ABSTRACT

We have determined the effect of glutathione (GSH) depletion on the cytotoxicity of three nitrogen mustards, six platinum complexes, and mitomycin C in a human ovarian carcinoma cell line. GSH levels in COLO 316 cells were depleted by exposure of cell monolayers to 0.5 mM d,L-buthionine-S,R-sulfoximine. GSH depletion significantly potentiated the cytotoxicity of L-phenylalanine mustard, chlorambucil, and mechlorethamine as determined by clonogenic assay on plastic plates. The dose modification factors were 2.6, 2.6, and 1.9, respectively. The same level of GSH depletion had a minimal effect on the cytotoxicity of cis-diaminedichloroplatinum(II) (cis-DDP), carboplatin, dichloro(ethylenediamine)platinum(II), 1,2-diaminocyclohexylplatinum(II) malonate, and iproplatin. The dose modification factors of GSH depletion for these drugs were 1.4 or less. trans-Diaminedichloroplatinum(II) was, however, markedly potentiated by GSH depletion with a dose modification factor of 2.7. Mitomycin C was minimally potentiated by GSH depletion. We have also generated cis-DDP-resistant cells from COLO 316 and 2008 human ovarian carcinoma cells by in vitro selection with cis-DDP. These cis-DDP-resistant cells had identical levels of GSH as the parental cells. GSH depletion sensitized these cells only to the same degree as the parental cells and did not reverse the resistant phenotype. Our results indicate that intracellular GSH levels are not an important determinant of the cytotoxicity of cis-platinum(II) or cis-platinum(IV) complexes in COLO 316 and 2008 cells. In addition, altered GSH metabolism does not appear to be a component of the cis-DDP-resistant phenotype in these cells.

INTRODUCTION

Ovarian carcinoma is the fourth major cause of cancer mortality in females (1). Alkylating agents and cis-DDP have been used extensively to treat advanced stages of this disease (2, 3). Although i.p. administration of these drugs appears to improve greatly their therapeutic benefit (3–6), effective therapy is still hampered by dose-limiting toxicities and the inevitable emergence of drug resistance by the tumor (2). The use of biochemical modulators that can sensitize tumor cells to the cytotoxic effects of these agents or prevent or reverse the resistant phenotypes that develop is one means of improving the efficacy of these alkylating and platinating agents. However, little is known about the exact mechanisms of resistance to platinating agents such as cis-DDP or alkylating agents such as LPAM and the rational choice of a biochemical pathway to modulate remains difficult.

GSH has been shown to be an important determinant of the sensitivity of cells to a wide variety of drugs and cytotoxic treatments (7). Elevated GSH has also been shown to be a component of the drug-resistant phenotypes that emerge in cells exposed to a number of electrophilic drugs and especially to nitrogen mustards (8–16). GSH would also be expected to be an important determinant of the cytotoxicity of platinum compounds since these electrophiles react readily with thiol functionalities (17). Recent advances in the understanding of the biochemistry of GSH have brought forth a specific means of depleting cellular GSH levels with the γ-glutamylcysteine synthetase inhibitor, BSO (18). Depletion of elevated GSH levels with BSO in both L1210 mouse leukemia cells and human ovarian carcinoma cells that display LPAM resistance has been shown to resensitize these cells to LPAM cytotoxicity (15, 16).

Ovarian carcinoma is uniquely suited for biochemical modulation of tumor cell sensitivity since it is confined to the peritoneal cavity throughout much of its natural history. Administration of an appropriate biochemical modulator i.p. may offer a very large pharmacological advantage for the sensitization of ovarian carcinoma tissue compared to normal tissues. The i.p. administration of modulators of GSH levels such as BSO thus presents an attractive means of potentiating the cytotoxicity of alkylating and platinating agents in ovarian carcinoma.

We have previously shown that GSH depletion with BSO can sensitize two human ovarian carcinoma cell lines to LPAM cytotoxicity but that the effect on DDP cytotoxicity was minimal.5 We have now extended these studies on GSH depletion to a variety of alkylating and platinating agents in COLO 316 human ovarian carcinoma cells. In addition, in order to determine whether GSH plays a role in cis-DDP-resistant phenotypes, we have investigated the effect of GSH depletion on cis-DDP cytotoxicity in cis-DDP-resistant sublines of both COLO 316 and 2008 human ovarian carcinoma cells.

MATERIALS AND METHODS

Drugs and Chemicals. cis-DDP (clinically formulated), carboplatin, iproplatin, and 1,2-diaminocyclohexylplatinum(II) malonate were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). LPAM, chlorambucil, mechlorethamine, and trans-DDP were obtained from Sigma Chemical Co. (St. Louis, MO). Dichloro(ethylenediamine)platinum(II) was obtained from Alfa Products (Danvers, MA). Platinum compounds were prepared in 0.9% saline and the concentrations were standardized by atomic absorbance spectrophotometry. LPAM prepared in 75% ethanol containing 55 mM HCl and chlorambucil prepared in ethanol were stored at −20°C. LPAM solutions were standardized by spectrophotometry (r

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2 Clayton Foundation Investigator. To whom requests for reprints should be addressed.
3 Clayton Foundation Investigator.
4 The abbreviations used are: cis-DDP, trans-DDP, cis- and trans-diaminedichloroplatinum(II); GSH, glutathione; LPAM, L-phenylalanine mustard; BSO, d,L-buthionine-S,R-sulfoximine.

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Mechlorethamine was prepared in water and stored frozen. Mitomycin C, obtained from Bristol Myers Co. (Syracuse, NY), was prepared in water, stored frozen, and standardized spectrophotometrically (λ = 21,800 at 365 nm). BSO, obtained from Chemical Dynamics Corp. (South Plainfield, NJ), was prepared as a 0.1 μM stock solution in water and stored at 4°C.

Cell Lines. Two human cell lines, 2008 (19) and COLO 316 (20), established from patients with serous cystadenocarcinoma of the ovary were used in these studies. Both lines were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM freshly added L-glutamine, and 1% Fungi-bact (Irvine Scientific, Santa Ana, CA). Cultures were equilibrated with humidified 5% CO₂ in air at 37°C. Cells in exponential growth were used in all experiments.

cis-DDP-resistant sublines of 2008 and COLO 316 cells, designated 2008/DDP and COLO/DDP respectively, were generated by exposure of cells to media containing 1 μM cis-DDP. When cells recovered from this treatment and resumed log growth, the exposure was repeated. These cells were already approximately 2-fold resistant after one treatment as measured by the ratio of the 50% inhibitory concentrations. The cis-DDP-resistant cells used in these studies received four cis-DDP exposures each and the resistance was stable in the range of 2.5 to 3.0 over the course of the experiments.

Drug Treatments and Colony Assays. To deplete human ovarian 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 to 28 h, the media was removed from both the control and GSH-depleted flasks and cells were harvested by treatment with 0.05% trypsin-EDTA for 10 min. Harvested cells were resuspended in media, counted by hemacytometer, and the remainder prepared for GSH analysis. Fifty μl of drug stock solutions were added to triplicate plates at each drug concentration. Drug plates received diluent alone. Plates were incubated in humidified 5% CO₂ in air, and after 10 days for 2008/DDP cells or 14 days for COLO 316 cells plates were fixed with methanol and stained with Giemsa. Colonies of over 50 cells were counted macroscopically. Cloning efficiencies were 25 to 30% for control and GSH-depleted COLO 316 and COLO/DDP cells and 43% for control and GSH-depleted 2008/DDP cells.

GSH Determinations. GSH was measured in cell extracts by the method of Reed et al. (21) and modified as previously described. Since the GSH levels are reported normalized to cell number, the cell volumes of the parent and resistant cells were assumed to be the same. This assumption was justified since the resistant cells had a morphology identical to the parent cells and the monolayers grew to similar densities.

RESULTS

BSO exposure routinely depleted GSH to levels that were 13.0 ± 5.3% (SD) of control COLO 316 cells (Table 1). This amount of GSH depletion in COLO 316 cells lead to minimal potentiation of the cytotoxicity of cis-platinum(II) compounds, i.e., cis-DDP, dichloro(ethylenediamine)platinum(II), carboplatin, and 1,2-diaminocyclohexylplatinum(II) maleonate (Table 2). The dose modification factor was 1.4 or less for each of these platinum drugs. This potentiation was not statistically significant when the mean differences in the slopes of dose-response lines were tested for difference from zero. In one experiment, GSH depletion also gave minimal sensitization to the platinum(IV) compound iroplatin.

The same amount of GSH depletion that had minimal effects on the cytotoxicity of cis-platinum compounds, however, markedly potentiated the cytotoxicity of LPAM, chlorambucil, mechlorethamine, and trans-DDP (Table 2). The dose modification factors ranged from 1.9 to 2.7. This potentiation was significant at the 2.5% level of confidence or less. The cytotoxicity of the bioreductive alkylating agent mitomycin C was not significantly potentiated by GSH depletion in these cells (dose modification factor, 1.1).

cis-DDP-resistant cells were generated by repeated selection with 1 μM cis-DDP. COLO/DDP cells possessed 2.5-fold resistance and 2008/DDP cells had 2.7-fold resistance to cis-DDP. The GSH level in COLO/DDP cells was nearly identical to that of the parent cells (Table 1). Despite the fact that the GSH was not elevated, we examined whether GSH depletion could nonetheless reverse the DDP resistance. COLO/DDP cells were depleted by BSO exposure similar to that of the parent cells (Table 1). This depletion of their GSH levels with BSO treatment lead to minimal potentiation of cis-DDP cytotoxicity (Chart 1). The mean dose modification factor was 1.2 ± 0.2 (N = 3). The slight potentiation observed was identical to that found in the parental COLO 316 cells and did not reverse this resistant phenotype. Similar results were found with 2008/DDP cells; GSH levels of these cis-DDP-resistant cells were also nearly identical to that of the parent cells (Table 1). GSH depletion had no significant effect on DDP cytotoxicity with these cells as was found with the parental 2008 cells (Chart 2). The mean dose modification factor was 1.0 ± 0.1 (N = 4).

Table 2

Potentiation of alkylating and platinating agent cytotoxicity by glutathione depletion

Drug cytotoxicity was determined in control and glutathione-depleted COLO 316 human ovarian carcinoma cells by clonogenic assay on plastic. Glutathione was depleted with 0.5 μM buthionine sulfoximine.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose-modification factor</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-DDP</td>
<td>1.2 ± 0.2 (6)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>1.1 ± 0.1 (3)</td>
<td>Not significant*</td>
</tr>
<tr>
<td>Dichloro(ethylenediamine)-platinum(II)</td>
<td>1.4 ± 0.3 (3)</td>
<td>Not significant*</td>
</tr>
<tr>
<td>1,2-Diaminocyclohexylplatinum(II) maleonate</td>
<td>1.4 ± 0.2 (3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Iroplatin</td>
<td>1.1 (1)</td>
<td></td>
</tr>
<tr>
<td>trans-DDP</td>
<td>2.7 ± 0.3 (4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPAM</td>
<td>2.6 ± 0.5 (4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>2.6 ± 0.9 (6)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>1.9 ± 0.3 (3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1.1 ± 0.3 (5)</td>
<td>Not significant*</td>
</tr>
</tbody>
</table>

* Defined as the 50% inhibitory concentration of control cells divided by the 50% inhibitory concentration of GSH-depleted cells.
* Mean difference in the slopes of control and GSH-depleted dose-response lines were tested for significance difference from zero by Student’s t test.
* Mean ± SD.
* Numbers in parentheses, number of experiments.
* Not statistically significant at the 5% level or less.
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DISCUSSION

The potentiation of the cytotoxicity of anti-cancer agents with nontoxic biochemical modulators is an attractive approach for improving their efficacy. Ovarian carcinoma is ideally suited for the implementation of this approach since i.p. instillation of the biochemical modulator may offer a means of selectively potentiating drug cytotoxicity in neoplastic versus normal tissue. BSO is an effective depletion of cellular GSH levels that has been shown to reverse LPAM-resistant phenotypes and sensitize parent cells in both L1210 cells and certain human ovarian carcinoma cells (14–16). We have recently extended these observations to two other human ovarian carcinoma cell lines.5 GSH depletion was found to markedly potentiate LPAM cytotoxicity but had little effect on cis-DDP cytotoxicity. We have now determined the specificity of this effect for a variety of alkylating and platinating agents in COLO 316 cells. We have also examined the role of GSH in the cis-DDP-resistant phenotypes of COLO 316 and 2008 cells.

GSH depletion was found to potentiate significantly the cytotoxicity of the two aniline mustards, LPAM and chlorambucil, and the prototype nitroge mustard, mechlorethamine. The aniline mustards alkylate nucleophiles by an S_n1-like mechanism compared to mechloethamine which reacts through an S_n2-like mechanism (22, 23). For an S_n2 mechanism, if one reduces the concentration of one of the reactants (such as GSH) 10-fold, one then obtains 10-fold less product and consumes 10-fold less reactants (drug and GSH) in a given amount of time. Why depletion of GSH to levels 10 to 20% of controls gives only 1.9-fold sensitization for mechlorethamine is thus not clear since one would expect 5 to 10 times more drug to reach cytotoxic sites at this level of GSH depletion. The marked potentiation of these three drugs that contain β-chloroethylamine moieties, contrasted to the minimal potentiation of cis-platinum complexes and the aziridine-containing drug mitomycin C, suggests that the GSH modulated deactivation of the nitrogen mustards may be enzymatically mediated. The GSH levels covered by BSO depletion may be in the range of the $K_m$ of GSH for this enzyme. This would explain not only these problems in stoichiometry and the specificity for nitrogen mustards but also why tripling GSH levels (which are already in large excess over drug) leads to LPAM resistance (14–16).

None of the platinum(II) compounds in the cis configuration were significantly potentiated by GSH depletion. Chloride ligands versus carboxylato ligands in the labile cis positions thus seem to be unimportant in determining the effect of GSH on the cytotoxicity of these drugs. Iproplatin reportedly requires an intracellular reduction to a platinum(II) complex for activity (24). If this reduction is linked to GSH, then GSH depletion might be expected to antagonize iproplatin cytotoxicity. GSH depletion, however, had a minimal effect on iproplatin cytotoxicity and this effect was potentiation. Some of these platinum analogues such as carboplatin are clinically very promising. Our results suggest that GSH depletion as for cis-DDP will not be a generally useful means of potentiating the cytotoxicity of these drugs in ovarian carcinoma.

In contrast to the cis-platinum(II) compounds, the potentiation of trans-DDP cytotoxicity was dramatic. The 2.7-fold sensitization was similar to that of the mustard compounds. A chloride of trans-DDP is more easily substituted than is a chloride of cis-
DDP due to the trans effect, i.e., the chloride in trans-DDP is a better trans labilizer than is the ammine group in cis-DDP (25, 26). In this regard, trans-DDP is known to react more readily than cis-DDP with a variety of proteins (27–29). trans-DDP is also known to react four times faster than cis-DDP (30), although trans-DDP has been reported to react both more rapidly (31) and more slowly (32) than cis-DDP with DNA. The differential sensitization of cells to trans versus cis platinum(II) compounds may therefore be due to a kinetic effect. The reaction of trans-DDP with GSH may be rapid enough so that significant amounts of the drug is inactivated as it crosses the cytoplasm. The level of GSH would then be an important determinant of cytotoxicity. The reaction of cis-platinum(II) compounds with GSH on the other hand may be slow compared to trans-DDP such that large percentages of the applied drug dose reach the nucleus before inactivation by reaction with GSH can take place. GSH levels would then have little effect on cytotoxicity.

Alternatively the differential effect of GSH depletion on trans-versus cis-platinum(II) drug cytotoxicity may not be related to kinetic phenomena but to steric considerations. GSH may effect platinum drug cytotoxicity by quenching DNA monoadducts and preventing DNA inter- and intrastrand cross-link formation (33). The trans-DDP monoadduct will be much less sterically hindered than the cis-DDP monoadduct for substitution of the remaining chloride and may therefore be more easily quenched by GSH. Alterations in GSH levels would thus have a much greater effect on trans-DDP cytotoxicity.

Elevated intracellular GSH is a component of the resistant phenotypes that develop following selection of cells with LPAM (14, 16). We have been interested in whether a similar rise in GSH levels in cis-DDP-resistant cells is consistent with the fact that GSH is not a major determinant of cis-DDP resistance. The increase of GSH levels in cis-DDP-resistant cells failed to reverse their resistance. This lack of GSH use in cis-DDP-resistant cells is consistent with the fact that GSH is not a major determinant of cis-DDP cytotoxicity in parent cells. Alterations in GSH metabolism in response to cytotoxic drug insult while within the capability of these cells are therefore not selected for by 1 μM cis-DDP exposure and would appear to play little role in conferring cis-DDP-resistance in these particular phenotypes.

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Differential Potentiation of Alkylating and Platinating Agent Cytotoxicity in Human Ovarian Carcinoma Cells by Glutathione Depletion

Paul A. Andrews, Michael P. Murphy and Stephen B. Howell


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