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Sensitization Effect of L-2-Oxothiazolidine-4-carboxylate on Tumor Cells to Melphalan and the Role of 5-Oxo-L-prolinase in Glutathione Modulation in Tumor Cells

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ABSTRACT. 5-Oxo-L-prolinase (5-OPase) (EC 3.5.2.9) links the synthesis and metabolism of glutathione (GSH) in the γ-glutamyl cycle. Previous studies showed that L-2-oxothiazolidine-4-carboxylate (OTZ), a 5-oxo-L-proline analog that is metabolized by 5-OPase, can preferentially decrease the cellular GSH levels *in vivo* in rat mammary tumors and sensitizes the tumors to the alkylating agent melphalan. The present study investigated the biochemical mechanism of this effect in a human breast cancer cell line, MCF7. We found that OTZ decreased the GSH levels in MCF7 cells. When the cells were treated with OTZ plus melphalan, the cytotoxicity of melphalan was increased as compared with that of melphalan alone, and this effect could be reversed by the addition of glutamate, which is the product of 5-OPase reaction and a critical substrate in GSH synthesis. We concluded that OTZ increases melphalan toxicity by limiting glutamate production from 5-OPase for GSH synthesis. We also observed that the expression of 5-OPase in the stably transfected MCF7 cells decreased the cellular GSH contents, sensitized the cells to melphalan toxicity, and diminished the sensitizing effect of OTZ. Furthermore, exposure to the GSH-depleting agent buthionine sulfoximine led to increased expression of 5-OPase in both MCF7 cells and the peripheral blood mononuclear cells of patients. These results indicate a critical interaction between cellular GSH levels and 5-OPase activity that could be important in GSH modulation in therapeutic settings. BIOCHEM PHARMACOL **56**;6:743–749, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 5-oxo-L-prolinase; L-2-oxothiazolidine-4-carboxylate; glutamate; glutathione; melphalan; sensitization to chemotherapy

GSH accounts for 90% of the non-protein thiol in cells and plays a critical role against electrophilic challenge of both endogenous and exogenous origins [1–3]. 5-OPase§ (EC 3.5.2.9) is one of the five enzymes in the γ-glutamyl cycle, an interrelated series of reactions involved in the synthesis and degradation of GSH. By converting 5-OP, the metabolite of the γ-glutamyl moiety in GSH, to L-glutamate, a substrate required in the first step of GSH synthesis, 5-OPase links the synthesis pathway and the metabolism pathway of GSH in the γ-glutamyl cycle [4–6]. However, the γ-glutamyl cycle is by no means a closed and self-regulated cycle. Under normal conditions, cells do not necessarily depend on 5-OPase for the maintenance of GSH content, since 5-OP has no significant feedback inhibition on GSH synthesis, and glutamate from many other sources, such as diet and protein degradation, is usually available and sufficient for this purpose [6, 7]. Rare cases of an inborn deficiency of 5-OPase have been reported, which are associated with a variety of clinical manifestations but do not necessarily lead to GSH deficiency [7, 8].

OTZ was developed as a cysteine prodrug to deliver cysteine intracellularly via the function of 5-OPase [9]. It has been shown that OTZ can increase the cellular GSH level in normal cells [9–12] and tissues [13–15], while paradoxically not altering or decreasing cellular GSH in tumor cells [11] and tissues [15, 16]. This results in both the protection of normal cells from, and the sensitization of tumors to, the toxicity of some chemotherapeutic agents whose detoxification involves GSH [9–16]. The mechanisms of these contradictory effects of OTZ in normal versus tumor cells have not been investigated thoroughly, which limits the further exploitation and clinical application of this mechanism.

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§ *Abbreviations:* BSO, buthionine-(*R*,*S*)-sulfoximine; IC_{50}, the concentration of a drug required for 50% inhibition of cell growth; MTT, 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5-OP, 5-oxo-L-proline; 5-OPase, 5-oxo-L-prolinase; and OTZ, L-2-oxothiazolidine-4-carboxylate.

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Following our findings that OTZ can preferentially decrease cellular GSH levels *in vivo* in rat mammary tumors and sensitizes tumors to the alkylating agent melphalan [15, 16], we discovered a significantly decreased 5-OPase protein level in some tumors as compared with the paired normal tissues from the same patient [17]. We suggested that this difference in 5-OPase expression between normal and tumor tissues could be one of the underlying mechanisms for the selective effect of OTZ. In the present report, using the breast epithelial carcinoma cell line MCF7, we further studied the biochemical mechanism by which OTZ sensitizes tumor cells to the toxicity of melphalan, a nitrogen mustard alkylating agent used in cancer chemotherapy [1]. We also investigated the significance of 5-OPase function in the response of tumor cells to GSH stress and the potential to achieve chemosensitization in tumor cells by increasing cellular 5-OPase expression.

MATERIALS AND METHODS

Reagents

Melphalan, glutamate, OTZ, and BSO were from the Sigma Chemical Co. Concentrations of OTZ and glutamate that cause less than 15% cell death and have no significant effect on the cell growth rate were considered non-toxic and were used in cell-killing experiments. These concentrations were determined by MTT assays and confirmed by comparing the growth rate of cells under study in the drug-free medium with that in the medium containing the chemical, single or in combination, at these concentrations. Results (data not shown) showed that the non-toxic concentration for OTZ and glutamate was 25 mM, and this concentration was used in the MTT assays. BSO was used at a concentration of 100 μM. Buffer A was composed of 50 mM Tris, pH 7.3, 0.1 mM EDTA, 2 mM dithiothreitol, and 5 mM 5-OP [18].

Clinical Specimens

Peripheral blood samples were obtained from patients undergoing a phase II trial of BSO pre-melphalan treatment for metastatic melanoma. This trial was undertaken in collaboration with The Cancer Therapeutic Evaluation Program, NCI. Patients were treated with a continuous infusion of BSO, an inhibitor of γ-glutamylcysteine synthetase [19], for 48 hr, to deplete the GSH levels in the tumors before they were treated with melphalan. Peripheral blood mononuclear cells were harvested both before and after the continuous BSO infusion, isolated by centrifugation through Ficoll–Paque (Pharmacia Biotech) for 30 min at 850 *g*. The suspended cells were collected and washed with PBS. The cell pellet was then lysed in Buffer A and processed for western blot and GSH assay, as indicated below.

Cell Culture and Tissue Preparation

Cell culture media and reagents were from GIBCO BRL (Burlington). All cells were maintained in a 37° tissue culture incubator in a 5% CO_2 atmosphere. MCF7 is a human mammary carcinoma cell line. MCF7ADR is a subline of MCF7 selected for resistance to Adriamycin® (500-fold). A2780 is a human ovarian carcinoma cell line. All cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 50 U/mL of penicillin/streptomycin. Cells were harvested by trypsin and lysed in ice-cold Buffer A. The protein concentrations of the cell-free extract were determined using the Bradford protein assay method (Bio-Rad).

MTT Assay

Cells were plated in 200 μL of medium at a density of 2500 cells/well in flat-bottom 96-well microtiter plates. Then the plates were incubated for 24 hr, and the medium was replaced with fresh medium containing either melphalan alone or a mixture of melphalan, OTZ, or glutamate, as indicated in Results. After a further 48 hr of incubation, the MTT assay was performed as previously described, and the plates were read for the density of formazan at 570 nm [20]. The IC_{50} values of melphalan were determined according to the absorbency reading and were defined as the drug concentration required for 50% inhibition of cell growth as compared with controls (non-treated).

Western Blot

Protein samples (40–60 μg) were electrophoresed on 7.5% polyacrylamide gels and were transferred to nitrocellulose membranes (Corning Costar) following standard procedures. An enhanced chemiluminescence (ECL) western blotting protocol (Amersham) was followed. The membranes were blocked overnight in 5% milk in PBS, and then were blotted with the primary antibody (rabbit anti-rat 5-OPase antibody [17]) at 1:50,000 dilution for 3 hr at room temperature, and with the secondary antibody [goat anti-rabbit IgG (H + L) Horseradish Peroxidase Conjugate; Bio-Rad] at 1:2,000 dilution for 1 hr at room temperature.

GSH Assay

Cells growing in the log phase at 80–90% confluence were harvested, lysed in 10 mM HCl by three consecutive freeze–thaw cycles, and deproteinized in 3% (w/v) 5-sulfosalicylic acid. After centrifugation for 2 min at 23,500 *g*, the protein-free supernatant was used for GSH assay according to a previously described GSH reductase assay [21].

Stable Transfection

5-OPase cDNA in the pT7-7 plasmid was a gift from Dr. Esther Breslow in the Department of Biochemistry, Cornell

University Medical College. The cDNA was then subcloned into a mammalian expression vector pRc/CMV (Invitrogen Corp.) under the control of a CMV promoter. Then the construct was transfected into MCF7 cells using LIPOFECTAMINE Reagent (Life Technologies). The cells were transfected for 6 hr in serum-free medium before being changed into complete medium for 48 hr. Then the transfected cells were split at 1:10, and the stable transfectants were selected and maintained by culturing in 700 μg/mL of G418 (Mediatech Inc.).

RESULTS

Sensitization Effect of OTZ on Tumor Cells to Melphalan Toxicity

MTT assays on MCF7 cells demonstrated that when OTZ was administered in combination with melphalan and maintained in the medium through the 48-hr incubation, it significantly sensitized the cells to melphalan toxicity (Fig. 1A). The sensitization ratio (SR = the concentration of melphalan for a cell killing rate over that for the same cell killing rate in the presence of OTZ) at the IC_{20} concentration of melphalan [SR_{20}(OTZ)] was 98.6 ± 49.1-fold, and that at the IC_{50} dose of melphalan [SR_{50}(OTZ)] was 27.8 ± 10.3-fold.

To investigate the role of GSH in this effect of OTZ, two experiments were performed in MCF7 cells. (1) The cells were treated with OTZ (10 mM), and the cellular GSH levels before and after the addition of OTZ were examined. The results showed a constant depletion of cellular GSH, by approximately 20% at 5 hr (Fig. 1B) and 24 hr (data not shown). (2) OTZ is a competitive inhibitor of 5-OPase and inhibits the physiologic function of 5-OPase, which is the conversion of 5-OP to glutamate [9]. To test whether the OTZ sensitization effect is due to glutamate depletion by OTZ, MTT assays were performed with the addition of glutamate. The results showed that the sensitization effect of OTZ to melphalan was reversed by glutamate, while glutamate alone affected neither melphalan toxicity (Fig. 1C) nor cell growth rate (data not shown). Substituting melphalan with chlorambucil rendered the same results (data not shown).

OTZ also sensitized A2780 cells to melphalan toxicity, and this effect was reversed by the addition of glutamate, as in MCF7 cells (data not shown).

Effect of 5-OPase on GSH Concentration and on Cellular Response to Melphalan and Melphalan Sensitizers

To understand the relation between 5-OPase and cellular GSH levels, we stably transfected MCF7 cells with the mammalian expression vector pRc/CMV containing 5-OPase cDNA. A resultant clone, ST5, was selected and characterized in comparison with the control, an MCF7 clone transfected with pRc/CMV alone. ST5 cells were shown to have a 5.5-fold higher 5-OPase protein level

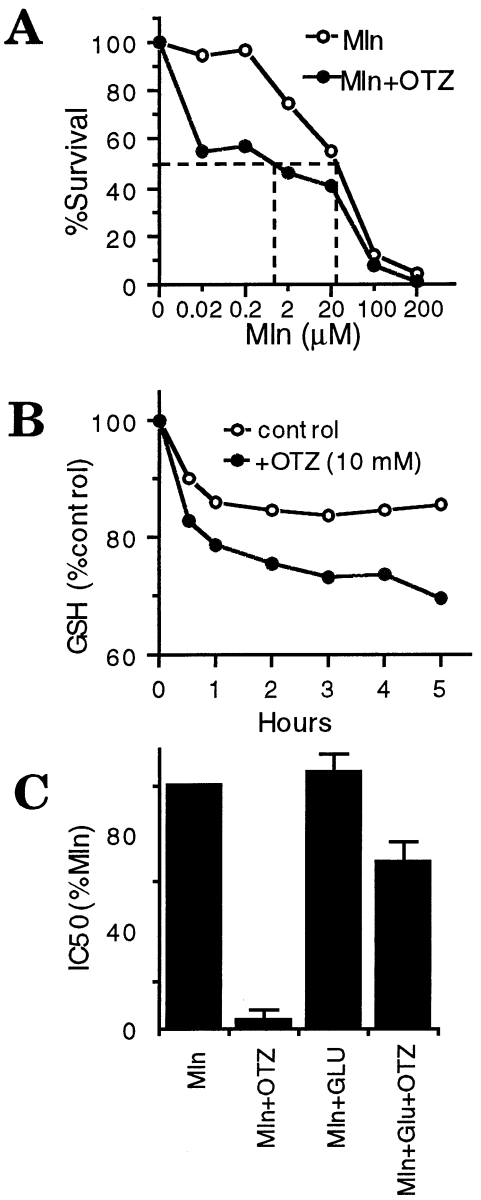

FIG. 1. Mechanism of OTZ sensitization effect on melphalan toxicity in MCF7 cells. (A) A typical MTT experiment on MCF7 cells administered melphalan alone or the combination of melphalan and OTZ. (B) Effect of OTZ on the cellular GSH level in MCF7 cells. Values are given as the percentage of GSH concentration at time points over that at time 0. The GSH level at time zero was 73.4 ± 3.9 nmol/mg. The experiments in panels A and B were performed at least three times. (C) Comparison of the melphalan IC_{50} values of MCF7 cells treated with melphalan or melphalan in combination with OTZ and/or glutamate. A representative experiment is shown. Results are means ± SD of three measurements. The IC_{50} value of the control cells treated with melphalan alone (Mln) was 28.8 ± 5.7 μM. Other values are the percentages of the IC_{50} value of melphalan in different combinations over that of melphalan alone. Statistical analysis: In panel A, cells treated with Mln + OTZ were significantly different from control cells treated with melphalan alone at concentrations of 0.02 to 20 μM melphalan, $P < 0.05$. In panel B, treated cells were significantly different from control cells at all time points except time 0, $P < 0.05$.

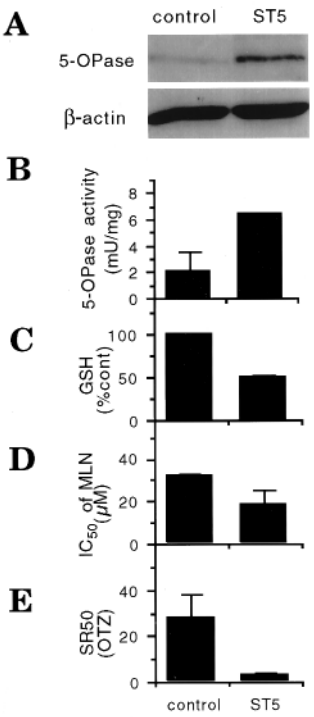

FIG. 2. Comparisons between ST5 cells, the MCF7 cells stably transfected with 5-OPase cDNA, and the control transfectant (transfected with vector only). All values in panels B–E are means ± SD of at least three measurements. (A) Western blot showing the 5-OPase level in the two cell lines with β-actin as the loading control. (B) 5-OPase activity. (C) Cellular GSH content. The GSH value in the control was 56.3 ± 8.2 nmol/mg. The value for ST5 cells is presented as the percentage of the control. (D) IC_{50} value of melphalan. (E) Sensitization ratio of OTZ (SR(OTZ)) to melphalan toxicity.

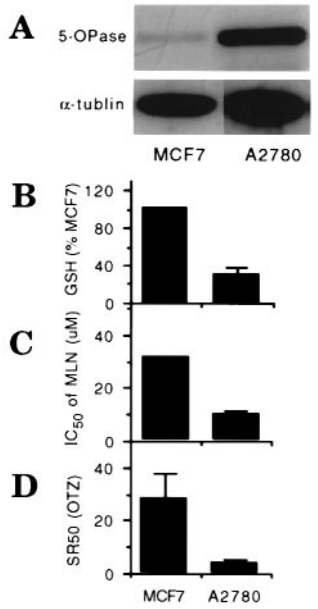

FIG. 3. Comparison of MCF7 cells and A2780 cells. (A) Western blot showing the 5-OPase level with α-tubulin as the loading control. (B) GSH values presented as the percentage of the GSH levels in MCF7 cells (52.8 ± 16.1 nmol/mg). (C) IC_{50} values of melphalan. (D) Sensitization ratio of OTZ (SR(OTZ)) to melphalan. Values in panels B–D are means ± SD of at least three measurements.

(Fig. 2A), 3.1-fold higher 5-OPase activity (Fig. 2B), and an almost 50% reduction of cellular GSH (Fig. 2C). MTT assays showed a 43% decrease in the IC_{50} value of melphalan (Fig. 2D) and an 11.0-fold decrease in the SR_{50}(OTZ) (Fig. 2E) as compared with the control.

The relationship between 5-OPase level and cellular GSH level was also examined in different cell lines to test our findings in the above transfection study. Comparisons between MCF7 and A2780 cells were made with the results from GSH assays, MTT assays, and Western blot experiments. A Western blot showed a 43.6-fold higher 5-OPase level in A2780 cells than in MCF7 cells (Fig. 3A). A2780 cells had a 3.3-fold lower cellular GSH level (Fig. 3B) and a 3.3-fold lower IC_{50} value of melphalan (Fig. 3C) than MCF7 cells. The SR_{50}(OTZ) in A2780 cells was 3.1 ± 2.3-fold, in contrast to that of MCF7 cells [SR_{50}(OTZ) = 27.8 ± 10.3-fold] (Fig. 3D).

Effect of GSH Depletion on 5-OPase Expression

Three patients in an ongoing clinical trial in metastatic melanoma were treated for 48 hr with continuous infusion of BSO, an inhibitor of γ-glutamylcysteine synthetase [19], to deplete the GSH levels in the tumors before they received melphalan. GSH assays showed that the BSO treatment decreased the GSH levels in the peripheral blood mononuclear cells of the patients by 8.0, 38.8, and 45.1%, respectively, in a 48-hr period. In the first two patients, western blot analysis revealed no detectable 5-OPase in the cells either before or after the BSO treatment. A third patient, who had the greatest GSH depletion (45.1%), had no detectable 5-OPase in the cells before, but showed a significant level of 5-OPase after the BSO treatment (Fig. 4A).

The effect of decreased cellular GSH on 5-OPase expression was examined in MCF7 cells *in vitro*. The exposure of MCF7 cells to 100 μM BSO alone resulted in 38 and 86% reduction of the cellular GSH level in 4 and 24 hr, respectively. Western blot showed that the 5-OPase level in these cells increased transiently by 3.1-fold after a 4-hr exposure (Fig. 4B), and returned to the control level at 24 hr. Independently, the exposure of MCF7 cells to 25 mM OTZ alone led to 26 and 16% reduction of the cellular GSH level in 4 and 24 hr, respectively. Western blot showed that the 5-OPase level in these cells was not changed significantly at 4 hr, but had an almost 2-fold increase at 24 hr (Fig. 4C).

The cellular GSH level of MCF7ADR cells is 50% of that of MCF7 cells [22]; we also observed a 3.5-fold increase of the 5-OPase level in MCF7ADR cells as compared with that in MCF7 cells (Fig. 4D).

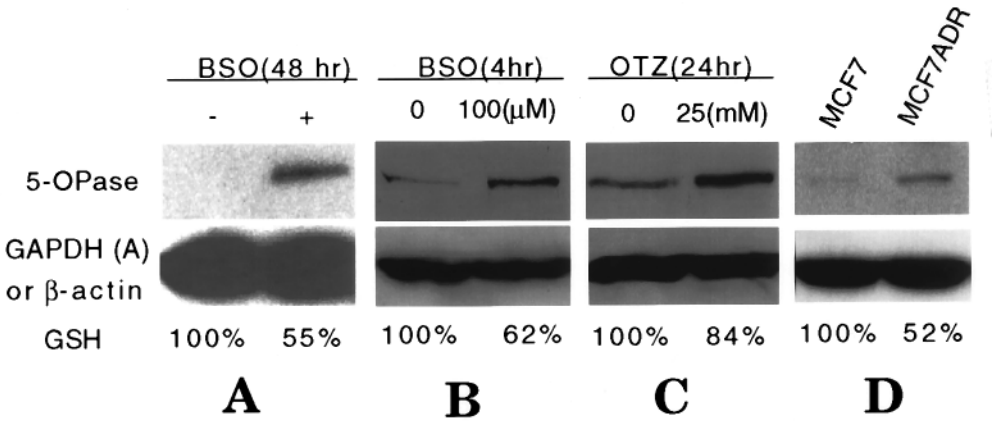

FIG. 4. Effect of cellular GSH depletion on cellular 5-OPase levels as studied by western blots. (A) 5-OPase level in the peripheral blood mononuclear cells of a patient before and 48 hr after BSO treatment. (B) 5-OPase level in MCF7 cells untreated and treated with 100 μM BSO for 4 hr. (C) 5-OPase level in MCF7 cells untreated and treated with 25 mM OTZ for 24 hr. (D) 5-OPase level in MCF7 cells and MCF7ADR cells. The loading control was GAPDH in panel A and β-actin in the other panels. GSH values are expressed as a percentage of the untreated (panels A–C) or the wild-type (panel D) MCF7 cells.

DISCUSSION

The present study focused on two issues in relation to 5-OPase: 1) the *in vitro* effect of OTZ on melphalan toxicity to tumor cells and its underlying mechanism; and 2) the significance of 5-OPase in cellular GSH modulation.

Following our previous observation that OTZ can sensitize rat mammary tumors to melphalan toxicity *in vivo* [15, 16], we investigated this effect *in vitro* in the human mammary carcinoma cell line MCF7. We found that OTZ sensitized MCF7 cells to melphalan toxicity. We considered three possible mechanisms, either alone or in synergy, for this effect: 1) OTZ itself is toxic; 2) OTZ promotes the uptake of melphalan by its transporters; and 3) OTZ interferes with the cellular detoxification mechanism against melphalan. First, OTZ has been shown to be, generally, a nontoxic chemical and has been used in previous studies at concentration of 10–70 mM [11, 12, 23–25]. We chose the concentration of OTZ with less than 15% cytotoxicity in all our MTT experiments. Therefore, the toxicity of OTZ alone cannot account for the 27.8-fold decrease in the IC_{50} value of melphalan. Second, we also performed MTT experiments using chlorambucil instead of melphalan, and the same sensitization effect of OTZ was observed. Since chlorambucil enters cells by passive diffusion and does not depend on an amino acid transporter in the manner of melphalan [26], an effect on the uptake system is not likely. The third possibility was then explored in greater detail.

The important role of GSH in melphalan detoxification is well documented. High levels of cellular GSH often add to cellular resistance to melphalan [1, 27–29]. In MCF7 cells, decreased GSH levels, as a result of BSO treatment, can sensitize cells to melphalan [30]. OTZ is converted to cysteine by 5-OPase and thus acts as a competitive inhibitor of the 5-OPase conversion of 5-OP to glutamate [9, 31]. Therefore, OTZ may exert opposite effects on cellular GSH levels depending on the state of the cells: under normal physiologic conditions, cysteine is the rate-limiting substrate in GSH synthesis [9, 15] and OTZ is, accordingly, a promoter for GSH synthesis; however, in tumor cells where glutamate has been found to be required and rate-limiting for cellular functions [32–34], OTZ may act to deplete or limit glutamate production from 5-OPase and, therefore, become a limiting factor for GSH synthesis. This hypothesis is strongly suggested by the following evidence from our study: 1) OTZ was shown to decrease moderately the cellular GSH in MCF7 cells; 2) the sensitization effect of OTZ on melphalan toxicity can be reversed by the addition of glutamate; 3) the fact that glutamate alone did not alter significantly melphalan toxicity to MCF7 cells excludes the possibility that glutamate alone rescued the cells from melphalan toxicity; and 4) the cells cultured in OTZ-containing medium did not show a significant change in the proliferation rate as compared with the control (our unshown data and [34]). Therefore, the contribution of decreased cell growth, due to decreased glutamate, to the sensitization effect of OTZ is not likely to play a role. Together, our results suggest that OTZ sensitizes MCF7 cells to melphalan toxicity due to, at least in part, depletion of cellular GSH content, which is achieved by inhibiting the glutamate production by 5-OPase. It is also possible that its effect on glutamate may act on other critical pathways as well. In fact, the depleting effect of OTZ on cellular GSH, and the sensitization effect of OTZ on chemotherapy toxicity, have been reported in some previous studies in other model systems, but the mechanism was not understood [15, 16, 35].

Because OTZ requires 5-OPase for activation, the degree of the OTZ sensitization effect depends on the interaction between 5-OPase and cellular GSH regulation. Therefore, we examined the significance of 5-OPase for cellular GSH modulation by studying the effect of 5-OPase activity on cellular GSH levels and, conversely, how the changes of cellular GSH levels affect 5-OPase function. 5-OPase links

the metabolism and synthesis of GSH in the γ-glutamyl cycle, and while it may decrease the cellular GSH content by promoting GSH degradation, it also facilitates GSH synthesis by providing glutamate for GSH synthesis [4–6]. The net effect of 5-OPase on cellular GSH levels depends on the particular state of the cells. Transfected MCF7 cells (ST5), which express 5.5-fold higher 5-OPase, demonstrated both a moderate reduction in GSH concentration and a moderate sensitization to melphalan, while the sensitization effect of OTZ to melphalan was reduced significantly. In A2780 cells, the role of GSH in melphalan detoxification has been well documented [36, 37]. Consistent with our observation in transfected MCF7 cells, in A2780 cells, which have a higher endogenous 5-OPase level, there is a lower GSH level, moderately higher sensitivity to melphalan, and much lower SR_{50}(OTZ) of A2780 cells to melphalan. Notwithstanding the fact that MCF7 and A2780 are obviously quite distinctive cell types, these relationships appear strikingly similar to our data with the transfected cells. They both indicate that a higher 5-OPase level results in decreased cellular GSH concentration and a smaller effect of OTZ sensitization to melphalan. At the same concentration of OTZ, higher 5-OPase levels in ST5 and A2780 cells diminished the inhibitory effect of OTZ on 5-OPase and resulted in a much lower SR_{50}(OTZ) as compared with that of MCF7 cells.

Our results from both the mononuclear cells of cancer patients treated with BSO and MCF7 cells treated *in vitro* with BSO or OTZ showed that decreasing the cellular GSH level may increase the expression of 5-OPase protein in these cells, suggesting that: 1) changes in cellular GSH level may regulate 5-OPase expression; and 2) the amount of glutamate spared from GSH synthesis by BSO has no significant product inhibition on 5-OPase in MCF7 cells. These results indicate that GSH depletion may up-regulate the expression of 5-OPase for faster GSH synthesis or turnover. Direct study of the factors that regulate 5-OPase expression, either directly or indirectly, remains speculative until the regulatory sequences of its gene are isolated.

Our data suggest that OTZ, acting through 5-OPase, can exert dual effects on cellular GSH levels: increasing it by providing cysteine, or decreasing it by inhibiting glutamate production from 5-OP. The dominant effect may depend on which substrate is rate-limiting for GSH synthesis in the cells in question. In normal cells, cysteine is the rate-limiting substrate [9, 15], and OTZ increases GSH in normal cells by providing cysteine [2, 9]. In tumor and other cells under oxidative stress, glutamate may become the rate-limiting factor for GSH synthesis [32–34], and OTZ may act by further limiting the glutamate production from 5-OP via 5-OPase. On the other hand, 5-OPase can also display dual effects on cellular GSH levels since it both promotes GSH degradation and provides glutamate for GSH synthesis. Which effect of 5-OPase prevails also depends on the particular situation of the cells. In the tumor cells we studied, OTZ sensitized the cells to melphalan toxicity by GSH reduction and limiting glutamate. The degree of the sensitization was related to the amount of 5-OPase protein in the cells. Both higher endogenous 5-OPase levels in A2780 and MCF7ADR cells and overexpressed exogenous 5-OPase in transfected MCF7 cells attenuated the sensitization effect of OTZ on melphalan toxicity. Given our recent finding that in some human tissues 5-OPase is significantly lower in tumor compared with normal tissues [17], our current data may have therapeutic importance.

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