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# Glutathione depletion increases the cytotoxicity of melphalan to PC-3, an androgen-insensitive prostate cancer cell line

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**Summary.** Prostate cancer that is androgen-insensitive is unresponsive to a wide spectrum of cytotoxic agents, including all of the alkylating agents. Since a major pathway for the detoxification of the alkylating agents is conjugation with glutathione (GSH), GSH depletion has proved to be effective as a technique to restore melphalan sensitivity in melphalan-resistant cancer cell lines. However, the effect of GSH depletion has not been widely studied in tumor cell lines that have not developed resistance due to previous exposure to alkylating agents. Thus, we decided to investigate GSH depletion as a technique to increase melphalan cytotoxicity to PC-3 cells, an androgen-insensitive prostate cancer line. After 2 and 6 h incubation with 0.25–5 μM melphalan, virtually no effect was observed on either clonogenic lethality or MTT viability until 5 μM exposures. A 24-h incubation of the cells with 100 μM buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, reduced the GSH content by 70%–75%. Following GSH depletion, an increase in clonogenic lethality and a decrease in MTT viability occurred after exposure to concentrations as low as 0.25 μM. The dose modification factor ranged from 2.9 after 2 h incubation to 4.5 at 6 h. These results provide support for additional studies in prostate cancer for further investigation of GSH depletion as a technique to induce sensitivity to alkylating agents in this chemotherapy-resistant tumor.

## Introduction

Prostatic cancer is the second leading cause of cancer deaths in men in the United States and resulted in 32,000 deaths in 1991 [35]. At diagnosis, up to 30%–50% of patients have disseminated disease [20]. Currently, therapy of disseminated disease is palliative and consists of testicular androgen suppression [11]. However, hormonal therapy is effective only against tumors that are androgen-dependent. Despite an initial response in 70%–80% of patients, most with systemic disease relapse within 1–2 years after initial endocrine therapy [22], with their mean survival being less than 40 weeks after relapse [34]. Many antineoplastic drugs, including melphalan, have been studied individually in the treatment of hormone-refractory disease, with the majority being either ineffective or, at best, marginally effective [6, 26, 29, 38]. Unlike in many other carcinomas, combination chemotherapy has not produced any increase in survival or objective response [21, 25, 28].

There is increasing interest in developing techniques to improve the response to currently available antineoplastics. Most of these have been directed to understanding and then modulating resistance mechanisms in tumors that have become resistant to chemotherapy. An example of this is found with carcinoma of the ovary, which, after showing an initial response to melphalan, rapidly becomes resistant. One technique employed to restore sensitivity of resistant tumors to melphalan is the inhibition of glutathione-S-transferase (GST) [37]. An increase in GST has also been found to be associated with increased tumor resistance to alkylating agents. Partial inhibition of GST by ethacrynic acid sensitized human melanoma cells to the cytotoxicity of melphalan [16]. Another possible source of resistance, particularly to alkylating agents, is an increase in the glutathione (GSH) content of the tumor. Subsequent depletion of GSH will restore the initial alkylating-agent sensitivity [5]. The most common method used to deplete GSH is inhibition of synthesis. This is accomplished by buthionine sulfoximine (BSO), which competes for cysteine on the active site of γ-glutamylcysteine synthase, a key enzyme in the synthesis of GSH [12].

Melphalan is a bifunctional alkylating agent that primarily exerts its cytotoxicity by forming DNA cross-linkages [15]. Although melphalan is active against a wide variety of neoplasms, it is only marginally active as a single agent in the treatment of prostatic cancer [17]. The cytotoxicity of melphalan has been increased by reducing

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the intracellular GSH content of a variety of tumor cell lines such as rhabdomyosarcoma [32], human ovarian carcinoma [9, 12, 14], and medulloblastoma [10].

Two studies have examined the role of GSH in the resistance of prostate cancer to chemotherapy. The first demonstrated increased GSH content and GST activity in chemotherapy-resistant as compared with -sensitive cell lines [30], and the second found that decreasing GSH increased the cytotoxicity of monoaziridinylputrescine toward a prostatic cancer cell line [31]. On the basis of the favorable results reported for the effect of GSH depletion in increasing the cytotoxicity of melphalan to other epithelial carcinomas, we investigated the effect of GSH depletion on the cytotoxicity of melphalan to an androgen-insensitive prostate cell line, PC-3.

## Materials and methods

*Cells.* PC-3 cells (ATCC CRL 1435) were maintained in RPMI media with 10% fetal calf serum supplemented with 0.2 mM L-glutamine and 2.57 Units insulin/ml. Streptomycin (100 μg/ml) and penicillin G (100 Units/ml) were added to sterile media to prevent bacterial contamination. All of the reagents used for cell culturing were purchased from Sigma Chemicals (St. Louis, Mo.). Streptomycin was purchased from Gibco (Detroit, Mich.) and penicillin G, from Bristol Myers Squibb (Evansville, Ind.).

*Reagents and chemicals.* Melphalan was provided courtesy of Alfred Guaspari, Burroughs Wellcome Co. (Research Triangle Park, N. C.). BSO, sulfosalicylic acid, *N*-ethylmorpholine, and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma. The monobromobimane used in the GSH assay was purchased from Calbiochem (La Jolla, Calif.).

*Analytical methods.* The GSH content in the PC-3 cells was determined by the bromobimane derivativization method of Anderson [3] for sulfhydryls. After homogenization by sonication in 0.1 M phosphate buffer (pH 7.0), the lysate was centrifuged at 12,500 g for 5 min. Then, 80 μl 5% sulfosalicylic acid was added and the mixture was centrifuged as before. To 48 μl of the supernatant was added 136 μl distilled water and 40 μl 1 M *N*-ethylmorpholine. The solution was mixed in a 2-ml plastic conical vial and 5 μl (78 μg) monobromobimane was added. This was gently mixed, covered with aluminum foil, and mixed for 20 min. Then, 6.9 μl glacial acetic acid was added to stop the reaction. The sample was covered with aluminum foil and stored at −10° C until assayed, usually within 1 week.

High-performance liquid chromatographic (HPLC) analysis was conducted on a 15×5.7-cm C18 reverse-phase column (Supelco) with fluorometric detection (excitation wavelength, 350 nm; emission wavelength, 454 nm). GSH was eluted isocratically at approximately 8 min using methanol in water (15:85, v/v) with 0.15% acetic acid (pH 4.0). After GSH had eluted, the column was washed with methanol in water (80:20, v/v) for 20 min.

*Viability and lethality assays.* Viability was assessed by the ability of a cell to metabolize MTT to the formazan derivative with subsequent fluorometric determination [27]. After the seeding of $5\times10^3-5\times10^5$ cells/well on a 96-well plate and an incubation with either melphalan (0–10 μM) or melphalan+BSO for 24 h, 10 μl of a 10-mg/1-ml solution of MTT in normal saline was added to each well and the incubation was continued for an additional 4 h. The media was aspirated and 100 μl of a solution of 90% isopropyl alcohol in 0.1 *N* HCl was added. The plate was gently rocked for 3–5 min to dissolve the formazan. The plates were then read at 570 nm in a plate reader (Titertek Multiskan, Flow Laboratories, McLean, Va.). Values were calculated as the percentage of fluorescence as compared with control levels (no melphalan, 100%).

Lethality was assessed using a modification of the limiting-dilution technique for clonogenic growth [2, 39]. In this method, following trypsin removal of the cells from flasks in which the cells were grown to confluence, between 2 and $6\times10^5$ cells were added to each well of a six-well plate and incubated for 24 h to exponential growth (35%–55% confluence) either with or without 100 μM BSO (BSO in this concentration was found to have no effect on clonogenic lethality). After the cells had reached exponential growth, 0.25–5 μM melphalan was added and the incubation was continued for an additional 2 or 6 h. For the GSH-depletion experiments, BSO was also present during the melphalan incubation. At the end of the incubation period, the cells were released with trypsin, pelleted, and resuspended in fresh media. Each pellet was reconstituted with RMPI media to a concentration of $6\times10^4-2.5\times10^5$ cells/ml. A series of eight serial 5-fold dilutions were made. Using a 96-well flat-bottom microtiter plate and RMPI as the incubation media, an aliquot of 100 μl of each dilution was plated into a row of 6 wells containing 100 μl medium/well. The plates were incubated for 2 weeks in room air with 5% $CO_2$ at 37° C, whereupon they were removed and each well was assessed for the presence or absence of clonogenic growth (minimum of ten cells in a colony).

Data are expressed in the figures as the mean values (±SEM) for four to five replicate experiments. The 50% growth-inhibitory concentration ($IC_{50}$) used in the calculation of the dose modification factor (DMF) was extrapolated from a straight line formula derived from the log survival data. The plating efficiency for the PC-3 cells was estimated at approximately 20%–25%.

## Results

### *Melphalan lethality to PC-3 cells*

In the clonogenic assay, following 2 h incubation there was virtually no effect of exposures to 0.25 and 0.5 μM melphalan (Fig. 1). Even at 5 μM melphalan exposures, the surviving fraction was 39%. The longer 6-h exposure increased lethality at exposure concentrations of 0.5 μM and greater, with less than 20% survival being observed after 6 h exposure to 2.5 μM melphalan.

### *BSO reduction of GSH content*

Prior to the GSH-depletion portion of this study, as the initial step we determined that incubation of the PC-3 cells for 24 h in concentrations of up to 500 μM BSO had no

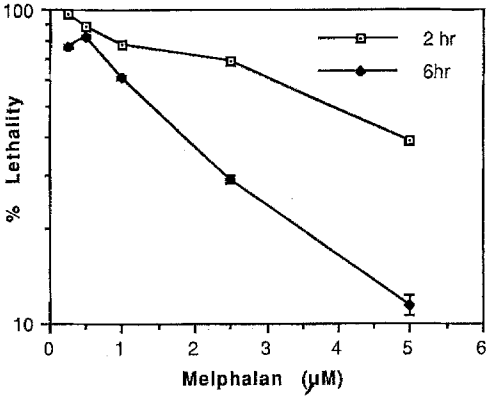


**Fig. 1.** Survival of PC-3 cells exposed to 0.25–10 μM melphalan for 0–24 h. Survival was determined by clonogenic assay as described in Materials and methods. Data represent mean values for 3–5 replicate experiments

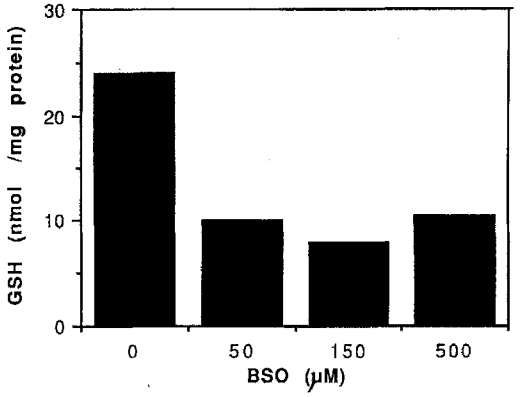


**Fig. 2.** GSH content of PC-3 cells exposed to BSO for 24 h

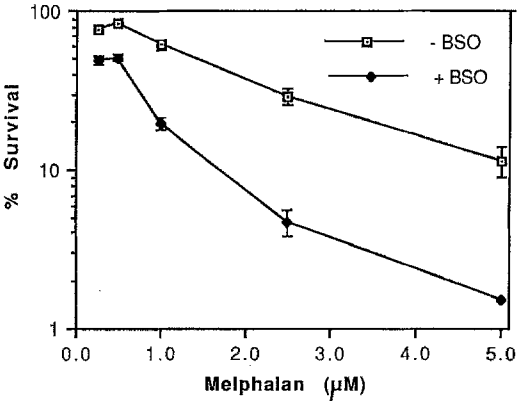


**Fig. 4.** As for Figure 3 except incubation with melphalan was for 6 h

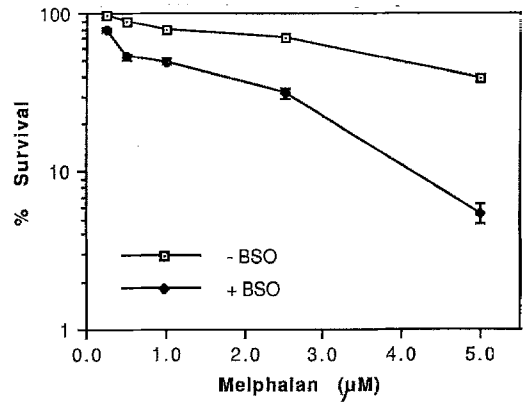


**Fig. 3.** Survival of GSH-deficient (+BSO) and -sufficient (–BSO) PC-3 cells exposed to 0–5 μM melphalan for 2 h

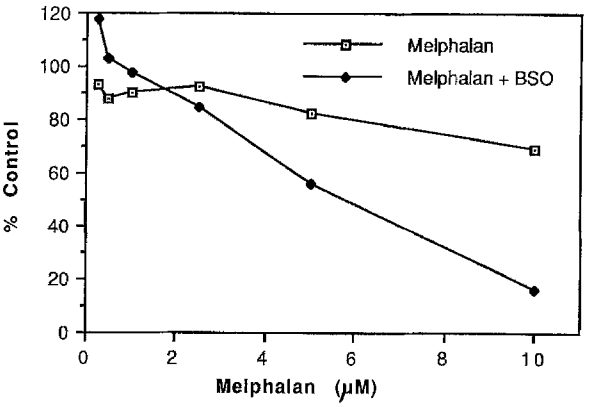


**Fig. 5.** Viability determined by MTT of GSH-sufficient and -deficient PC-3 cells exposed to 0.25–10 μM melphalan for 24 h. Data are expressed as the percentage of fluorescence, with that in non-exposed GSH-sufficient or -deficient cells representing 100%

effect on cell survival by the clonogenic assay. Subsequent incubation of $5 \times 10^6$ cells with 50–500 μM for 24 h reduced the GSH content by 65%–75% (Fig. 2). On the basis of these results we decided to use a 24-h exposure to 100 μM BSO as the time and exposure concentration for the GSH-depletion melphalan cytotoxicity experiments.

*Effect of GSH depletion on melphalan lethality and viability*

Following 24 h incubation with 100 μM BSO, the PC-3 cells were exposed to 0.25–5 μM melphalan for 2 and 6 h and cytotoxicity was determined by clonogenic assay as described above. After exposure of the GSH-depleted cells to 2.5 μM melphalan for 2 h there was 31% survival as compared with 69% survival without BSO (Fig. 3). The difference between GSH-depleted cells and those with normal GSH content was even more pronounced after 6 h continuous melphalan exposure, with a difference in lethality being observed at 1 μM (Fig. 4). A smaller difference in lethality between the GSH-depleted and normal cells was observed after 6 h exposure to concentrations as low as 0.25 μM melphalan.

Similar but less dramatic results were found in the MTT assay for viability. There was no effect on viability of exposures of up to 2.5 μM melphalan. However, following GSH depletion there was a marked decrease in viability as compared with cells with normal GSH content that became evident at 2.5-μM melphalan exposures (Fig. 5).

*$IC_{50}$ and DMF*

The $IC_{50}$ of the GSH-sufficient cells ranged from 4.0 μM after 2 h exposure to 2.0 μM after 6 h (Table 1). On the other hand, GSH-depleted cells had substantially lower $IC_{50}$ values ranging from 1.38 μM after 2 h melphalan incubation to 0.44 μM after 6 h. This resulted in a 2.9- to 4.5-fold increase in lethality (DMF) in the GSH-depleted cells as measured by the clonogenic assay.

## Discussion

Single- or multiple-agent chemotherapy for nonlocalized carcinoma of the prostate has proved to be disappointing, with even the most favorable studies reporting disease stabilization rather than objective regression or remission. No study to date has reported that chemotherapy resulted in prolongation of survival. Intracellular GSH depletion as a technique to increase the cytotoxicity response to various chemotherapeutic agents of a variety of tumor cell lines has been the subject of a number of studies for the past

**Table 1.** $IC_{50}$ and dose-modification factor values for PC-3 cells exposed to melphalan following BSO-mediated GSH depletion

| Hours | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | –BSO | +BSO | DMF |
| 2 | 4.00 | 1.38 | 2.90 |
| 6 | 2.00 | 0.44 | 4.55 |

DMF, Dose-modification factor ($IC_{50}$ in the absence of BSO)/($IC_{50}$ with 100 μM BSO)

10 years. The majority of these studies have focused on the bifunctional alkylating agents, although this technique has also been shown to be useful in increasing the cytotoxicity of doxorubicin and platinum complexes [7, 8, 25, 33]. Depletion of GSH has been proven successful in increasing the cytotoxicity of melphalan both to carcinomas in culture and to tumors in situ [5, 9, 10, 12, 16, 32]. Because of those favorable data, we decided to evaluate the hypothesis that GSH depletion would increase the cytotoxicity of melphalan to an androgen-insensitive prostate cancer cell line, PC-3. This particular cell line was chosen because of the likelihood that if chemotherapy is to have an impact on survival following metastasis, the drug(s) would need to be active against androgen-insensitive cancer cells, the predominant prostate cell remaining after androgen suppression.

Not unexpectedly, we found the PC-3 cells to be relatively resistant to melphalan-induced lethality by clonogenic assay. The failure to observe any melphalan effect until exposures reached 2.5 μM contrasts with results reported by other investigators for a number of other cancer cell lines. The $IC_{50}$ of melphalan to a variety of human melanoma cell lines was approximately 0.1 μM following a 1-h exposure [19]. The $IC_{50}$ was less than 1 μM in two human ovarian cancer cell lines [5], both of which showed an effect on survival with melphalan exposures of 1 μM or less. This compares with our results of an $IC_{50}$ in the PC-3 cells of 4.0 μM after 2 h exposure to melphalan and 2.0 μM after 6 h. Thus, the sensitivity of PC-3 cells to melphalan is lower than that of other human tumor cell lines, particularly those not previously exposed to melphalan, and appears to be similar to that of melphalan-resistant tumor cell lines [5].

Our determination that a 100-μM concentration of BSO was required for GSH inhibition is similar to the results obtained using ovarian cancer cell lines [23]. It is possible that had we incubated the cells for a longer time with BSO, i.e. 48 h, we could have achieved even greater GSH depletion than the 70% that we found for the concentrations tested.

Incubation of the cells pretreated with BSO with similar concentrations of melphalan resulted in an increase in clonogenic lethality after both 2- and 6-h incubations. At both incubation times the difference between the GSH-normal and -depleted cells was evident beginning at 1 μM, with the 6-h incubation greater than the 2-h. In a separate experiment we found that the difference in lethality was even greater if the incubation was continued for 24 h. Those data are not presented in this report because we did not determine if melphalan metabolism resulted in a cytotoxicity-significant reduction in melphalan content in the media. Obviously, this potential problem with exposure concentrations is greatly reduced with shorter incubation periods such as those we have reported in this paper.

The increase in lethality (DMF) observed in the GSH-depleted PC-3 cells as compared with those not pretreated with BSO was 2.9 and 4.55 after 2- and 6-h incubations, respectively (Table 1). This compares favorably with the DMF values reported by Hamilton et al. [14] in ovarian carcinoma cell lines after GSH depletion, which ranged from 3.4 in a melphalan-resistant cell line to 6.8 in a non-melphalan-resistant line. GSH depletion also reduced the exposure time required for melphalan lethality.

We also decided to employ the MTT assay to determine viability (mitochondrial function) as differentiated from lethality as an additional method to evaluate the effect of GSH depletion on melphalan cytotoxicity. In the GSH-depleted cells, there was a decrease in viability after 24 h exposure beginning with a melphalan concentration of 2.5 μM. Because of our failure to detect any effect on MTT viability at 5 μM without BSO, we increased the exposure concentration to 10 μM for those experiments. No effect on viability was observed at any melphalan exposure concentration for non-GSH-depleted cells.

There are few data evaluating the concentration of melphalan in solid tumors following either oral or intravenous administration. Differences in vascularization and tumor density as well as individual patient melphalan pharmacokinetics make it difficult to extrapolate our in vitro findings to in vivo results. Similarly, few studies have investigated the in vivo ability of BSO to deplete GSH in solid tumors, a necessary requirement for there to be clinical usefulness for this melphalan-potentiation technique in prostate cancer. However, the concentrations that produced cytotoxicity in the PC-3 cells, 0.5–2 μM (0.15–0.6 μg/ml) were in the range achieved clinically following intravenous administration. The peak plasma concentration of melphalan reported after intravenous administration ranged from 0.7 to 7 μg/ml [1, 4, 24, 36]. However, it is clear that without GSH depletion, oral melphalan would not achieve a blood concentration similar to that required for cytotoxicity in vitro. The combination of two agents such as melphalan and doxorubicin that are partially or wholly dependent on GSH detoxification coupled with GSH reduction increases the cytotoxicity of each, offering an interesting potential for increased therapeutic effectiveness which has not been widely studied. An example of this potential is that reported by Jevtoric-Todorovic and Guenthner [19], who pretreated melanoma cells with doxorubicin and carmustine (BCNU) followed by exposure to 0.05–20 μM melphalan. The doxorubicin BCNU-pretreated cells in which the GSH was depleted by 60% were 10 times more sensitive to melphalan than were cells not exposed to doxorubicin BCNU.

The clinical usefulness of GSH depletion in treating neoplasms that have become resistant to alkylating agents is currently under investigation, with the initial studies focusing on ovarian carcinoma. The data we obtained in the androgen-insensitive prostate cancer cell line PC-3 provides support for the extension of similar GSH-depletion

studies as a technique with potential to produce an objective response in metastatic prostate cancer to alkylating agents in general and, specifically, to melphalan.

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