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Sandra E. Brooks · Timothy T. Korbut
Norman P. Dupuis · Sylvia A. Holden
Beverly A. Teicher

Cytotoxicity of antitumor platinum complexes with L-buthionine-(*R, S*)-sulfoximine and/or etanidazole in human carcinoma cell lines sensitive and resistant to cisplatin

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Abstract Human 2008 ovarian carcinoma cells and the C13 CDDP-resistant subline and human MCF-7 breast carcinoma cells and the MCF-7/CDDP CDDP-resistant subline were exposed to L-buthionine-(*S, R*)-sulfoximine (50 μ*M*) for 48 h prior to and during exposure for 1 h to the antitumor platinum complexes, *cis*-diamminedichloroplatinum(II), carboplatin or D,L-tetraplatin and/or to etanidazole (1 m*M*) for 2 h prior to and during exposure for 1 to the antitumor platinum complexes. These modulators alone did not significantly alter the cytotoxicity of CDDP toward either parental line. A twofold enhancement in cytotoxicity was observed with carboplatin in the 2008 cells and with D,L-tetraplatin in both parental lines with the single modulators. The modulator combination (buthionine sulfoximine/etanidazole) was very effective along with D,L-tetraplatin in both the MCF-7 parent and MCF-7/CDDP cell lines where at the higher platinum complex concentrations there was 1.5 to 3 logs increased killing of cells by the drug plus the modulators compared with the drug alone. Similarly, when C13 cells were exposed to CDDP (100 μ*M*) or D,L-tetraplatin (100 μ*M*) along with buthionine sulfoximine and etanidazole there was a 2-log increase in cell killing compared with exposure to the platinum complex alone. Treatment of each of the four cell lines with buthionine sulfoximine decreased both the non-protein and total sulfhydryl content of the cells. Treatment with the combination of modulators did not produce a further decrease in cellular sulfhydryl content compared with buthionine sulfoximine alone. The total sulfhydryl content in MCF-7 cells and 2008 cells exposed to buthionine sulfoximine and etanidazole was 58% and 31% of normal and the total sulfhydryl content of MCF-7/CDDP cells and C13 cells treated the same way was 54% and 23% of normal, respectively. DNA alkaline elution was used to assess the impact of exposure to the modulators, buthionine sulfoximine and etanidazole, alone and in combination on the cross linking of DNA by the antitumor platinum complexes in the MCF-7 and MCF-7/CDDP cell lines. Overall, the increases in DNA cross linking factors were greater in the MCF-7 cells than in the MCF-7/CDDP cells. These results indicate a possible clinical potential for this modulator combination.

Key words Buthionine sulfoximine · Etanidazole · Antitumor platinum complexes · Tetraplatin

Introduction

The clinical efficacy of platinum-based chemotherapy in ovarian and breast cancer is frequently compromised by the development of drug resistance or dose-limiting toxicity. The development of resistance and cross-resistance to chemotherapy is a major clinical problem in the treatment of breast and ovarian cancer [7, 33, 47, 49, 51]. Glutathione is the major intracellular non-protein sulfhydryl compound. One function of glutathione is to reduce the cytotoxic effects of endogenous or exogenous electrophiles, including chemotherapeutic alkylating agents [26, 41]. L-Buthionine-(*R, S*)-sulfoximine (BSO) is a selective inhibitor of γ-glutamylcysteine synthetase, causing depletion of glutathione in vitro and in vivo [25, 27, 66]. Calcutt and Connors [10] initially demonstrated the role of glutathione in mediating tumor cell sensitivity to the antitumor alkylating agent melphalan. Studies with

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S.E. Brooks
Department of Obstetrics and Gynecology, Division of Gynecological Oncology, University of Massachusetts Medical Center, Worcester, MA 01655, USA

T.T. Korbut · N.P. Dupuis · S.A. Holden · B.A. Teicher (✉)
Division of Cancer Pharmacology, Dana-Farber Cancer Institute and Joint Center for Radiation Therapy, 44 Binney Street, Boston, MA 02115, USA

L1210 leukemia cells and human ovarian carcinoma cell lines have demonstrated glutathione-mediated resistance to melphalan and reversal of this resistance following glutathione depletion mediated by BSO in both sensitive and resistant cells [9, 14, 24, 29, 37, 42, 48].

Glutathione has also often been implicated in the mechanism of resistance to platinum antitumor agents [2, 3, 9, 10, 14, 17, 21, 22, 24–29, 33, 36, 37, 39, 41, 42, 47–49, 56, 57, 60–62, 65, 66, 69–71, 76, 77, 80]. Previous studies have shown that glutathione depletion by BSO pretreatment can increase the sensitivity of cells to the cytotoxic actions of antitumor platinum complexes, that the degree of sensitization correlates with the degree of glutathione depletion and that greater sensitization may occur with Pt(IV) drugs (D,L-tetraplatin) than with Pt(II) drugs (cisplatin, carboplatin) [15, 31, 43, 44].

The 2-nitroimidazole radiosensitizer, etanidazole (ETA), is also a hypoxic cell-selective cytotoxic agent [16, 74] and a chemosensitizer or modulator of some antitumor drugs [30, 59, 72]. The mechanism(s) by which ETA act as a chemosensitizer are not known. The most important effects appear to occur at the level of the cell and may involve interaction of a metabolite of the 2-nitroimidazole with the alkylating agent in the vicinity of the DNA [20, 38, 40, 53, 59, 78]. Isobologram analysis [73] has been used to examine the possibility that ETA potentiates the tumor cell killing and tumor growth delay of cyclophosphamide, *cis*-diamminedichloroplatinum(II) (CDDP), and carboplatin alone and in combination in the FSaIIC fibrosarcoma. Bone marrow granulocyte-macrophage progenitor (CFU-GM) survival has been used to estimate the effects of the combinations on a representative normal tissue. The results of these preclinical studies suggest that ETA could significantly improve the therapeutic efficacy of antitumor alkylating agents individually or in combination [75].

The current study was undertaken to evaluate the ability of BSO and ETA in combination to potentiate the cytotoxicity of antitumor platinum complexes in human breast and ovarian tumor cell lines sensitive and resistant to CDDP.

Materials and methods

Drugs

CDDP was purchased from Sigma Chemical Co. (Milwaukee, Wis.). Carboplatin was purchased at the Dana-Farber Cancer Institute Pharmacy. D,L-Tetraplatin was a gift from Upjohn Co. (Kalamazoo, Mich.). BSO and ETA were gifts from Dr. Jill Johnson, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md.).

Cell lines

MCF-7 breast carcinoma

The MCF-7 human carcinoma of the breast cell line is estrogen receptor-positive. MCF-7 has been used extensively as a model for in vitro studies of breast carcinoma [68]. MCF-7 human breast carcinoma cells grow as monolayers in Dulbecco's minimal essential medium supplemented with antibiotics, L-glutamine, and 10% fetal bovine serum. This cell line has a plating efficiency of 25–40%.

2008 ovarian carcinoma

The 2008 cell line was established from a patient with serous cystadenocarcinoma of the ovary [4]. Human ovarian 2008 carcinoma cells grow as monolayers in RPMI-1640 medium supplemented with 5% fetal bovine serum, L-glutamine and antibiotics. The 2008 cell line was a gift from Dr. Paul Andrews.

CDDP-resistant cell lines

The MCF-7/CDDP-resistant cell line was established by weekly 1-h exposures to escalating concentrations of CDDP [69, 70, 76]. After 14 months of treatment, CDDP-resistant sublines were cloned from the treated cultures. Resistant sublines were screened for degree of resistance, similarity of generation time to that of the parent line, and relative stability of resistance (at least 2 months). Every 2 months, a vial of early-passage cloned cells was used to ensure that all experiments were carried out with the same subline [69].

The 2008/C13 CDDP-resistant cell line was established after 13 monthly selections of chronic exposure to escalating concentrations of CDDP [2]. The 2008/C13 cell line was a gift from Dr. Paul Andrews.

Survival experiments

The parental or CDDP-resistant cells in exponential growth were exposed to various concentrations of CDDP, carboplatin or D,L-tetraplatin for 1 h alone; along with exposure to BSO (50 μM) for 48 h prior to and during exposure to the platinum complex; along with exposure to ETA (1 mM) for 2 h prior to and during exposure to the platinum complex; or along with both BSO and ETA as described above. After treatments in medium with serum, the cells were washed three times with phosphate-buffered 0.9% saline and suspended by treatment with 0.25% trypsin. The cells were plated in duplicate at three dilutions for colony formation. After 2 weeks, the colonies were visualized by staining with crystal violet, and colonies of 50 or more cells were counted and compared with vehicle-treated control cells. Results were expressed as the surviving fraction of treated cells compared with vehicle-treated control cells [70, 76].

Sulfhydryl measurements

Parent cells and each of the resistant sublines in exponential growth (4×10^6) were lysed in 2 ml 5% perchloric acid. For the fluorescence assay, 0.1 ml sample was added to 3 ml 0.1 M potassium phosphate buffer containing 5 mM EDTA (pH 8.0), then 0.15 ml OPT (o-phthaldialdehyde; Aldrich, Milwaukee, Wis.) solution (1 mg/ml OPT in methanol) was added. Fluorescence was measured with an excitation wavelength of 350 nm and at an emission wavelength of 420 nm. The calibration curve was linear from 0.05 to 10 nmol of reduced glutathione (GSH/ml) [32, 76]. The data shown are the average of five determinations.

Non-protein and total sulfhydryl content were determined using a modification of the Ellman method [79, 82]. Protein sulfhydryl content was determined from the difference between the total sulfhydryl content and non-protein sulfhydryl content. Each measurement was repeated at least three times.

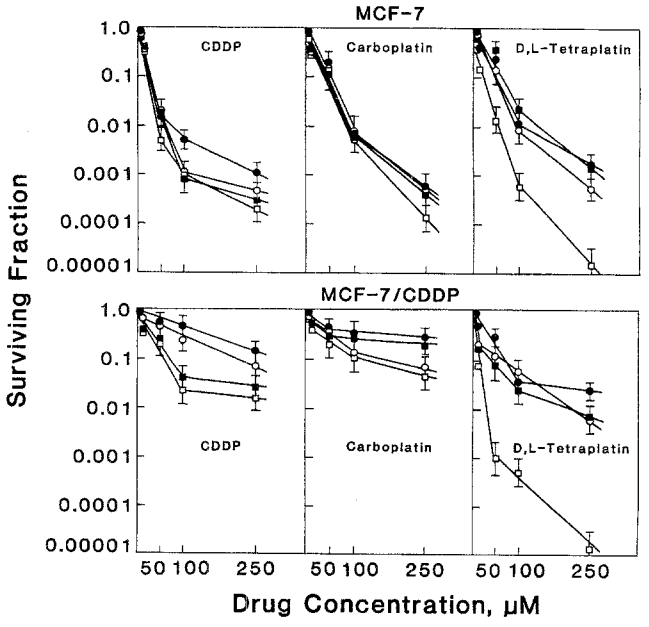

Fig. 1 Survival of MCF-7 human breast carcinoma cells and MCF-7/CDDP cisplatin-resistant human breast carcinoma cells exposed for 1 h to an antitumor platinum complex (●), to BSO (50 μ*M*) for 48 h prior to and during exposure to an antitumor platinum complex (○), to ETA (1 m*M*) for 1 h prior to and during exposure to an antitumor platinum complex (⊨) or to BSO (50 μ*M* for 48 h) and ETA (1 m*M* for 2 h) and an antitumor platinum complex as described above (⩓). Points are the means of three experiments, (*bars* are SEM)

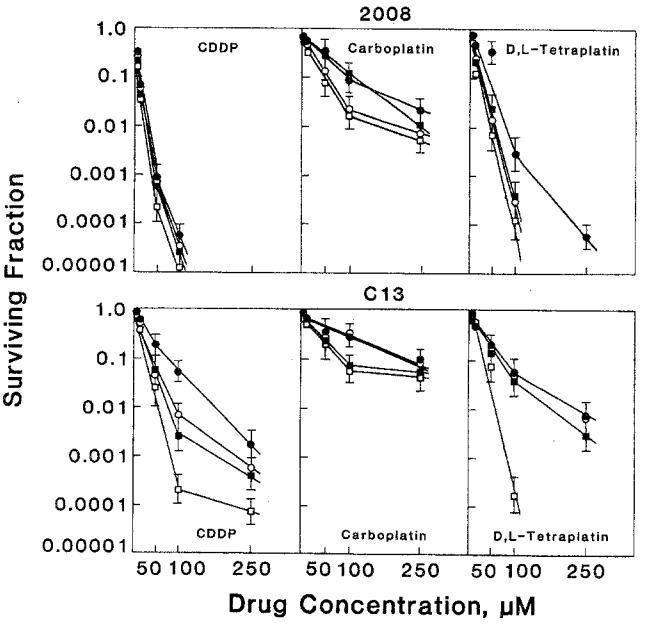

Fig. 2 Survival of 2008 human ovarian carcinoma cells and C13 cisplatin-resistant human ovarian carcinoma cells exposed for 1 h to an antitumor platinum complex (●), to BSO (50 μ*M*) for 48 h prior to and during exposure to an antitumor platinum complex (○), to ETA (1 m*M*) for 1 h prior to and during exposure to an antitumor platinum complex (⊨) or to BSO (50 μ*M* for 48 h) and ETA (1 m*M* for 2 h) and an antitumor platinum complex as described above (⩓). Points are the means of three experiments (*bars* are SEM)

DNA alkaline elution

Alkaline elution was performed by standard procedures [70, 76, 83, 84]. Samples were counted on an LS 7000 Beckman scintillation counter. Each point was measured in three independent experiments. The cross-linking factor was calculated as:

$$\frac{\log(\text{irradiated control/control})}{\log(\text{irradiated drug/control})}$$

Results

Upon exposure to CDDP, steep log-linear survival was maintained in the MCF-7 parent cell line through 2.5 logs of cell killing, but in the MCF-7/CDDP cell line through only 0.5 logs of cell killing after exposure to CDDP (Fig. 1). The kinetics of killing versus drug concentration in both the MCF-7 and MCF-7/CDDP cell lines slowed at higher concentrations of CDDP. The MCF-7/CDDP cell line was very resistant to carboplatin compared with the MCF-7 cell line, but maintained much of the sensitivity of the MCF-7 parent cell line to D,L-tetraplatin. The concentration of CDDP that killed 90% (1 log) of MCF-7 cells was 40 μ*M* and of MCF-7/CDDP cells was 250 μ*M* giving a relative resistance of about 6.3-fold. Exposure of the MCF-7 cells or MCF-7/CDDP to BSO (50 μ*M*) for 47 h prior to and during the 1-h exposure to the antitumor platinum complexes produced, in general, small increases in cell killing compared with the platinum complexes alone. Exposure of the MCF-7 cells or MCF-7/CDDP cells to ETA (1 m*M*) for 1 h prior to and during the 1-h exposure to the antitumor platinum complex also produced, overall, small increases in cell killing by the antitumor platinum complexes. Exposure to the combination of modulators (BSO, 50 μ*M*, 48 h; ETA, 1 m*M*, 2 h) produced a marked enhancement in the cytotoxicity of D,L-tetraplatin toward both MCF-7 cells and MCF-7/CDDP cells. At D,L-tetraplatin concentrations from 50 to 250 μ*M*, the addition of BSO and ETA resulted in 1.5 to 2 logs of increased cell killing compared with the drug alone. This modulator combination was also very effective along with D,L-tetraplatin in the MCF-7/CDDP cell line where over the platinum complex concentration range from 50 to 250 μ*M* there was 2 to 3 logs increased killing of cells by the drug plus the modulators compared with the drug alone.

The human 2008 ovarian carcinoma cells were very sensitive to CDDP showing log-linear cell killing through the 5-log range of sensitivity examined (Fig. 2). The corresponding CDDP-resistant subline designated C13 also showed log-linear killing by CDDP through 3 logs but with a much shallower slope on the survival curve. The concentration of CDDP required to kill 1 log (90%) of the 2008 cells was 80 μ*M* resulting in a relative resistance of 3.3-fold. Exposure of 2008 cells

Table 1 Protein and non-protein sulfhydryl content of MCF-7, MCF-7/CDDP, 2008 and C13 cells after exposure to BSO and/or ETA. Cells in exponential growth (4×10^6) were lysed in 2 ml 5% perchloric acid. For the fluorescence assay, 0.1 ml sample was added to EDTA (pH 8.0), then 0.15 ml of an OPT (*o*-phthaldialdehyde; Aldrich, Milwaukee, Wis.) solution (1 mg/ml OPT in methanol) was added. Fluorescence was measured with an excitation wavelength of 350 nm and at an emission wavelength of 420 nm. The calibration curve was linear from 0.05 to 10 nmol/ml of reduced glutathione. The data shown are the average of five determinations. Nonprotein and total sulfhydryl content were determined using a modification of the Ellman method. Protein sulfhydryl content was determined from the difference between the total sulfhydryl content and nonprotein sulfhydryl content. Each measurement was repeated at least three times. The figures in parentheses are the percentage decrease in nonprotein sulfhydryl content compared with untreated cells.

Treatment group	Sulfhydryl level (nmol/10^7 cells) Protein-SH	Non-protein-SH	Protein-SH	Non-protein-SH
	MCF-7 cells		MCF-7/CDDP cells	
Controls	537	203	636	262
BSO, 50 μM,48 hrs.	414	39(81)	449	80(70)
ETA, 1 mM, 2 hrs.	471	146(28)	607	167(36)
BSO/ETA	390	38(81)	445	42(84)
	2008 cells		C13 cells	
Controls	361	275	364	338
BSO, 50 μM, 48 hrs.	128	74(73)	127	75(78)
ETA, 1 mM, 2 hrs.	333	266(3)	213	235(31)
BSO/ETA	123	72(74)	107	56(83)

or C13 cells to BSO (50 μ*M*) for 47 h prior to and along with the 1-h exposure to the antitumor platinum complexes resulted, in general, in small increases in the cytotoxicity of the platinum antitumor agents. Exposure of the 2008 cells or C13 cells to ETA (1 m*M*) for 1 h prior to and during exposure to the antitumor platinum complexes also produced small increases in the cytotoxicity of the platinum antitumor agents with the greatest enhancement in cytotoxicity being seen in C13 cells exposed to CDDP. The combination of BSO and ETA was most effective in increasing the cytotoxicity of the antitumor platinum complexes especially in C13 cells exposed to CDDP or to D,L-tetraplatin. In the C13 cells exposed to 100 μ*M* of CDDP there was a 2-log increase in cell killing with the modulator combination with the drug compared with the drug alone. Similarly, when C13 cells were exposed to D,L-tetraplatin (100 μ*M*) along with BSO and ETA there was a 2-log increase in cell killing compared with exposure to D,L-tetraplatin alone.

Exposure of each of the four cell lines to the modulators, BSO and ETA, singly or in combination, produced marked changes in the protein, and especially the non-protein, sulfhydryl content of the cells (Table 1). The total sulfhydryl content of the MCF-7 cells was 740 nmol/10^7 cells while that of the MCF-7/CDDP cells was 898 nmol/10^7 cells. Treatment with BSO (50 μ*M*) for 48 h reduced the non-protein sulfhydryl content of the MCF-7 cells to 19% of normal and of the MCF-7/CDDP cells to 30% of normal. This treatment also decreased the protein sulfhydryl content of the cells so that the total sulfhydryl content of the MCF-7 cells was 453 nmol/10^7 cells (61% of normal) and of the MCF-7/CDDP cells was 529 nmol/10^7 cells (59% of normal). Exposure to ETA (1 m*M*) for 2 h resulted in smaller changes in cellular sulfhydryl content, so that cellular non-protein sulfhydryl content was reduced to 72% and 64% of normal and total cellular sulfhydryl content was reduced to 83% and 86% of normal in the MCF-7 cells and MCF-7/CDDP cells, respectively. The combination of BSO and ETA did not increase the overall sulfhydryl depletion from that obtained with BSO alone. After treatment with the modulator combination the total cellular sulfhydryl content of the MCF-7 cells was reduced to 58% of normal and of the MCF-7/CDDP cells was reduced to 54% of normal.

The total cellular sulfhydryl content of the 2008 cells was 636 nmol/10^7 cells while that of the C13 cells was 702 nmol/10^7 cells. The sulfhydryl content of the two ovarian carcinoma cell lines was more sensitive to exposure to BSO than the breast carcinoma cell lines. Treatment of 2008 cells with BSO (50 m*M*) for 48 h resulted in a decrease in non-protein content to 27% of normal and a decrease in total sulfhydryl content to 32% of normal. Treatment of C13 cells in the same way produced a decrease in non-protein sulfhydryl content to 22% of normal and a decrease in total sulfhydryl content to 29% of normal. ETA (1 m*M*, 2 h.) did not alter the sulfhydryl content of the 2008 cells but decreased the non-protein sulfhydryl content of the C13 cells to 69% of normal and decreased the total protein sulfhydryl content of the C13 cells to 64% of normal. As was seen in the MCF-7 cell lines, treatment with the combination of modulators, BSO and ETA, did not produce a further decrease in cellular sulfhydryl content compared with BSO alone. The total sulfhydryl content in 2008 cells exposed to BSO and ETA was 31% of normal and the total sulfhydryl content of C13 cells treated the same way was 23% of normal.

Table 2 Relative DNA crosslinking factors for human MDF-7 and MCF-7/CDDP breast carcinoma cells exposed to an antitumor platinum complex in the presence or absence of BSO and/or ETA. Exposure to the antitumor platinum complexes, BSO (50 μ*M*, 48 hrs.) and ETA (1 m*M*, 2 h) was as given in the legend of Fig. 1

Treatment	Relative DNA crosslinking factor	
	MCF-7 cells	MCF-7/CDDP cells
CDDP (50 μM, 1 h)	4.0	1.1
BSO/CDDP	8.2	1.8
ETA/CDDP	4.8	1.5
BSO/ETA/CDDP	17.0	3.0
Carboplatin (50 μM, 1 h)	2.4	1.5
BSO/carboplatin	9.0	2.8
ETA/carboplatin	7.0	2.0
BSO/ETA/carboplatin	10.6	4.7
D,L-tetraplatin (50 μM, 1 h)	5.6	5.5
BSO/D,L-tetraplatin	8.4	7.3
ETA/D,L-tetraplatin	6.8	6.6
BSO/ETA/D,L-Tetraplatin	13.0	10.6

The three antitumor platinum complexes studied produce similar bifunctional lesions in cellular DNA. DNA alkaline elution was used to assess the impact of exposure to the modulators, BSO and ETA, alone and in combination on the crosslinking of DNA by the antitumor platinum complexes in the MCF-7 and MCF-7/CDDP cell lines (Table 2). In both cell lines and with each of the three antitumor platinum complexes there was an increase in DNA crosslinking when the cells were exposed to BSO (50 μ*M*, 48 h) in addition to the platinum complex. In each case there was a small increase in DNA crosslinking when the cells were exposed to ETA (1 m*M*, 2 h) along with the platinum complex. Treatment with the combination of modulators along with the antitumor platinum complexes resulted in the highest levels of DNA crosslinking as determined by alkaline elution. DNA alkaline elution was carried out immediately upon completion of the 1-h exposure to the platinum complexes, allowing no time for DNA repair.

Discussion

The resistance of malignant cells to antitumor platinum complexes appears most often to be multifactorial [2, 3, 5, 70, 76, 77]. Even in tumor cells sensitive to the antitumor platinum complexes, however, the bifunctional reaction of the drugs with the critical target molecule, DNA, is inefficient. One of the most frequently observed changes in tumor cells that have developed resistance to CDDP is increased intracellular glutathione [3, 70, 76]. Exposure of each of the four cell lines to BSO for 48 h markedly decreased the glutathione content in the cells; however, the increase in the cytotoxicity of the antitumor platinum complexes under these conditions was relatively modest.

The combination of CDDP and cyclophosphamide has been well established in the treatment of ovarian carcinoma [1, 11]. In recent years, several clinical trials have been conducted comparing [19] the efficacy of carboplatin with CDDP alone [8, 34, 55, 67] and in combination with cyclophosphamide [1, 11] in ovarian carcinoma. Complete response rates in these clinical trials are still in the range of 20–35%, indicating that there is much room for improvement. A phase I clinical trial has been carried out to determine the maximum tolerated dose of ETA that can be administered along with carboplatin and cyclophosphamide in the treatment of advanced ovarian cancer [58]. A tolerated dose and schedule was found for ETA that results in serum levels of ETA comparable to those that produce chemosensitization in murine model systems. There was evidence of increased hematologic toxicity with the complete treatment regimen compared with cyclophosphamide and carboplatin alone.

Several studies have suggested that the combination of BSO with antitumor alkylating agents increases toxic effects in the host [22, 39, 57, 65]. Cardiac toxicity, hepatotoxic effects and enhanced nephrotoxic effects were observed following administration of BSO and several antitumor alkylating agents [22, 36, 65]. The timing of glutathione depletion to allow maximal enhancement of the therapeutic effect while minimizing the toxic effect will need to be identified for optimal use of BSO/alkylating agent combination therapy, perhaps using glutathione monoethyl ester as a normal tissue protector [39, 56, 71]. In preclinical in vivo studies, BSO plus melphalan has been shown to result in a significant increase in median survival over that produced by melphalan alone [21, 61, 62]. A phase I clinical trial of BSO administered in repeated short infusions along with melphalan has shown that the combination can be administered safely, but is not highly effective in producing glutathione depletion [6]. Another phase I clinical trial with BSO

administered by continuous infusion along with melphalan is currently underway [45].

The modulatory effects of BSO with the several organic antitumor agents described above led to increases in overall toxicity. The focus here is on platinum-based antitumor agents. BSO and ETA potentiate the cytotoxicity of these drugs by different mechanisms. It is possible through careful scheduling of the modulators that increased tumoricidal activity without increased normal tissue toxicity could be achieved using BSO/ETA and an antitumor platinum complex. The modulator combination, BSO plus ETA, was most effective at increasing the cytotoxicity of D,L-tetraplatin in all four of the cell lines studied. Tetraplatin is a second generation platinum complex in which platinum is in the 4 + oxidation state and which exists in two isomeric forms, D and L [54]. Most preclinical studies, both in vitro [18, 35, 46, 50, 54] and in vivo [52, 81], have been conducted with a racemic mixture (1 : 1 D:L) of the two tetraplatin isomers. In several in vitro studies, tetraplatin has been shown to be a more potent cytotoxin than CDDP and the degree of cross-resistance of CDDP-resistant cell lines to tetraplatin is less than to other platinum-containing anticancer agents, especially carboplatin [35, 46, 50]. D-Tetraplatin was, in general, a more potent cytotoxic agent in cell culture than CDDP or carboplatin, as determined by comparison of the drug concentration required to kill 1 log of cells [76]. Both tetraplatin isomers are rapidly biotransformed [12, 13, 23] and in rats the racemic mixture has been shown to be less nephrotoxic than CDDP [63, 64]. Thus tetraplatin may have substantial clinical potential, and may be especially effective when used in combination with BSO and ETA.

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