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Differential Sensitization of Human Ovarian Carcinoma and Mouse L1210 Cells to Cisplatin and Melphalan by Glutathione Depletion

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SUMMARY

We have investigated the role of glutathione in determining the macromolecular binding and cytotoxicity of cisplatin (DDP) and melphalan (LPAM) in human ovarian carcinoma cells and DDP-resistant L1210 mouse leukemia cells. Glutathione reacted avidly with DDP in normal saline with a bimolecular rate constant of 16.2 $M^{-1}hr^{-1}$. Glutathione had no effect on the rate of hydrolysis of LPAM, consistent with the S_N1-like reaction mechanism of LPAM. Glutathione protected calf thymus DNA and bovine serum albumin from DDP platination and LPAM alkylation. Glutathione also protected nuclei isolated from human ovarian carcinoma cells from DDP platination. The importance of intracellular glutathione in determining the cytotoxicity of DDP and LPAM was assessed by depletion of glutathione with buthionine sulfoximine in three cell types. Exposure to 0.5 mM buthionine sulfoximine for 20–28 hr depleted glutathione to levels that were 10–20% of control levels. COLO 316 and 2008 human ovarian carcinoma cells, and ZCR9 mouse leukemia cells were all sensitized to LPAM cytotoxicity by this level of glutathione depletion. The dose modification factors, defined as the IC_{50} control cells/IC_{50} depleted cells, were: 2.6 ± 0.5 for COLO 316 cells, 1.6 ± 0.1 for 2008 cells, and 2.1 ± 1.1 for ZCR9 cells. In contrast, glutathione depletion had a minimal effect on DDP cytotoxicity in these cells with dose modification factors of: 1.2 ± 0.2 for COLO 316 cells, 0.8 ± 0.3 for 2008 cells, and 1.1 ± 0.1 for ZCR9 cells. The differential potentiation of DDP and LPAM cytotoxicity by glutathione depletion in these cells, despite the similar protection that glutathione affords macromolecules from drug binding, suggests that there are fundamental differences in the intracellular interaction of these electrophilic drugs with glutathione.

The principal intracellular nonprotein thiol GSH is involved in a number of biochemical pathways essential to cellular survival, such as detoxification of reactive electrophils, scavenging of free radicals and degradation of peroxides, synthesis of deoxynucleotides, transport of γ-glutamyl-amino acids, and repair of certain types of DNA damage (1–3). The elucidation of the diverse roles of this ubiquitous tripeptide has brought forth selective means for altering its intracellular concentration both *in vitro* and *in vivo* (1). Considering the multiplicity of functions attributable to GSH, it is not surprising that such modulations of GSH levels can have a dramatic effect on the therapeutic efficacy of many cytotoxic treatments, as recently reviewed (4).

Elevated concentrations of intracellular thiols and GSH in particular have been shown to be a component of drug-resistant phenotypes that develop in malignant cells exposed to a variety of electrophilic antitumor drugs such as LPAM (5–8), mechlorethamine (9, 10), methyl-bis-(β-chloroethyl)-amine N-oxide (11), merophan (12), and doxorubicin (Adriamycin) (13, 14). Vistica and co-workers (15, 16) have shown that LPAM-resistant L1210 mouse leukemia cells could be completely resensitized to LPAM by depletion of their elevated GSH levels through either nutritional deprivation of cysteine or exposure to BSO, a specific competitive inhibitor of the first step in GSH synthesis. Green *et al.* (8) and Hamilton *et al.* (14) have recently extended these studies to LPAM, DDP, and Adriamycin-resistant human ovarian carcinoma cells. The fact that BSO appears to be nontoxic *in vivo* means that BSO may have tremendous clinical potential in chemotherapeutic strategies designed for improving antitumor potency at given levels of systemic toxicity or for sensitizing drug-resistant tumor populations.

LPAM and DDP are two of the most important drugs for the treatment of ovarian carcinoma (17, 18). The effectiveness of

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ABBREVIATIONS: GSH, glutathione; DDP, *cis*-diamminedichloroplatinum(II); LPAM, L-phenylalanine mustard; BSO, DL-buthionine-*S*,*R*-sulfoximine; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; FAA, graphite furnace atomic absorption spectrometry; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetate.

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both drugs is limited by toxic side effects and by the rapid appearance of drug resistance in the initially responsive tumor (19, 20). DDP is an electrophil that reacts extensively with cellular nucleophils and generates DNA intrastrand and interstrand crosslinks, the presumed cytotoxic lesions. Like LPAM (21), DDP reacts with thiols such as GSH (22, 23), and one would predict that GSH should play a role in determining cellular sensitivity to DDP by preventing platination of critical loci. We have investigated the ability of two ovarian carcinoma cell lines *in vitro* to be sensitized to LPAM and DDP killing by GSH depletion with BSO. We have also conducted these studies on a DDP-resistant L1210 mouse leukemia line to ascertain whether this DDP-resistant phenotype could be reversed by BSO. To understand better the mechanism involved in sensitization, we also investigated the role GSH plays in preventing the covalent binding of LPAM or DDP to isolated DNA, BSA, and nuclei.

Materials and Methods

Drugs and chemicals. Clinical vials of DDP were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). Vials were reconstituted with sterile water and diluted in sterile 0.9% saline. BSO, obtained from Chemical Dynamics Corp. (South Plainfield, NJ), was prepared as a 0.1 M stock solution in water, stored at 4°, and replaced every 6–8 weeks. LPAM, iodoacetic acid, 2,4-dinitrofluorobenzene, hexadecyltrimethylammonium bromide, GSH, calf thymus DNA, BSA, and equine liver glutathione *S*-transferase (EC 2.5.1.18) were obtained from Sigma Chemical Co. (St. Louis, MO). LPAM stock solutions were made in 75% ethanol with equimolar HCl and stored at −20°. Labeled LPAM, 3-[*p*-(bis(2-chloro-[^{14}C]ethyl)amino)phenyl]-L-alanine (14.2 mCi/mmol), was obtained from the Stanford Research Institute (Menlo Park, CA) through Dr. Robert R. Engle of the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute. 1,10-Phenanthroline was from Eastman Kodak C. (Rochester, NY). HPLC grade methanol was from Fisher Scientific Co. (Fair Lawn, NJ). Phosphate-buffered saline tablets were obtained from Oxoid USA, Inc. (Columbia, MD). All other chemicals were reagent grade or better.

Cell lines. Two human cell lines, 2008 (24) and COLO 316 (25), established from patients with serous cystadenocarcinoma of the ovary were used in these studies. Both lines were grown as monolayer cultures in Roswell Park Memorial Institute 1640 media supplemented with 10% heat-inactivated fetal calf serum, 2mM freshly added L-glutamine, and 1% Fungi-bact (Irvine Scientific, Santa Ana, CA). Cultures were equilibrated with humidified 5% CO_2 in air at 37°. Cells in exponential growth were used in all experiments.

An L1210 mouse leukemia line, ZCR9, made resistant to DDP by repeated mutagenesis with methylnitrosourea and selection with DDP (26), was obtained from Dr. Leonard A. Zwelling of the National Cancer Institute. These cells were cross-resistant to LPAM and were maintained in suspension culture in the same media described above for the ovarian lines, except with 15% fetal calf serum.

Drug treatments and colony assays. To deplete human ovarian carcinoma cells of GSH, BSO was added to flasks of cells to give a final concentration of 0.5 mM. Control flasks received no additions. After 20–28 hr, the media were removed from both the control and the GSH-depleted flasks and cells were harvested by treatment with 0.05% trypsin-EDTA for 10 min. Harvested cells were resuspended in media and counted by hemacytometer; then, an aliquot was removed for assay of colony-forming ability, and the remainder was prepared for GSH analysis as described below. Five ml of suspended cells were plated onto 60-mm polystyrene tissue culture dishes (Corning Glass Works, Corning, NY). Fifty μl of either DDP or LPAM stock solutions were added to triplicate plates at each drug concentration. Control plates received diluent alone. Plates were incubated in humidified 5% CO_2 in air; and after 10 days for 2008 cells, or 14 days for COLO 316 cells, plates were fixed with methanol and stained with Giemsa. Colonies of over 60 cells were counted macroscopically. Cloning efficiencies were 47.6 ± 12.0% (N = 12) and 41.2 ± 15.9% (N = 12) for control and GSH-depleted 2008 cells, respectively, and 28.2 ± 5.7% (N = 12) and 28.1 ± 4.9% (N = 12) for control and GSH-depleted COLO 316 cells, respectively.

The ZCR9 cells were seeded at 5 × 10^5 cells/ml and BSO was added to give a final concentration of 0.5 mM. After 20–28 hr, cells were harvested by centrifugation, resuspended in media, and counted by hemacytometer, after which an aliquot was removed for soft agar colony-forming assay and the remainder was prepared for GSH analysis. Three ml of suspended cells (300 cells/ml) were placed in sterile tissue culture tubes, treated with 30 μl of appropriate stock solution of either DDP or LPAM, and placed in an incubator. After 1 hr of drug exposure, the tubes were centrifuged, and the supernatant was replaced with 3 ml of media at 43° containing 0.3% agar. One-ml aliquots were plated onto gridded 35-mm tissue culture dishes (Lux, Miles Laboratories, Naperville, IL), left at room temperature for 1–2 hr, incubated at 37° in humidified 7.5% CO_2 in air, and assessed for colony formation after 7 days. Cloning efficiencies were 44.9 ± 20.5% and 33.6 ± 22.2% (N = 8) for control and GSH-depleted cells, respectively.

Levels of statistical significance were determined with a two-tailed paired Student's t test.

High pressure liquid chromatography. A Waters Associates (Milford, MA) HPLC equipped with a WISP autosampler and either a model 440 fixed wavelength absorbance detector or a model 450 variable wavelength detector was used for all analyses. Loss of native DDP by reaction with GSH was followed by solvent-generated anion exchange chromatography (27) on a 10 cm × 8.0 mm μBondapak C_{18} cartridge (10 μm particle size) radially compressed in a Z-module (Waters). The Z-module was fitted with a 4 mm × 22 mm guard column (Waters) packed with Corasil C_{18} (Waters, 37–50 μm particle size). The mobile phase was 0.1 mM hexadecyltrimethylammonium bromide at a flow rate of 2.0 ml/min. DDP was detected at 300 nm.

LPAM hydrolysis was monitored on a 30 cm × 3.9 mm μBondapak C_{18} column (Waters) also fitted with a guard column as described above. The mobile phase was 2% acetic acid/methanol, 1:1 (v/v), pumped at 1.2 ml/min (28). LPAM, monohydroxy-LPAM, and dihydroxy-LPAM were detected by absorbance at 263 nm at 6.6 min, 4.6 min, and 4.0 min, respectively.

GSH determinations. GSH was measured in cell extracts by the method of Reed *et al.* (29). Cells were centrifuged from media, washed once in 10 ml of 0.9% saline, and resuspended in 1.0 ml of 0.9% saline. An aliquot was removed for determination of cell number with a Coulter counter (model ZBI) and the remainder was lysed by rapidly freezing in a dry ice/ethanol bath. Samples were stored at −70° until derivatized for HPLC analysis. Samples were thawed at 37° treated with 100 μl of 9.2 M perchloric acid, and pelleted at 1500 × g for 10 min. One ml of the supernatant was removed and rapidly derivatized with 50 μl of iodacetic acid (20 mg/ml H_2O), 50 μl of 1,10-phenanthroline (5 mg/ml ethanol), and 1.0 ml of 2.0 M KOH, 2.4 M $KHCO_3$. After 1 hr in the dark, the samples were treated with 1.0 ml of 1.5% (v/v) 2,4-dinitrofluorobenzene in 95% ethanol and allowed to stand stoppered in the dark overnight. The samples were centrifuged at 1500 × g for 10 min to pellet the crystalline precipitate, and the supernatant then was transferred to sample vials for loading into the WISP. An injection of 50 μl was chromatographed in a 25 cm × 4.6 mm 3-aminopropyl Spherisorb column (Custom LC, inc., Houston, TX) as described (28) with minor modifications.

Bindings to DNA, BSA, and nuclei. Calf thymus DNA, purified by chloroform/phenol extraction and ethanol precipitation, was dissolved in 4 mM NaCl, 0.01 M potassium phosphate buffer, pH 7.0, at approximately 0.1 mg/ml (300 μM deoxynucleotide concentration). DDP (10 mM), prepared in dimethylformamide, was added to DNA solutions containing either 0 or 2.0 mM GSH to give a final platinum/deoxynucleotide molar ratio of 0.1. These solutions were incubated in

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a 37° water bath and 0.5-ml aliquots were removed at the indicated times to microcentrifuge tubes. Aquation and binding of DDP to DNA were immediately quenched by the addition of 50 μl of 3.0 M sodium chloride and precipitation with 1 ml of −20° 100% ethanol. The precipitated DNA was washed several times with ethanol and then dissolved and hydrolyzed with 1 ml of 1.0 N HCl at 70° for 2 hr (30). Nucleobase concentration was determined by absorbance at 260 nm using a molar absorbance coefficient of 8900 (30). Pt was determined by FAA (31). For analysis of LPAM binding to DNA, [^{14}C]LPAM was added to a final concentration of 45 μM (3.68 mCi/mmol). At the indicated times 0.5 ml was removed to a microcentrifuge tube and the DNA precipitated with 1.0 ml of −20° ethanol. After two ethanol rinses, the DNA was hydrolyzed with 1.0 N HCl at 70° and the DNA-bound radioactivity was determined by liquid scintillation counting in Scinti-Verse Bio-HP (Fisher).

BSA was dissolved in 4.0 mM NaCl, 0.1 M potassium phosphate buffer, pH 7.0, at 40 mg/ml with and without 2.0 mM GSH. DDP was added to the solutions to give a final concentration of 33 μM. The solutions were incubated at 37°. At the indicated times, 0.5-ml aliquots were removed and immediately centrifuged at 2000 × *g*, 4°, through Amicon CF-25 ultrafiltration cones. The ultrafiltrate was analyzed for Pt by FAA. For analysis of LPAM binding to BSA, [^{14}C]LPAM was added to BSA solutions to give a final concentration of 0.1 mM (16.7 μCi/mmol). At the indicated times, 1.0 ml was removed and added to 1.0 ml of ice-cold 12% TCA (w/v). The precipitate was collected on glass microfiber filters (Whatman GF/C) and rinsed sequentially with water, methanol, and ether. The TCA-precipitable radioactivity on the filters was determined by liquid scintillation counting in Omnifluor (4 g/liter of toluene).

Nuclei were prepared by lysing a suspension of trypsinized 2008 cells in 0.25 M sucrose, 4 mM NaCl, 0.01 M potassium phosphate, pH 7.0, with 0.2% Triton X-100 for several min. The resultant suspension was filtered through four layers of gauze and centrifuged at 20 × *g* for 3 min. The supernatant was discarded and the nuclei pellet was resuspended in the sucrose, NaCl, phosphate buffer. The nuclei were centrifuged again, resuspended in 4 mM NaCl, 0.01 M potassium phosphate, pH 7.0, buffer, and counted by hemacytometer. DDP was added to the nuclei suspensions (8×10^5 nuclei/ml) containing either 0 or 2.0 mM GSH to give 50 μM final concentrations and incubated at 37°. At appropriate times, 1.0-ml aliquots were removed, centrifuged at 1000 × *g*, 4°, for 10 min, and resuspended in cold phosphate-buffered saline; then, nuclei were counted by hemacytometer. The nuclei were sedimented again and digested with aqua regia for 24 hr at 37°. Digests were analyzed for Pt by FAA.

Results

Effect of GSH on DDP stability and LPAM hydrolysis. The rate of loss of 3.0 mM DDP in 0.9% saline at 37° was followed by solvent-generated anion exchange HPLC. As expected, there was no loss of parent drug in saline alone over 24 hr. The presence of 5.0 mM GSH, however, caused a rapid loss of DDP (data not shown). The second order rate constant was 16.2 $M^{-1}hr^{-1}$, similar to reported values for other thio compounds reacting with DDP (32).

The hydrolysis of 0.1 mM LPAM in 0.01 M potassium phosphate (pH 7.0), 4.0 mM NaCl, at 37° was monitored by HPLC. The rate of loss of LPAM was first order with an apparent first order rate constant of 0.73 hr^{-1}. The presence of 2.0 mM GSH had no effect on the rate of loss of LPAM (data not shown). Addition of 1.5 units/ml of glutathione *S*-transferase with 5.0 mM GSH also had no effect on the rate of loss of LPAM. Although GSH had no effect on the rates, the yield of the mono- and dihydroxy hydrolysis products of LPAM was approximately 75% higher in the solutions containing GSH. No evidence of an LPAM-GSH adduct was found by HPLC.

Effect of GSH on drug binding to BSA. BSA in potassium phosphate buffer (pH 7.0), 4.0 mM NaCl was incubated with either DDP or [^{14}C]LPAM in the presence or absence of 2.0 mM GSH and the binding of drug to protein was determined as described in Materials and Methods. As shown in Fig. 1A, the presence of GSH diminished the binding of Pt to BSA. At 6 hr, there was 20% less Pt bound to BSA when GSH was present. GSH had a similar protective effect for BSA against LPAM alkylation (Fig. 1B). After 6 hr of incubation, the amount of LPAM bound to TCA-precipitable protein was 55% less in the solutions with GSH.

Effect of GSH on drug binding to DNA. Since DNA is the presumed cytotoxic target for both DDP and LPAM, we next assessed the ability of GSH to protect calf thymus DNA from DDP platination and LPAM alkylation. Consistent with the BSA experiments, GSH protected naked DNA from DDP binding (Fig. 2A). In tubes with 2.0 mM GSH present, the DDP bound to DNA at 24 hr was only 23% of controls. A small amount of the measured Pt could be attributed to co-precipitation of the GSH-DDP adduct(s) along with the DNA by ethanol. Parallel solutions of DDP and GSH without DNA were used to subtract out these background values. GSH afforded a similar protective effect against LPAM alkylation of DNA (Fig. 2B). After 3 hr of reaction, the LPAM bound to DNA in the solution with GSH was only 31% of the LPAM bound in the control solution. These experiments consistently gave a peak in the binding of LPAM to the DNA. The loss of bound drug between 6 and 24 hr may be the result of spontaneous depurination of alkylated bases.

Effect of GSH on DDP binding to nuclei. To define further the protective effect of GSH against DDP platination, we investigated the ability of GSH to prevent the platination of isolated chromatin. Nuclei isolated from 2008 human ovarian carcinoma cells were incubated with DDP in the absence and presence of GSH. As seen with the isolated BSA and calf thymus DNA experiments, there was less binding of DDP to chromatin with added GSH (Fig. 3).

Effect of intracellular GSH depletion on DDP and LPAM cytotoxicity. COLO 316 cells had intracellular GSH levels of 46.0 ± 15.9 nmol/10^6 cells ($N = 15$) that were depleted to 13.0 ± 5.3% ($N = 10$) of control cultures in 20–28 hr by 0.5 mM BSO. The role of intracellular GSH in determining COLO 316 sensitivity to DDP and LPAM was assessed by the ability of GSH-depleted cells versus undepleted cells to form colonies on plastic during exposure to these drugs. As reported for parent and LPAM-resistant L1210 mouse leukemia cells and human ovarian carcinoma cells (8, 14–16), this human ovarian line was markedly sensitized to LPAM by GSH depletion (Fig. 4). The extent of this sensitization was calculated by testing the mean difference in the dose response slopes ($N = 4$) of control versus depleted cells for significant difference from 0. The sensitization of COLO 316 to LPAM by GSH depletion was significant at $p < 0.01$. The IC_{50} of untreated cells was 0.21 ± 0.05 μM LPAM ($N = 4$) and the mean dose modification factor (IC_{50} control/IC_{50} treated) was 2.6 ± 0.5 for GSH-depleted cells. The effect of GSH depletion on sensitivity to DDP was less pronounced (Fig. 4). The mean of the differences in the dose response slopes of control versus depleted cells ($N = 6$) was significantly different from 0 at $p < 0.05$. The IC_{50} for DDP in this cell line was 0.055 ± 0.012 μM ($N = 6$) and the mean dose modification factor was 1.2 ± 0.2 for GSH-depleted cells.

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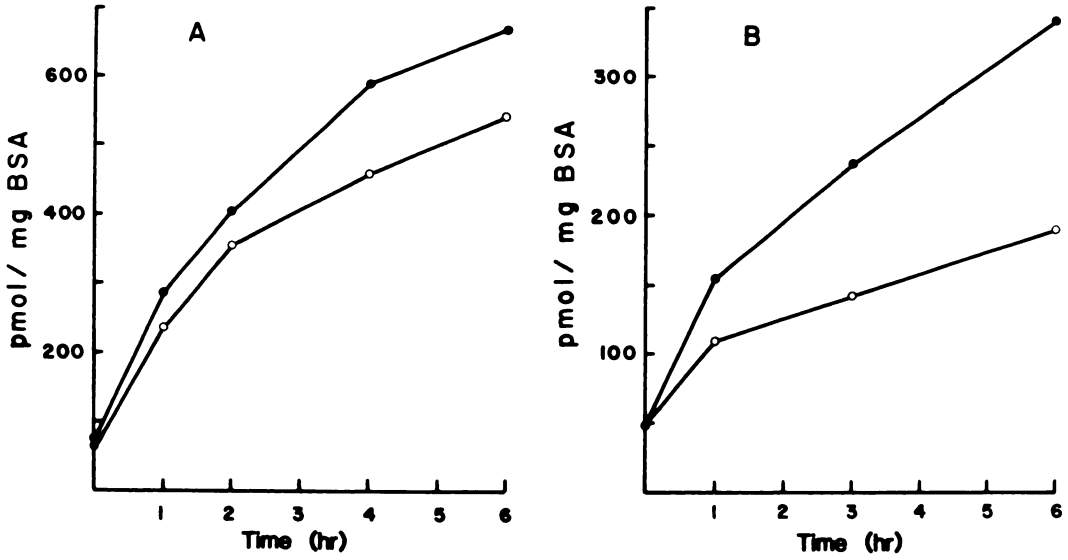

Fig. 1. Effect of GSH on the binding of DDP and LPAM to BSA. DDP (33 μM) or [^{14}C]LPAM (0.1 mM) was incubated with BSA (40 mg/ml) at 37° with or without GSH. A. Ultrafilterable Pt was determined by atomic absorption spectroscopy: ●, control; ○, with 2.0 mM GSH. B. TCA-precipitable ^{14}C was determined by liquid scintillation counting: ●, control; ○, with 2.0 mM GSH.

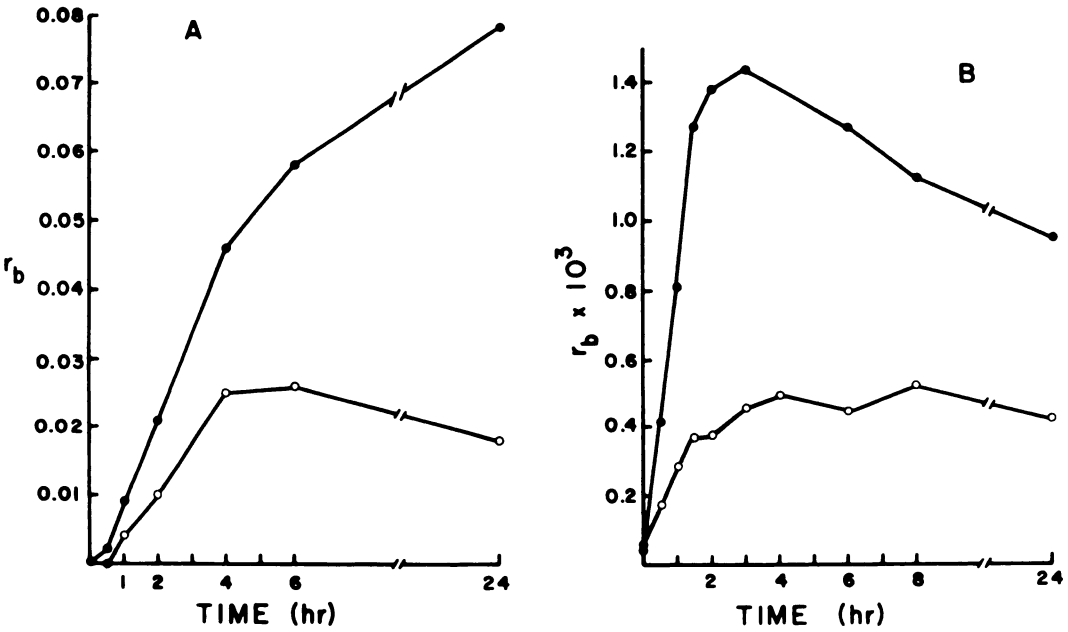

Fig. 2. Effect of GSH on binding of DDP and LPAM to calf thymus DNA. DDP (33 μM) or [^{14}C]LPAM (45 μM) was incubated with DNA (100 μg/ml) with or without GSH. r_b, the molar ratio of drug to deoxynucleosides. A, DNA-bound Pt; B, DNA-bound [^{14}C]LPAM: ●, control; ○, with 2.0 mM GSH.

Like COLO 316 cells, the 2008 cells were also dramatically sensitized to LPAM by GSH depletion (Fig. 5). The intracellular GSH level was 15.3 ± 4.8 nmol/10^6 cells (N = 13) and BSO-treated cultures were depleted to 11.2 ± 3.4% (N = 9) of control levels.[1] This level of depletion gave a mean difference in the dose response slopes of control versus depleted cells that was significantly different from 0 at $p < 0.05$. The mean LPAM dose modification factor for GSH-depleted cells was 1.6 ± 0.1 (N = 4). Depletion of GSH was, however, without effect on the sensitivity of 2008 cells to DDP (Fig. 5). The mean difference in the dose response slopes of control versus depleted cells (N = 4) was not significantly different from 0. The mean DDP dose modification factor was 0.8 ± 0.3 for GSH-depleted cells. The IC_{50} of DDP was 0.15 ± 0.04 μM (N = 4) for 2008 cells.

The ability of cells to recover their GSH may be a factor in determining their sensitivity to DDP and LPAM under our depletion protocol. GSH rebound was assessed in log-phase cultures of COLO 316 and 2008 cells. Following depletion with 0.5 mM BSO for 24 hr, the GSH in COLO 316 cells began to rise 9 hr after BSO was removed from the medium and reached 50% of control levels at 12 hr post-depletion (Fig. 6). As shown in Fig. 6, the rebound of GSH in 2008 cells was much more sluggish; the levels rose to 33% of control cells at 24 hr but did not recover further up to 48 hr post-depletion. In comparison, however, 2008 cells depleted with 0.05 mM BSO recovered their GSH much more rapidly. GSH levels in these cells rose to 75% of control cells at 12 hr.

[1] These cells are smaller than COLO 316 cells and the GSH levels should not be compared in relation to the relative cytotoxicities of DDP and LPAM.

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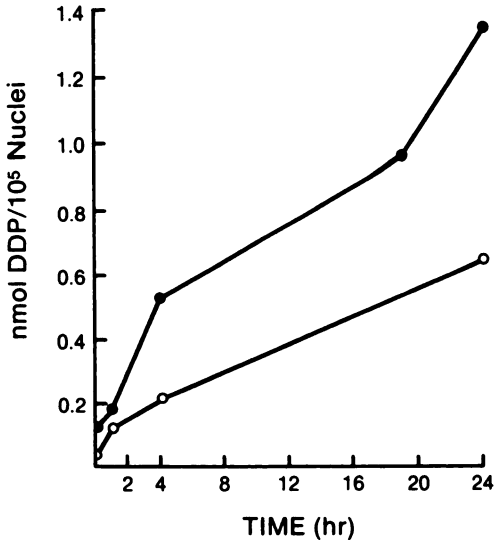

Fig. 3. Effect of GSH on binding of DDP to isolated nuclei. Nuclei were isolated from human ovarian carcinoma cells and incubated with 50 μM DDP with or without added GSH: ●, control; ○, with 2.0 mM GSH.

To determine whether GSH depletion could confer DDP sensitivity to a DDP-resistant phenotype, we also studied the ZCR9 mouse leukemia line. GSH levels in these cells were the same as those of the parent K25 cells, 5.4 ± 1.3 nmol/10^6 cells ($N = 11$), and were depleted to 18.3 ± 7.9% of control levels by 0.5 mM BSO after approximately 24 hr. As reported for LPAM-resistant L1210 cells (L1210/PAM) and their parent cells (15, 16), these L1210 cells were sensitized to LPAM as determined by colony-forming ability in soft agar (Fig. 7A). The mean difference in the dose response slopes of control versus depleted cells was significantly different from 0 at $p < 0.1$. The IC_{50} for LPAM was 12.8 ± 6.3 μM ($N = 4$), similar to a previous report (26). The mean dose modification factor for GSH-depleted cells was 2.1 ± 1.1 ($N = 4$). GSH depletion had no effect on ZCR9 sensitivity to DDP, however (Fig. 7B). The mean difference in the dose response slopes was not significantly different from 0. The IC_{50} for DDP was 7.8 ± 2.7 μM ($N = 5$), in agreement with previous reports (26), and the mean dose modification factor for depleted cells was only 1.1 ± 0.1.

Discussion

Recent studies have shown that BSO depletion of GSH potentiates the cytotoxicity of LPAM in L1210 and human ovarian carcinoma cells and effectively reverses the LPAM resistance of cells with elevated GSH (8, 14–16). We have extended these studies to two other human ovarian carcinoma cell lines and a DDP-resistant L1210 cell line. We have also investigated the effect of GSH depletion on the cytotoxicity of DDP, another electrophilic drug of great utility in the treatment of ovarian carcinoma.

As expected, kinetic studies indicated that GSH reacted rapidly with DDP in normal saline. GSH can thus be expected to compete with other cellular nucleophils for substitution of the chloride ligands of DDP or the aqua ligands of the aquated species. In contrast, GSH had no effect on the rate of loss (hydrolysis) of LPAM. This is consistent with the S_N1-like mechanism of reaction of aniline mustards with nucleophils (21, 33). Interestingly, the solutions of LPAM with GSH produced higher amounts of the mono- and dihydroxy hydrolysis products than did control solutions of LPAM alone. Higher amounts of these products have also been observed in cells with elevated GSH content (6). The reasons for this are not immediately clear since the reaction of GSH with LPAM is expected

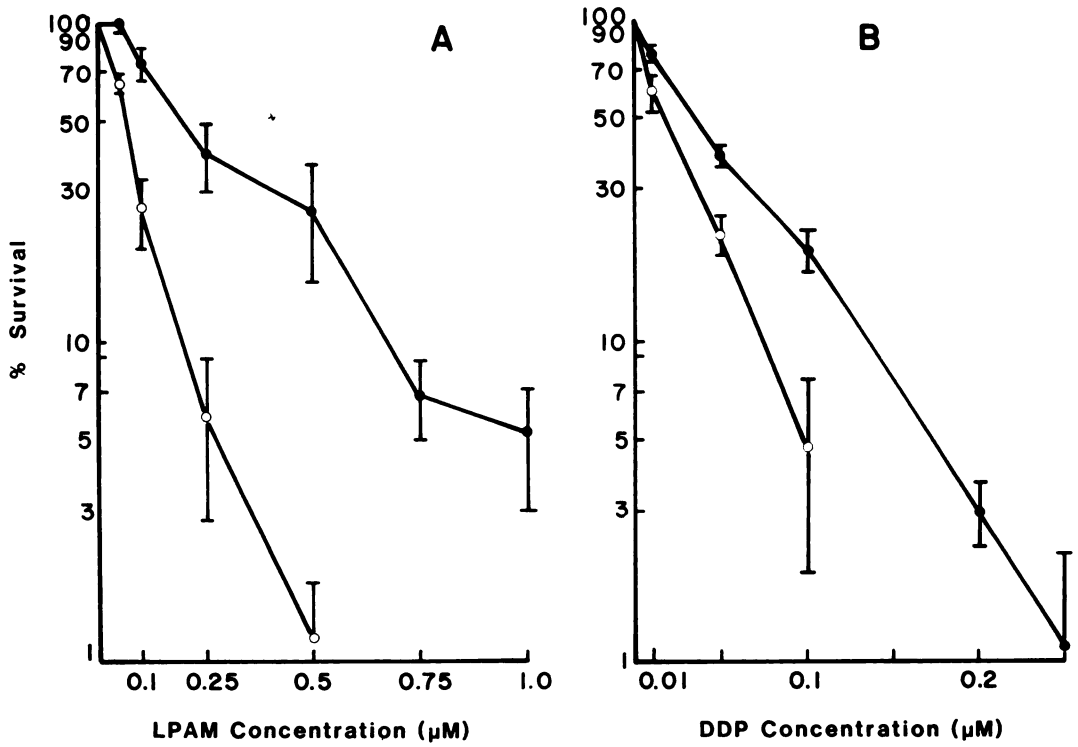

Fig. 4. Typical dose response of COLO 316 cells to DDP and LPAM following depletion of intracellular GSH with BSO. Drug cytotoxicity was determined by clonogenic assay on plastic. A. Dose response to LPAM: ●, control; ○, GSH-depleted cells. B. Dose response to DDP: ●, control; ○, GSH-depleted cells. *Points* are the mean of triplicate plates; *bars* are the standard error.

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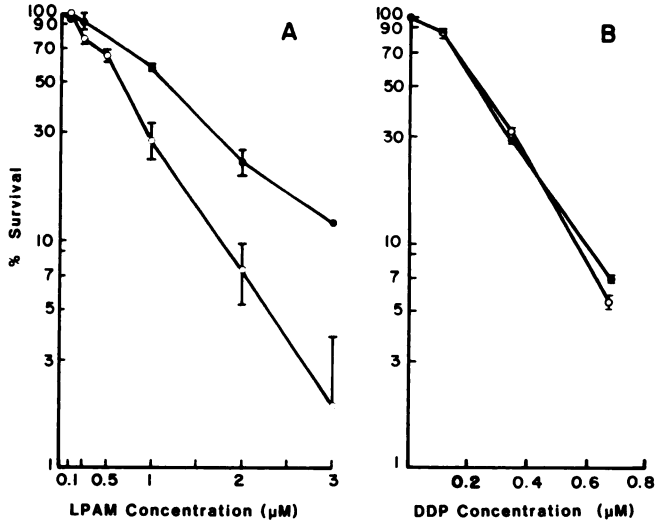

Fig. 5. Typical dose response of 2008 cells to DDP and LPAM following depletion of intracellular GSH as determined by clonogenic assay on plastic. A. Dose response to LPAM: ●, control; ○, GSH-depleted cells. B. Dose response to DDP: ●, control; ○, GSH-depleted cells. *Points* are the mean of triplicate plates; *bars* are the standard error.

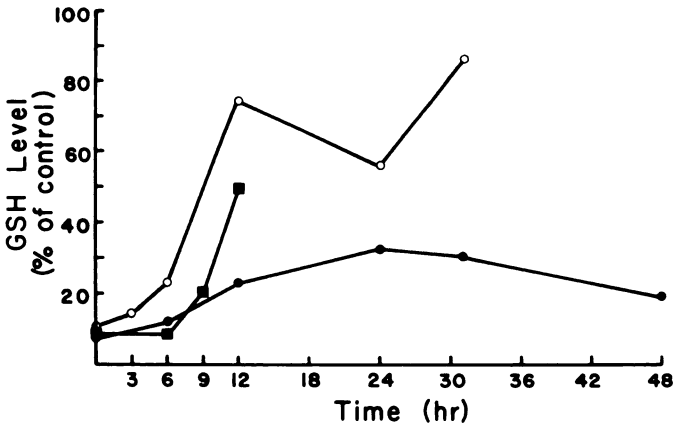

Fig. 6. Rebound of GSH in COLO 316 and 2008 cells following GSH depletion. Log-phase cells were exposed to 0, 0.05, or 0.5 mM BSO for 24 hr and then the medium was replaced with 20 ml of fresh medium without BSO. Flasks were harvested at the indicated time after changing the medium and GSH was determined as described in Materials and Methods. GSH levels are expressed as percentage of the control cells which received no BSO. ●, 2008 cells exposed to 0.5 mM BSO; ○, 2008 cells exposed to 0.05 mM BSO; ■, COLO 316 cells exposed to 0.5 mM BSO.

to generate a stable thioether linkage. The identification of a GSH-LPAM adduct, however, has not yet been reported, and we have seen no evidence for its formation by HPLC.

Consistent with the rapid reaction of DDP with GSH, we have found that GSH can protect macromolecules from DDP platination. GSH protected naked DNA, BSA, and isolated nuclei from platination. Despite these effects, GSH seems to play only a small role in determining DDP cytotoxicity. Reduction of GSH content by 5- to 10-fold with the nontoxic γ-glutamylcysteine synthetase inhibitor, BSO, in 2008, COLO 316, and ZCR9 cells was sufficient to sensitize them to LPAM but led to minimal sensitization to DDP. We have also found that depletion of GSH in human lymphoblastoid cells, WI-L2, by growth in cysteine-free media does not sensitize these cells to DDP either.[2] In contrast to our results with these cell lines, other studies have found that GSH depletion will potentiate DDP cytotoxicity in some cells (14, 34). This suggests that the role of GSH in intracellular DDP metabolism may vary between cell lines.

The reasons for the lack of effect of GSH depletion on DDP cytotoxicity in these three cell lines are not clear. One possibility was that the GSH rebounds rapidly after cells are removed from BSO and protects the cells from delayed DDP damage. DDP-induced DNA-interstrand crosslinking peaks 6–12 hr after drug exposure (26). The GSH levels in COLO 316 cells do not begin to rise until 6 hr after removal from 0.5 mM BSO; GSH was approximately 50% of control at 12 hr post-depletion. In 2008 cells the GSH did not rise past 20% of control cells for 12 hr and remained below 33% of control cells for 48 hr. The fact that COLO 316 cells with the more rapid rebound of GSH were more sensitized to DDP than were the 2008 cells argues against the GSH rebound as being an important factor in ameliorating potentiation of DDP cytotoxicity. The fact that LPAM-induced DNA-interstrand crosslinking has a time course similar to that of DDP (26) and yet the same levels of GSH depletion gave significant sensitization also makes this explanation seem unlikely. Alternatively, GSH may not protect a critical DDP target. Scanlon *et al.* (35) have suggested that DDP may exert its cytotoxicity at the plasma membrane by inhibiting methionine transport. Thus, although GSH undergoes rapid efflux and turnover, a 10-fold decrease in intracellular levels of GSH may have no effect on extracellular GSH levels surrounding and protecting critical sites on the plasma membrane.

Another explanation for the minimal effects of GSH depletion on DDP cytotoxicity may be that the GSH-DDP adduct generated intracellularly is still capable of forming cytotoxic lesions. We have previously found that the mixture of GSH-DDP adducts generated extracellularly is not cytotoxic to WI-L2 human lymphoblastoid cells (23); however these species may simply not be transported into cells and, if generated intracellularly, may indeed be cytotoxic. We have found that the reaction of GSH with DDP generates a complex mixture of products that sequentially interconverts with time.[3] The cytotoxicity of each of these intermediates and whether they are formed intracellularly is unknown. In support of the explanation that a GSH-DDP adduct is still capable of forming cytotoxic lesions is the observation that GSH enhances the rate at which DDP alters the tertiary structure of superhelical DNA (36). The sulfur atom of GSH is expected to labilize the amine ligand *trans* to it. This position and the remaining chloride ligand thus have the potential for generating crosslinks in the *cis* conformation. It will thus be important to measure the effect of GSH modulations on the net levels of both intra- and interstrand crosslinks produced by DDP in order to clarify the exact reason for the lack of effect of GSH levels on DDP cytotoxicity.

Similar to the protection afforded macromolecules from DDP platination, GSH also protected naked DNA and BSA from alkylation by LPAM. However, unlike DDP, depletion of GSH in 2008, COLO 316, and ZCR9 cells led to significant sensitization to LPAM cytotoxicity. This indicates that GSH suc-

[2] M. Shea and P. A. Andrews, unpublished data.
[3] P. A. Andrews, unpublished data.

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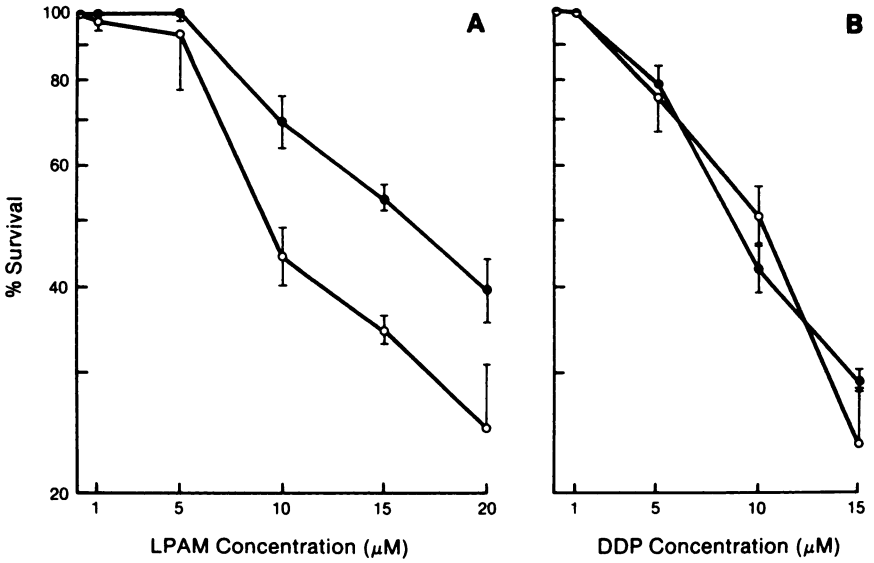

Fig. 7. Typical dose response of ZCR9 cells to DDP and LPAM as determined by clonogenic assay in soft agar following depletion of intracellular GSH. A. Dose response to LPAM: ●, control; ○, GSH-depleted cells. B. Dose response to DDP: ●, control; ○, GSH-depleted cells. *Points* are the mean of triplicate plates, and *bars* are the standard error.

cessfully competes with intracellular targets for the reactive LPAM-immonium ion intermediate. The mechanistic basis for the potentiation may thus be that GSH depletion allows more of the reactive species to reach its cytotoxic target, presumably DNA. Alternative explanations for the mechanism of the potentiation of LPAM cytotoxicity by GSH depletion need to be considered. Considering the importance of GSH in maintaining membrane integrity, it is conceivable that depletion of GSH may cause enhanced uptake of LPAM through changes induced at the membrane level. GSH functions in deoxynucleotide synthesis and apparently in the repair of certain types of DNA damage (2, 3); depleted GSH could thus possibly alter repair of LPAM-induced DNA lesions. The differential potentiation of DDP and LPAM cytotoxicity by GSH depletion in these cells, despite the similar protection that GSH affords macromolecules from drug binding, suggests that there are fundamental differences in the intracellular interaction of these electrophilic drugs with GSH.

We have observed that potentiation of LPAM cytotoxicity occurred in cells in which the GSH was depleted to only 20% of control cells. No correlation of the degree of sensitization has been observed with the range of depletion from 5 to 20%. At these levels of depletion, GSH should still be present in large excess over the intracellular levels of drugs, even considering the fact that LPAM is concentrated 5- to 10-fold in the cell (37). If the mechanism of the effect of GSH on LPAM cytotoxicity is purely a kinetic one, i.e., GSH is acting as a nucleophilic sink for LPAM, then it is unclear why a 5-fold decrease in GSH should lead to potentiation or why a 3-fold increase in GSH levels, as is found in LPAM-resistant cells (8, 14), leads to antagonism. Clarifying these discrepancies and further defining the exact mechanisms for the effects of GSH depletion on DDP and LPAM cytotoxicity will be important for improving the effectiveness and usefulness of GSH depletors such as BSO in the clinic. These investigations are being pursued.

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