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ENHANCED MELPHALAN CYTOTOXICITY IN HUMAN OVARIAN CANCER *IN VITRO* AND IN TUMOR-BEARING NUDE MICE BY BUTHIONINE SULFOXIMINE DEPLETION OF GLUTATHIONE

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Abstract—The development of acquired resistance to alkylating agents frequently limits the effectiveness of chemotherapy in the treatment of ovarian cancer. While the resistance to alkylating agents is multifactorial, the association of drug resistance with elevations in glutathione (GSH) is of potential clinical relevance since there exist pharmacologic means to lower intracellular GSH levels. We have used *in vitro* and *in vivo* models of human ovarian cancer to demonstrate that selective inhibition of GSH synthesis with L-buthionine-*S*,*R*-sulfoximine (L-BSO) leads to a lowering of GSH levels and an increase in cytotoxicity of the alkylating agent melphalan. In the human ovarian cancer cell line NIH:OVCAR-3, derived from a patient clinically refractory to alkylating agents, L-BSO resulted in a 3.6-fold enhancement of melphalan cytotoxicity. This cell line was also adapted for intraperitoneal growth in athymic nude mice. In this *in vivo* model, in which the mice die of massive ascites and intraabdominal carcinomatosis, L-BSO given orally in drinking water for 5 days decreased GSH levels in the tumor cells by 96% compared to a 79 and 86% reduction in GSH levels in the bone marrow and gastrointestinal mucosa respectively. Lowering of GSH levels with BSO was not accompanied by an increase in lethality for melphalan in non-tumored nude mice. However, in tumor-bearing nude mice, a single melphalan (5 mg/kg) treatment following GSH depletion with L-BSO was markedly superior to treatment with melphalan alone, producing a 72% increase in median survival time. Furthermore, L-BSO treatment of human bone marrow cells prior to melphalan exposure had little effect on melphalan toxicity as assessed in a CFUc-GM assay. These results suggest that treatment with the GSH synthesis inhibitor BSO may preferentially enhance the cytotoxic effects of alkylating agents against human ovarian cancer and overcome acquired resistance.

Alkylating agents have a broad spectrum of clinical activity in the treatment of both hematologic malignancies and solid tumors. However, their effectiveness is frequently limited by the development of drug resistance. The consequences of alkylating agent resistance are particularly evident in the treatment of ovarian cancer [1]. While the majority of advanced ovarian cancer patients respond to alkylating agents, most patients become resistant to therapy and only 20–25% of patients have long-term disease-free survival [2]. The mechanisms responsible for the development of resistance to alkylating agents in ovarian cancer have only been partially characterized, although studies in other tumor systems have indicated that alkylating agent resistance is multifactorial and may involve drug transport, metabolism, and/or repair of damaged DNA [3–5].

We have demonstrated recently that a relationship exists between intracellular glutathione (GSH¶) levels and cytotoxicity to melphalan, cisplatin, and irradiation in human ovarian cancer cells lines [6–8]. Cell lines with resistance induced *in vitro* to either melphalan or cisplatin have a 2- to 3-fold elevation in intracellular GSH levels compared to the sensitive cell lines from which they were derived. Furthermore, when GSH levels are lowered with buthionine sulfoximine (BSO), a synthetic amino acid analog which specifically inhibits γ-glutamylcysteine synthetase, there is increased cytotoxicity of melphalan and cisplatin in both the drug sensitive and resistance cell lines [8]. In addition, depletion of GSH is associated with the reversal or cross-resistance to irradiation and some chemotherapeutic agents present in cell lines with acquired resistance to either melphalan or cisplatin [6–8]. These *in vitro* data suggest that the pharmacological manipulation of GSH levels may be of clinical benefit in the treatment of ovarian cancer.

It has been demonstrated previously that BSO administration to mice leads to a transient reduction in GSH levels in many normal tissues, including liver and kidney [9]. For BSO to increase the therapeutic index of alkylating agents requires increased cytotoxicity in tumor cells compared to normal tissues. To evaluate the potential of BSO for the treatment

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¶ Abbreviations: GSH, glutathione; BSO, buthionine sulfoximine; DMF, dose modifying factor; and MST, median survival time.

of ovarian cancer, we examined the effects of BSO plus melphalan in (1) an ovarian cancer cell line (NIH: OVCAR-3) established from a patient refractory to therapy with cisplatin and cyclophosphamide, (2) a xenograft model of human ovarian cancer (derived from OVCAR-3) which is characterized by the production of ascites and intraabdominal carcinomatosis, and (3) a CFUc-GM assay of human bone marrow cells. The demonstration of increased survival in the nude mouse model of human ovarian cancer and the absence of increased toxicity of BSO plus melphalan in human bone marrow cells or in the *in vivo* nude mouse system support the presence of a potentially clinically relevant differential effect of GSH reduction upon alkylating agent cytotoxicity in tumor cells compared to normal tissues. Such a differential effect is necessary to achieve a therapeutic advantage for the combination of BSO plus melphalan compared to merely increasing the dose of melphalan.

MATERIALS AND METHODS

Materials. D,L-Buthionine-*S*,*R*-sulfoximine (BSO) and the L-isomer (L-BSO) were obtained from Chemalog (Chemical Dynamics Corp., South Plainfield, NJ). The intravenous formulation of melphalan (Burroughs Wellcome Co., Research Triangle Park, NC) was obtained from the Investigational Drug Branch, Division of Cancer Treatment, National Cancer Institute. RPMI 1640, fetal bovine serum, penicillin/streptomycin, and glutamine were obtained from the Grand Island Biological Co., Chagrin Falls, OH.

Experimental models of human ovarian cancer. NIH:OVCAR-3 is the cell line derived by us from the malignant ascites of a patient with adenocarcinoma of the ovary refractory to combination chemotherapy with adriamycin, cyclophosphamide, and cisplatin. The characteristics of the cell line have been described previously in detail [10]. Briefly, the cells grow as an irregular cobblestone-like monolayer, clone in agarose, have an abnormal karyotype, contain androgen and estrogen receptors, estrogen inducible progestagen receptors, and form subcutaneous tumors in nude athymic mice [10, 11]. The cell line is more resistant to adriamycin, cyclophosphamide, and cisplatin compared to cell lines from previously untreated patients [7].

OVCAR-3 is maintained in RPMI 1640 medium containing fetal bovine serum (10%, v/v), insulin (0.25 units), penicillin (100 units), streptomycin (100 μg/ml) and glutamine (0.3 mg/ml). Passage of the cells is accomplished at weekly intervals with trypsin/EDTA (0.05%/0.02%, w/v) with split ratios of 1:3 to 1:6 and cultures are maintained at 37° in a humidified atmosphere of CO_2 (5%, v/v) in air.

We have adapted OVCAR-3 through *in vitro* and *in vivo* selection procedures to grow intraperitoneally in nude athymic mice, resulting in a highly representative *in vivo* model of human ovarian cancer [12]. The mice develop massive ascites and widespread intraabdominal solid tumors with surface invasion of intraperitoneal organs. The tumor is passaged *in vivo* to subsequent hosts by transfer of cells obtained from the ascites. Implantation of 40 million cells results in death from intraabdominal carcinomatosis by day 50 which allows adequate time for assessment of any therapeutic effects prior to the time when nude mice are at an increased risk to die from infections (>120 days). The malignant cells *in vivo* have the same histology, karyotype, and receptor characteristics as OVCAR-3 *in vitro*. The ascites supernatant fraction and the serum of OVCAR-3 bearing animals also have high concentrations of the CA125 ovarian cancer antigen [13], further confirming the relevance of this model of human ovarian cancer.

GSH measurements. We have demonstrated previously that GSH content of tumor cells in tissue cultures is dependent on cell density [14]. Consequently, all comparisons of GSH levels of tumor cells were performed 3 days after subculture during logarithmic growth. Cultured cells (5×10^6) were harvested with trypsin/EDTA and, after washing with phosphate-buffered saline, were lysed by hypotonic shock. Total GSH was measured by the method of Tietze [15] on a protein-free filtrate of the lysate. GSH was determined similarly on ascites cells, bone marrow cells, and gastrointestinal mucosa removed from tumor-bearing nude mice (see below). For cultured tumor cells, ascites tumor cells, and bone marrow cells, the results are expressed as nmoles of GSH per 10^6 cells ± standard error of the mean of repeat determinations. For gastrointestinal mucosa, the results are expressed as nmoles of GSH per mg of DNA.

In vitro *cytotoxicity studies with* L-*BSO*. OVCAR-3 cells were plated in 24 culture dishes at 200,000 cells/25 cm^2 dish. After 24 hr, twelve cultures (series B) were switched to a medium containing L-BSO (50 μM), while a replicate series (A) received a standard medium change. After an additional 24 hr, melphalan (final concentration 0.05, 0.10, 0.2, and 0.5 μM) was added to individual cultures in the presence (series B) or absence of L-BSO (series A). After 24 hr, the cells were harvested with trypsin and cloned in agarose as previously described at 50,000 cells/dish [6, 10]. After 3 weeks of growth, colonies >60 microns containing more than 50 cells [16] were enumerated on an Omnicon FAS II Image Analysis System (Bausch & Lomb Co., Rochester, NY). GSH content of cells was also determined on replicate cultures (series A and B) without melphalan exposure but after BSO treatment (series B).

Depletion of GSH in tumor-bearing nude mice. The following studies were performed to determine the efficacy and feasibility of two methods of L-BSO administration to tumor-bearing mice. Initially, six mice with ascites were anethesized with ether, and time-release pellets containing 84 mg of BSO (Innovative Research of America, Rockville, MD) were surgically implanted in the peritoneal cavity. This procedure resulted in a 90% reduction of cellular GSH in ascites tumor cells (5.12 nmoles/10^6 cells vs 0.52 nmoles/10^6 cells after BSO). However, the necessity for general anesthesia and the high surgical mortality in athymic nude mice made such an approach relatively unsatisfactory for large scale animal survival studies with melphalan plus L-BSO. We subsequently evaluated the effect of L-BSO added to the drinking water of tumor-bearing animals upon

GSH levels in tumor cells, gastrointestinal mucosa, and bone marrow cells. The effects of L-BSO upon GSH levels in these two normal tissues were examined since gastrointestinal toxicity and myelosuppression are the dose-limiting toxicities of melphalan [17]. Six mice were inoculated intraperitoneally with 40×10^6 OVCAR-3 cells, and after 2 days L-BSO (30 mM) was added to the drinking water of three mice. Five days later the mice were killed between 1:00 and 3:00 p.m., and the tumor cells were harvested by peritoneal lavage. A 5-cm section of duodenum was removed and slit lengthwise, and the mucosa was scraped with a glass slide and transferred to iced phosphate-buffered saline [18]. Bone marrow cells were harvested from the tibia by first stripping the muscles from the bone and expressing the cells from the marrow cavity into iced buffer using a syringe and a 25-gauge needle [18]. Cell counts were obtained on the peritoneal washings and bone marrow collections while DNA was extracted from the mucosal scrapings by the method of Schneider [19], and the DNA content was determined by the method of Burton [20].

Effect of melphalan plus or minus L-BSO upon survival. The effect of L-BSO-mediated GSH depletion on melphalan-induced prolongation of survival in OVCAR-3 bearing mice was performed as follows: mice (N = 90) were inoculated i.p. with 50×10^6 OVCAR-3 ascites cells. Three days after tumor transplantation forty-five animals were placed on drinking water containing L-BSO (30 mM) which was continued through day 9. On day 8 after transplantation, fifteen mice from the group receiving L-BSO water and fifteen mice receiving plain water were treated with 5 mg/kg melphalan, i.p. An additional fifteen animals from each group received 10 mg/kg melphalan, i.p. Controls consisted of thirty tumored animals who received no melphalan and were maintained on either plain water (fifteen animals) or L-BSO-containing water (fifteen animals). All animals were followed daily for survival for up to 130 days. A separate experiment with an additional forty tumor-bearing mice was also performed. Twenty mice were placed on a L-BSO drinking water regimen from day 2 to 8, and ten of these mice received a single injection of 5 mg/kg melphalan, i.p., on day 7 after transplantation. The other twenty mice received plain drinking water, and ten mice in this group received a single dose of 5 mg/kg melphalan, i.p., also on day 7 after transplantation. Animals were followed for survival as in the previous experiment.

In separate toxicity studies, non-tumor-bearing nude mice (five to ten animals per group) were maintained on BSO drinking water (30 mM) for 4 days and treated with a single injection of 5–15 mg/kg melphalan with 15 mg/kg melphalan considered to be the maximum tolerated dose (MTD) of the drug.

CFUc-GM assay of human bone marrow cells. The effect of BSO upon melphalan cytotoxicity in human bone marrow cells was assessed in a CFUc-GM assay using bone marrow aspirates obtained from normal volunteers and from cancer patients between cycles of chemotherapy. Human bone marrow cells were maintained in liquid culture for 20–24 hr either in the absence or presence of BSO (25 μM), after which the cells were treated with melphalan at three different concentrations in the absence or continued presence of BSO. After a 4-hr exposure to melphalan (estimated to be 2–4 half-lives of the drug), the CFUc-GM assays were performed in semisolid agar by the method originally described by Pike and Robinson [21]. Briefly, bone marrow mononuclear cells were separated by Ficoll–Hypaque (1.070 g/ml) (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation and resuspended in Minimal Essential Medium containing 2.5% pooled human AB serum. Cell counts were by a hematocytometer and dilutions were made with McCoy's 5A medium. Cells that had been exposed previously to melphalan and BSO were then plated at 1×10^5 mononuclear cells/well in McCoy's 5A, 20% fetal calf serum, 0.3% agar and 10% colony-stimulating factor. The colony-stimulating factor was prepared fresh from human placental conditioned medium as previously described [22]. After 10–14 days of incubation at 37° in a humidified atmosphere of 5% CO_2 in air, colonies (≥40 cells) were scored using an inverted microscope.

Cytochemical analysis of the colonies was performed by the method of Kubota *et al.* [23]. CFUc-M (monocyte-macrophage) colonies were identified by staining with alpha-naphthyl butyrate esterase. CFUc-G (granulocyte) colonies were recognized by staining with naphthol chloroacetate esterase [24].

RESULTS

In vitro *effects of L-BSO upon melphalan cytotoxicity in OVCAR-3.* The GSH level in OVCAR-3 was 7.9 ± 1.3 nmoles/10^6 cells at the time cytotoxicity experiments were performed. While no correlate cell line from the same patient exists prior to therapy, the GSH value in OVCAR-3 was 4-fold greater than the GSH value in a cell line from a previously untreated patient (A2780: 1.9 nmoles/10^6). In previous studies we have reported that OVCAR-3 is 2.44 times larger than A2780 [25]; thus, if GSH values are normalized to cell volume, OVCAR-3's GSH content is 1.7 times that of A2780. L-BSO at a concentration which reduced cloning efficiency by less than 10% and had little effect on colony size depleted GSH levels to 0.52 ± 0.2 nmoles/10^6 cells (6.6% of control) in OVCAR-3. This degree of GSH depletion resulted in a shift in IC_{50} concentrations for melphalan from 0.44 to 0.12 μM (Fig. 1), with a resulting dose modification factor (DMF) (ratio of IC_{50} for melphalan/IC_{50} for melphalan + BSO) of 3.6.

Effect of L-BSO upon GSH levels in normal murine tissues and tumor cells in vivo. The effects of 5 days of administration of L-BSO (30 mM) in drinking water on GSH levels in tumor cells, bone marrow, and gastrointestinal mucosa are compared in Table 1. There was a 96% reduction of GSH levels in the tumor cells. Similarly, GSH levels in the bone marrow and gastrointestinal mucosa were reduced by 79 and 88%, respectively, following administration of L-BSO.

Effect of BSO upon survival and toxicity in tumor-bearing nude mice. The effects of a single injection

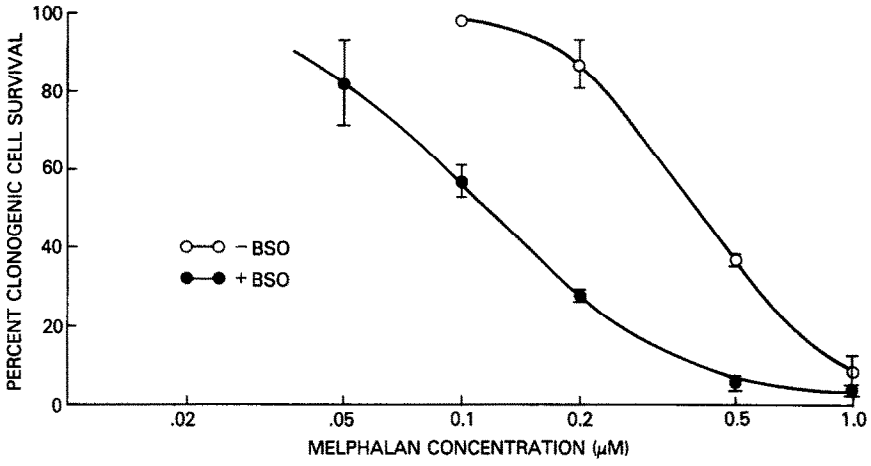

Fig. 1. Augmentation of melphalan cytotoxicity by L-BSO-mediated GSH depletion. Each point on the dose–response curves is the mean of six determinations performed on two separate occasions; bars are standard error of the mean. Percent clonogenic cell survival was calculated by $(C_p/C_a) \times 100$, where C_p represents colonies formed in the presence of melphalan, and C_a represents colonies formed in the absence of melphalan but in the presence of BSO for series "B". This exposure protocol results in less than a 10% reduction in clonogenic cell survival from BSO alone (50 μM) compared to cells maintained for 72 hr in standard growth medium. This BSO exposure resulted in reduction of GSH from 7.9 nmoles/10^6 cells in control cells to 0.52 nmoles/10^6 cells in BSO-treated cultures (>90%).

of melphalan (5 mg/kg or 10 mg/kg) upon survival of nude mice bearing the transplantable intraperitoneal human ovarian carcinoma are shown in Fig. 2. Melphalan was administered at a time when the tumor was well established in the host (day 8 after inoculation of 50×10^6 tumor cells, i.p.). In this model of human ovarian carcinoma, there was a dose-dependent effect of melphalan on survival. The median survival times (MST) with 5 and 10 mg/kg doses were 75 and 102.5 days, respectively, compared to 46 days for control animals. Administration of L-BSO for 5 days had no effect on survival in tumor-bearing mice (MST = 43 days). L-BSO treatment of tumor-bearing animals prior to the administration of melphalan (5 mg/kg) resulted in a MST of 105 days. Thus, a combination of 5 mg/kg melphalan and L-BSO was equal to efficacy to the dose of 10 mg/kg of melphalan alone. The addition of L-BSO to the 10 mg/kg melphalan regimen also resulted in an increase in survival compared to melphalan alone. Furthermore, there were 3/15 long-term survivors (>130 days) in this latter group of mice, whereas while there was only one long-term survivor in the group treated with melphalan (10 mg/kg) alone.

The results of this ninety animal experiment were confirmed in a separate experiment with forty animals in which the effect of 5 mg/kg melphalan was compared to the same dose of melphalan plus L-BSO. In this duplicate experiment, the MST for the control animals was again 46 days, whereas the MST for 5 mg/kg dose of melphalan was increased to 68 days. However, L-BSO had a greater potentiating effect with melphalan than observed in the previous experiment: median survival in animals receiving 5 mg/kg melphalan plus L-BSO had not been reached at 125 days at which time the experiment was terminated.

In non-tumor-bearing nude mice, administration of BSO in the same dose range as described for the tumor-bearing mice had no effect on the lethality of

Table 1. Effect of oral L-BSO on mouse tissue GSH content and OVCAR-3 ascites GSH content

Tissue	Control GSH content	GSH content after oral L-BSO	Percent reduction
Bone marrow (nmoles/10^6 cells)	0.33 ± 0.10	0.07 ± 0.02	79
Gastrointestinal mucosa (nmoles/mg DNA)	0.52 ± 0.11	0.06 ± 0.02	88
Ascites (nmoles/10^6 cells)	12.36 ± 1.51	0.54 ± 0.22	96

Nude mice were inoculated with 40×10^6 OVCAR-3 ovarian cancer cells, and after 2 days were placed on drinking water which contained 30 mM L-BSO. Five days later GSH levels were measured in tumor cells and in normal tissues. Values are mean ± one SD.

Table 2. Effect of BSO on melphalan myelosuppression in the CFUc-GM assay % Survival compared to control

	Median age	L-BSO	MEL, 0.05 μM	MEL, 0.05 μM, + L-BSO	MEL, 0.10 μM	MEL, 0.10 μM, + L-BSO	MEL, 0.50 μM	MEL, 0.50 μM, + L-BSO
Normal volunteers (N = 5)	22	100 ± 6	92 ± 4.4	92 ± 6.0	70 ± 9.8	71 ± 11.0	42 ± 8.5	37 ± 11.0
Chemotherapy patients (N = 4)	24	98 ± 1.4	77 ± 8.1	81 ± 11.0	60 ± 9	58 ± 8	30 ± 8.1	25 ± 12

Human bone marrow aspirates were incubated with and without BSO for 24 hr after which the cells were treated with melphalan at three different concentrations. CFUc-GM assays were performed, and the percent survival in colonies after exposure to melphalan ± BSO was assessed. Values are mean ± SEM.

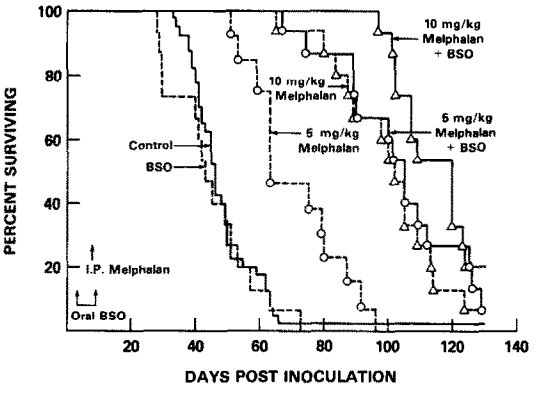

Fig. 2. Prolongation of survival of nude mice bearing intraperitoneal OVCAR-3 cells by L-BSO in combination with melphalan versus melphalan alone. Survival of untreated controls (——), L-BSO controls (---), melphalan-treated animals [5 mg/kg, (--0--); 10 mg/g, (--△--)], and animals treated with melphalan while receiving oral L-BSO [5 mg/kg, (—0—); 10 mg/kg, —△—] are shown above. Percent survival was calculated by $(C_T/15) \times 100$, where C_T represents animals surviving at the given day in the appropriate treatment or control group.

melphalan. The absence of any major effects of BSO upon melphalan toxicity in the non-tumored nude mice were confirmed by studies in the tumor-bearing nude mice. In these animals there was no acute lethal toxicity of L-BSO plus melphalan and there was only a minor degree of weight reduction in these animals (at day 8 of experiment mean weight was 18 g for BSO controls and BSO + melphalan 5 or 10 mg/kg) compared to those animals receiving standard drinking water (mean weight 23 g). This change was associated with a reduced fluid intake of approximately 50% for animals on BSO containing water.

Effect of BSO upon melphalan cytotoxicity in CFUc-GM assay. The effects of BSO upon melphalan cytotoxicity in CFU assay of bone marrow aspirate from normal volunteers and cancer patients is shown in Table 2. There was a dose-dependent reduction in colony formation following exposure of bone marrow cells to melphalan (0.05 to 0.5 μM), producing the same IC_{50} in bone marrows obtained from cancer patients undergoing chemotherapy as in marrows from normal volunteers. Incubation of the bone marrows with BSO for 24 hr prior to treatment with melphalan did not increase melphalan cytotoxicity significantly. The effect of BSO treatment upon GSH levels in human bone marrow was not determined due to insufficient amounts of marrow available for this study, since the major portion of the marrow was reserved for clinical and other research studies.

DISCUSSION

The results presented in this paper demonstrate that L-BSO can potentiate the cytotoxic effects of melphalan both *in vitro* in a human ovarian cancer cell line and *in vivo* in a nude mouse model of human ovarian carcinoma. In contrast to the improved survival in tumor-bearing nude mice, BSO did not

potentiate the lethality of melphalan in non-tumor-bearing mice nor did it increase significantly the cytotoxicity of melphalan in human bone marrows from volunteers and cancer patients as assessed in an *in vitro* CFUc-GM assay in agreement with previous studies on mouse bone marrow after GSH reduction by either diet [26] or BSO [27].

The mechanisms responsible for the potentiating effects of L-BSO upon melphalan cytotoxicity in human ovarian cancer cells have not been established. It is generally accepted that the critical cellular target for alkylating agents is DNA, although the exact molecular mechanisms by which interstrand DNA cross-links lead to cell death have not been established. Several investigators [4, 28, 29] have demonstrated a relatively consistent correlation between the sensitivity of various cell lines to bifunctional alkylating agents and the degree of interstrand cross-linking. There are multiple potential cellular mechanisms which could result in decreased cross-linking in alkylating agent resistant cells, including (1) a decrease in uptake of the alkylating agent, (2) metabolism of the active drug to a less cytotoxic intermediate, (3) interaction of the electrophilic alkylating agent with a non-critical nucleophile, thereby decreasing the amount of active species available for interaction with DNA, (4) increased repair of interstrand DNA cross-links, and (5) a quenching (or repair) of monoalkylated DNA prior to formation of cytotoxic interstrand cross-links by bifunctional alkylating agents. It is also possible that any combination of these mechanisms could account for alkylating agent resistance. Similarly, it is possible that the increased levels of GSH may exert a protective effect from alklylating agent cytotoxicity at numerous sites within the cell. Suzukake *et al.* [5] demonstrated that melphalan resistance in L1210 leukemia cells is accompanied by elevations in GSH and that melphalan is metabolized to a less cytotoxic intermediate (dihydroxy melphalan) at a greater rate than observed in drug-sensitive L1210 cells. We previously demonstrated that, in a human ovarian cancer cell line with acquired resistance to melphalan, there also is an increased formation of the inactive dihydroxy derivative of melphalan [6]. The enzymes responsible for this metabolic deactivation of melphalan have not been determined although it is possible that GSH linked *S*-transferases may play a role in this process. We previously reported that, in a cell line with acquired resistance to melphalan, there was a 20% increase in the activity of GSH linked transferases compared to the sensitive cell line from which it was derived [8].

It is also possible that increased levels of GSH decrease DNA alkylation and cross-link formation or facilitate repair of cytotoxic cross-links once they have been formed. Conceivably, GSH melphalan adducts are formed in the cytosol which decrease the availability of reactive species to interact with nuclear DNA protein complexes. Consistent with this hypothesis is the observation that GSH depletion in Chinese hamster ovary cells by misonidazole or diethylmaleate increases both the binding of melphalan to macromolecules and the formation of cross-links [30]. The direct assessment of the role of GSH in cross-link formation and repair in human ovarian cancer cells, however, has not been determined, and experiments aimed at establishing this relationship are currently in progress in our laboratory. The results also suggest that L-BSO selectively potentiates the cytotoxicity of melphalan in tumor cells compared to normal tissues. While the administration of L-BSO in drinking water to mice for 5 days led to similar reductions in GSH in tumor cells (96%), gastrointestinal cells (88%), and bone marrow cells (79%) (Table 1), the reduction in GSH in normal tissues did not lead to any increased lethality over the range of melphalan used. In addition, in non-tumored animals the estimated MTD of melphalan (15 mg/kg) in combination with BSO did not result in lethality. It is possible, however, that at higher melphalan doses the addition of BSO could increase the lethality of melphalan alone. The mechanisms responsible for this preferential effect of L-BSO plus melphalan on increasing cytotoxicity in tumor cells compared to normal tissues have not been determined. OVCAR-3 cells have a forty times higher intracellular GSH content than murine bone marrow cells (Table 1), and it is possible that tumor cells require higher levels of GSH for their homeostasis and that reductions in GSH make these cells more vulnerable to the cytotoxic effects of chemotherapy compared to normal tissues [31]. It is also possible that there are alternate mechanisms not associated with GSH levels which protect normal cells from the effects of alkylating agents which are absent or less functional in tumor cells.

While the exact mechanisms responsible for the effect of BSO upon potentiating the cytotoxicity of melphalan *in vitro* and *in vivo* in tumor cells have not been established, the demonstration that survival can be prolonged in a relevant model of human ovarian carcinoma suggests that L-BSO may be of therapeutic value in the treatment of patients with ovarian cancer. If L-BSO leads to a similar dose modifying factor in cancer patients such that the effective dose of alkylating agents or cisplatin can be increased 3- to 4-fold, then it may represent an important new therapeutic modality. We have demonstrated previously the clinical relevance of dose increases of this magnitude or less in phase II trials of high dose therapy with either cisplatin or the non-nephrotoxic cisplatin analog carboplatin in refractory ovarian cancer patients [32, 33]. In these trials the dose of platinum drugs was doubled and administered to patients who had progressive disease on standard dose cisplatin regimens. A 2-fold increase in the dose of cisplatin or carboplatin produced objective response rates of 32 and 35%, respectively, and a prolongation of survival was observed in those patients responding to treatment. Thus, if L-BSO can be safely administered to cancer patients, and if it produces the same dose modifying effect as observed in our experimental model systems of ovarian cancer, it may lead to improved therapy in those tumors in which there is a steep dose–response relationship. However, it must be emphasized that GSH depletion may increase the toxicity of some antineoplastic drugs (e.g. doxorubicin and nephrotoxic alkylating agents) as well as of those non-cancer drugs (e.g. acetominophen) in which GSH is required for metabolism and detoxification

[34]. Consequently, careful preclinical toxicology studies of BSO plus anticancer agents will be required prior to any clinical trials of drug combinations.

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