268% and 36%, respectively). This could be explained by an accumulated pool of intracellular GSH conjugate of cisplatin, which could not interact further with DNA. Depletion and enrichment of GSH also decreased and increased cisplatin efflux from the human tumor cells significantly. These results were supported by previous findings that showed that cisplatin was exported from cells in the form of a glutathione conjugate and cisplatin export is GSH-dependent (Ishikawa and Ali-Osman, 1993; Goto et al., 1995). The increase of cellular GSH content by transfection of  $\gamma$ -glutamylcysteine synthetase gene has also been shown to enhance the GS-X pump activity and to decrease cellular cisplatin accumulation (Kurokawa et al., 1995). From these results, it is conceivable that in cisplatin-treated cells, depletion of intracellular GSH by BSO could induce less formation of GSH conjugate of cisplatin. Thus, there was less cisplatin efflux through the GS-X pump. A larger proportion of cisplatin accumulated intracellularly may react with DNA causing increased DNA damages (DNA ICL) leading to potentiation of cisplatin cytotoxicity. On the other hand, enrichment of intracellular GSH by GSHe would have reversed effects.

In summary, glutathione conjugation of anticancer drugs catalyzed by GST and export of the glutathione conjugates represent sequential reactions in an overall multistep detoxification system for the elimination of many anticancer drugs, including cisplatin. GSH, GST, and GS-X pump play different roles in the system for detoxification of various substrates. Of these, intracellular GSH seemed to be the rate-limiting parameter in the detoxification of cisplatin in human liver tumor cells. Modulation of intracellular GSH content affects cisplatin cytotoxicity significantly, whereas inhibition of GST and GS-X pump activity by more than 50% had negligible effects. These results support the clinical approach in depleting GSH for more efficient sensitization of human tumor cells to cisplatin.

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