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A GLUTATHIONE DEPLETION SELECTIVELY IMPOSED ON μGLUTATHIONE *S*-TRANSFERASE OVERPRODUCING CELLS INCREASES NITROGEN MUSTARD TOXICITY

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Abstract—Glutathione (GSH) contributes to the detoxification of anticancer drugs through the operation of specific glutathione *S*-transferases (GST) and innate, or acquired, overexpression of this enzyme family has been frequently observed in tumor cell lines. In the GMA32 line of Chinese hamster fibroblasts, we showed that GSH starvation produced by exposing cells to buthionine sulfoximine (BSO) increased the toxicity of chlorambucil and melphalan, but not that of *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), cisplatine and doxorubicin. This indicates that efficient mechanisms of detoxification using GSH operate for chlorambucil and melphalan, but not for the other drugs in these cells. We then showed that GSH depletion could be selectively and transiently induced in the μGST overexpressing cell line derived from GMA32, HC474, by exposing cells to substrates specific to the overexpressed isozyme. Exposing cells to such a substrate, *trans*-stilbene oxide, does not alter the sensibility of GMA32 cells to melphalan and chlorambucil, but increases that of HC474 cells to these drugs, to an extent comparable to that obtained with BSO. This observation highlights the possibility of exploiting GST overexpression, a frequent feature of tumor cells, to selectively sensitize these undesirable cells to anticancer drugs.

Key words: glutathione; glutathione *S*-transferase; nitrogen mustards; buthionine sulfoximine; *trans*-stilbene oxide

GSH‡ and GSH enzymes play an important role in the detoxification of various electrophilic chemicals of exogeneous or endogeneous origin [1–5]. The contribution of GSH to spontaneous and acquired resistance to anticancer drugs has been established by the observation that the specific depletion of the GSH pool induced by BSO, an inhibitor of γGCS activity [6], partially reverses resistance. BSO also increases cell sensitivity to irradiation [7]. Moreover, administration of GSH precursors protects cells against irradiation and acetaminophen hepatotoxicity [8, 9].

Among the GSH enzymes, GSTs are thought to play a central role in the metabolism of many toxic electrophiles and reactive oxygen intermediates. GST-mediated conjugation of toxic or carcinogenic molecules usually represents a detoxification pathway [10, 11]. In mammals, cytosolic GSTs are dimeric proteins encoded by at least four distinct gene families, termed α, μ, π and θ [12–14]. The α, μ and θ gene families encode more than one peptide (subunit) and mature GSTs are homo—or heterodimers of subunits from the same family. GSTs are predominantly located in the liver but are also present in most, if not all, other tissues. The differential expression of GST genes is so pronounced that every organ has a different GST composition.

Permanent alterations of the GST pattern are frequently observed *in vivo* in untreated tumors: an extreme situation is the tumor-specific appearance of πGST in rat liver nodules [15]. This gene is also overexpressed in many other tumors [16]. However, its overexpression may be secondary to the deregulation of other genes [17]. In some human breast cancers, overexpression is the consequence of gene amplification, since at the 11q13 locus, the πGST gene and several protooncogenes are frequently co-amplified [18]. The biological significance of GST overexpression in tumors is unknown, but its importance has been implicated in the resistance to anticancer drugs [19]. The elevated expression of αGST isozymes has been frequently correlated with resistance to alkylating anticancer

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‡ Abbreviations: AMPD, AMP deaminase; BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; BSO, L-buthionine *S-R* sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5-5'-dithiobis-(2-nitrobenzoic acid); FITC, fluorescein isothiocyanate; γGCS, γglutamyl-cysteine synthetase; GSH, glutathione; GST, glutathione *S*-transferase; γGT, γglutamyl-transpeptidase; ID_{50}, dose required to reduce survival to 50% of the untreated cell population; ID_{90}, dose required to reduce survival to 90% of the untreated cell population; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer pH 7.4); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue); TSO, *trans*-stilbene oxide; CHO, Chinese hamster ovary.

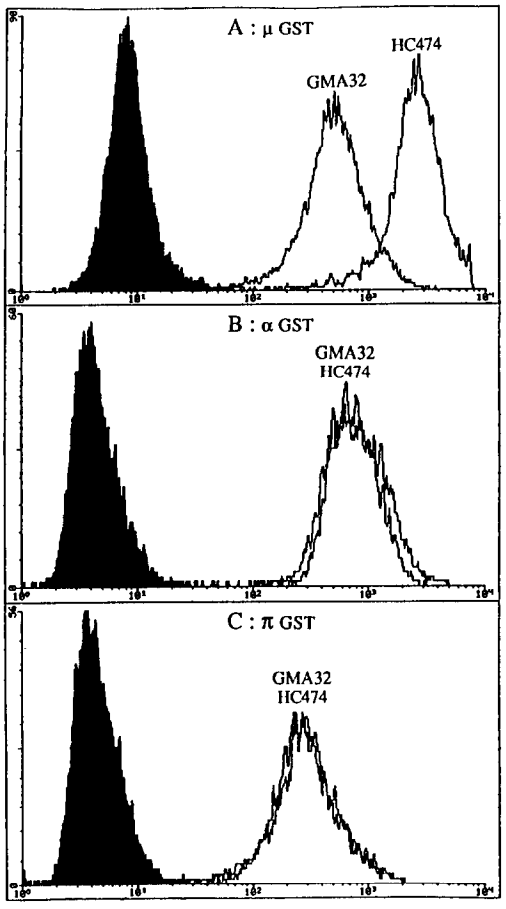

Fig. 1. GST content of populations of GMA32 and HC474 cells. Cells were incubated with rabbit antisera specific for either μ, α or πGSTs, then with donkey anti-rabbit IgG coupled to FITC. Negative control obtained by direct labelling of GMA32 and HC474 cells with the donkey anti-rabbit IgG coupled to FITC are completely superimposed (black histogram). (A) cells incubated with anti-μGST serum, (B) anti-αGST serum, (C) anti-πGST serum. The curves in B and C were obtained in the same experiment. Curves in A are from a separate experiment. Note that curves obtained with GMA32 and HC474 cells are superimposed in B and C.

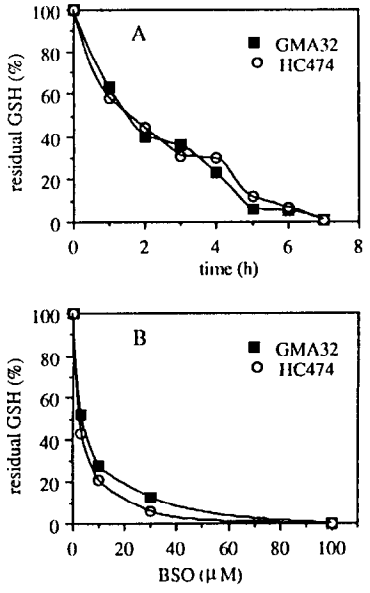

Fig. 2. Effect of BSO exposure on GSH levels in GMA32 and HC474 cells. (A) Time course of GSH depletion in cells exposed to 1 mM BSO, (B) residual GSH in cells exposed for 16 hr to various doses of BSO. In this experiment, 100% GSH represented 2.2 and 4.2 nmol/10^6 cells for GMA32 and HC474 cells, respectively.

drugs [20, 21]. Overexpression of πGST, first detected in Adr^RMCF-7, a multidrug resistant human breast cancer cell line [22], has also been observed in various other drug resistant lines. *In vitro* gene transfer experiments have shown that overexpression of α and πGST may, in cells from different tissues, contribute to resistance to anticancer drugs [23–25].

Since GSH and GSTs contribute to the detoxification of many anticancer drugs, two types of treatments have been used to improve the efficiency of these drugs. Both GSH depletion by BSO [26, 27] and GST inhibition by ethacrynic acid [28, 29] are currently under clinical trial [30, 29].

In this work, we explore another attractive possibility: exploiting the frequent overproduction of particular GST by cancer cells to selectively deplete them of GSH under conditions that do not alter the GSH pool in normal cells. We have used the Chinese hamster cell line GMA32 and its derivative HC474 cell line that overexpresses the μ family of GSTs through gene amplification [31, 32]. This mutant cell line is coformycin resistant due to the amplification of the AMPD2 gene that encodes the target enzyme of the drug. The amplification of the μGST gene has not been selected for, but results from, its close linkage to and consequent amplification with the AMPD2 gene. In this report, we first establish that BSO-induced GSH depletion increases the toxicity of nitrogen mustards in GMA32 cells, indicating that GSH contributes to the detoxification of these drugs in these cells. GSH depletion does not increase the toxicity of BCNU, cisplatine or doxorubicin. We then demonstrate that GSH depletion, with similar effects but restricted to μGST overproducing cells, can indeed be induced by exposing them to μGST substrates, such as TSO. TSO specifically increases nitrogen mustard toxicity towards HC474 cells but not that of BCNU, cisplatine or doxorubicin.

MATERIALS AND METHODS

Cell lines. Cells were routinely grown as monolayer in modified Eagle's medium containing twice the concentration of glucose, vitamins and amino acids (essential and non-essential) and supplemented with 10% horse serum [31]. The lack of mycoplasma was routinely tested by the Service des Mycoplasmes at the Pasteur Institute.

The cell lines used have previously been described [31]. Briefly, the parental line, GMA32, is a cloned

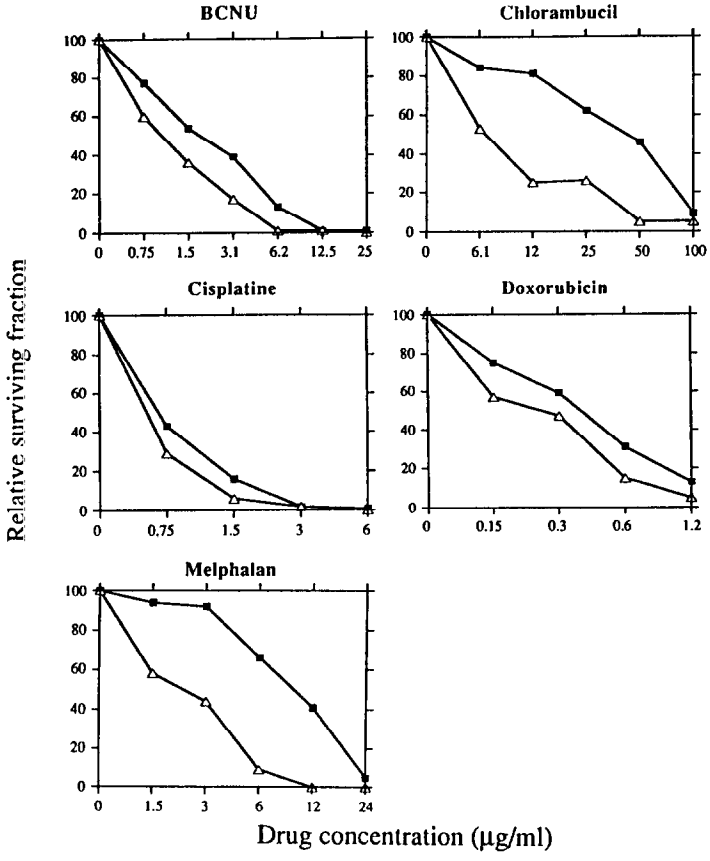

Fig. 3. Effect of BSO-induced GSH depletion on the susceptibility of GMA32 cells to anticancer drugs. After a 16 hr exposure of the cells to 100 μM BSO, the drugs were added and kept for 3 hr before washing off. The relative survival fraction was determined 3 days later by the MTT assay. (■) Untreated GMA32 cells, (△) GMA32 cells exposed to BSO.

deoxycytidine-kinase deficient derivative of the CCL39 line of Chinese hamster lung fibroblasts. HC4 and HC474 are coformycin-resistant clonal derivatives selected for their ability to survive 0.5 μg/mL and 25 μg/mL coformycin, respectively, in a mixture (10 μM azaserine, 50 μM adenine and 50 μM uridine) making the cells dependent on their AMPD activity for growth. HC4 and HC474 overexpress AMPD, the target enzyme for coformycin, and also overexpress μGST activity due to the co-amplification of these closely linked genes. Their GST activity towards CDNB is increased by 2.2 and 14 times, respectively [32].

Reagents. BSO, TSO, chlorambucil, melphalan, doxorubicin, DMSO, MTT, DTNB, NADPH, GSH and GSH reductase were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.), BCNU from Laboratoires Bristol (Paris, France), cisplatine from Lilly France (St Cloud, France) and monochlorobimane from Calbiochem (San Diego, CA, U.S.A.). Rabbit anti-αGST serum (MED 27 Yc), rabbit anti-μGST serum (MED 59 MUA) and rabbit anti-πGST serum (MED 25 PI) were obtained from Medlabs (Stillogran Co., Dublin, U.K.). Donkey anti-rabbit IgG coupled to FITC was obtained from Amersham (Les Ulis, France).

Detection of μGST by intracellular immunofluorescence. Cells were washed in PBS and fixed for 15 min at room temperature in 3.7% paraformaldehyde in PBS containing 0.03 M sucrose. They were then washed with PBS containing 1 mg/mL BSA and incubated for 1 hr with the rabbit anti-μGST serum at room temperature, washed again with PBS containing 1 mg/mL BSA and incubated for 45 min with donkey anti-rabbit IgG coupled to FITC. Cells were finally washed three times with PBS and resuspended by vigorous pipetting. Fluorescence analysis was performed on a FACSCAN (Becton Dickinson, Mountain View, CA, U.S.A.). All antibodies were diluted in PBS containing 1 mg/mL BSA.

Enzymatic determination of GSH. Total glutathione (GSH and GSSG) was determined by the enzymatic assay of Tietze [33] with minor modifications, allowing automatic measurement of numerous samples. In most experiments, exponentially growing cells (between 2×10^5 and 10^6 cells) were trypsinized, centrifuged and resuspended in 100 μL of 1 M perchloric acid, 1 mM EDTA. After 15–30 min at room temperature, extracts were deproteinized by centrifugation at 10,000 g and the supernatants were neutralized by addition of 5 M

Table 1. Relative residual GSH pool in cells exposed to different GST substrates in the presence of BSO

Compound	GMA32	HC474
CDNB	ND	ND
Monochlorobimane	ND	ND
p-nitrobenzylchloride	ND	ND
3,4-Dichloronitrobenzene	33%	14%
1,2-Epoxy-3-(*p*-nitrophenoxy) propane	28%	15%
Styrene oxide	45%	10%
2-Bromo-2-chloro-1,1,1-trifluoroethane	89%	83%
Cetremide	84%	92%
TSO	92%	71%
Hexachlorobutanediene	96%	72%

Duplicate suspensions of 5×10^5 cells in 1 mL culture medium were simultaneously exposed to 1 mM BSO and to 100 μM of the indicated substrate for 15 min at 37°, briefly centrifuged and the GSH content of pellets determined after perchloric acid extraction. Results are expressed as % of GSH present in cells exposed to BSO alone (1.7 and 4.4 nmol/10^6 GMA32 and HC474 cells, respectively). ND, not detectable.

KOH. Aliquots (up to 20 μL) of the supernatants were distributed in a 96-well microplate and 300 μL of 0.1 M potassium phosphate buffer pH 6.5 containing 1 mM EDTA, 0.1 mg/mL NADPH, 25 μg/mL DTNB and GSH reductase (0.1 U) were quickly added to each well. The increase in absorbance was recorded at 405 nm by an automatic reader (Diagnostics Pasteur, LP500). Standard GSH dilutions were included in each experiment which was done in duplicate and with extracts from two independent cell cultures. Results are expressed as % of GSH present in control cells. In several experiments using cells exposed to a drug for a short period of time, cells were trypsinized, resuspended at 5×10^5 cells in 1 mL of medium containing the drug for the indicated period of time and briefly centrifuged. The pellets were processed as described above.

Determination of antineoplasic drug susceptibility. Cells were plated at 300 cells per well in a volume of 100 μL of medium in 96-well microplates (Costar). Forty-eight hours later, 50 μL of medium supplemented or not with 300 μM TSO was added to each well. After an additional 3 hr at 37°, 50 μL of the drug dilutions to be tested were added for 2 or 3 hr at 37°. The medium was then replaced by 200 μL of fresh medium and the cells allowed to grow for 3 days. Cells were stained by the addition of 100 μg MTT per well in 50 μL PBS for 3 hr and, after removing the medium, the formazan crystals were dissolved in 100 μL DMSO. Absorbance at 510 nm was recorded [34]. Cells to be exposed to BSO were treated similarly except that BSO (100 μM final) was added 30 hr after plating and incubated a further 18 hr before the addition of drug dilutions. All determinations were done in triplicate. Blanks corresponded to wells containing culture medium without cells. Fluctuations between independent experiments did not exceed 20%.

RESULTS

GST content

The GST content of exponentially growing GMA32 and HC474 cells was analysed by flow cytometry after labeling with antisera specific for either the μ, α or π class of GST. As shown in Fig. 1, both cell lines gave a single homogeneous peak of fluorescence with each antiserum, indicating a constant distribution of the GSTs throughout the cell cycle. This figure also shows the largely increased μGST content of HC474 cells as compared to that of GMA32 cells, whereas the level of α and πGST is similar in both cell lines. This indicates that the overexpression of only the μ component of the GST family is responsible for the 14-fold increase in GST activity towards CDNB detected in HC474 extracts [32].

GSH level and turnover

GSH pools of 1.9 and 4.0 nmoles/10^6 cells were determined in exponentially growing GMA32 and HC474 cells, respectively, by the Tietze recycling assay. The GSH pool was reduced to a similar extent and had a similar half-life (2 hr) for both cell lines when exposed to the γGCS inhibitor, BSO, at 1 mM concentration (Fig. 2A). Moreover, the same dose of 3 μM BSO added for 16 hr was necessary to reduce the GSH pool by 50% in either cell line (Fig. 2B).

Role of GSH in anticancer drug susceptibility

To determine if GSH is significantly involved in drug detoxification, GMA32 cells were exposed to 100 μM BSO for 16 hr to completely deplete their GSH pool before checking their susceptibility to BCNU, chlorambucil, cisplatine, doxorubicin and melphalan. Figure 3 shows that GSH depletion increased the susceptibility of GMA32 cells to melphalan and chlorambucil, indicating that GSH contributes to the detoxification of these two drugs. In contrast, the same treatment had only a minor effect on the susceptibility of these cells to BCNU, cisplatine and doxorubicin.

Depletion of GSH by GST substrates

Since GSH depletion increased nitrogen mustard toxicity, it was tempting to try to selectively induce such a depletion in μGST overproducing cells. This led us to investigate the ability of μGST substrates to deplete the GSH pool more rapidly in HC474 than in GMA32 cells. In a first screen, cells were exposed for 15 min at 37° to such substrates in the presence of BSO to inhibit GSH biosynthesis. As shown in Table 1, exposing cells to cetremide or 2-bromo-2-chloro-1,1,1-trifluoroethane did not affect the GSH pool of either line. In contrast, nearly complete GSH depletion was obtained within 15 min in both lines when exposed to 100 μM CDNB, or *p*-nitrobenzylchloride. This result was expected for CDNB, because the total GST activity with this chemical (0.26 and 3.60 μmol/mg/min for GMA32 and HC474, respectively, as determined *in vitro*) predicted complete GSH depletion within a few seconds. Other chemicals, such as 3,4-dichloro-nitrobenzene, 1,2-epoxy-3-(*p*-nitrophenoxy) propane and styrene oxide, largely depleted the GSH

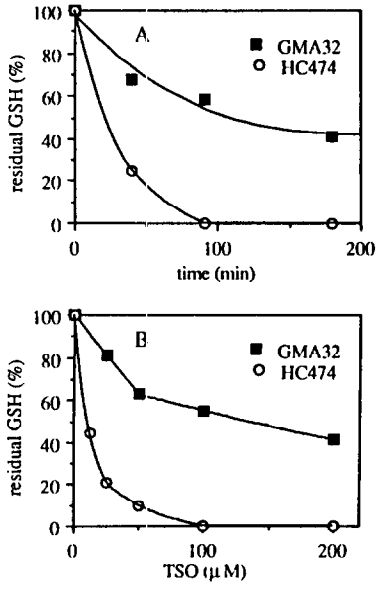

Fig. 4. Short term effect of TSO exposure at 37° on GSH pools of GMA32 and HC474 cells. (A) Time course of GSH depletion in cells exposed to 0.1 mM TSO, (B) residual GSH in cells exposed for 3 hr to various doses of TSO. In this experiment, 100% GSH represented 2.3 and 3.9 nmol/10^6 cells for GMA32 and HC474 cells, respectively.

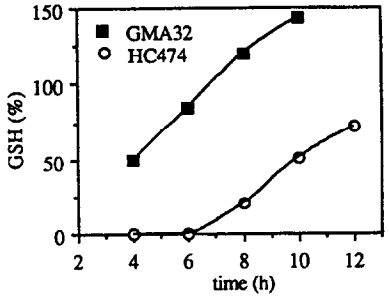

Fig. 5. Spontaneous release of GSH depletion induced by TSO. At time 0, 100 μM TSO was added to the culture medium and kept throughout the experiment. Results are expressed as the percentage (%) between the GSH content of TSO treated and untreated cells. The GSH content at the beginning of the experiment was 2.0 and 3.75 nmol/10^6 cells for GMA32 and HC474 cells, respectively.

pool in HC474 by more than 50% during this short period of treatment as well as in GMA32 cells, although to a lesser extent. However, two substrates—TSO and hexachlorobutanediene—did not significantly alter the GSH pool in GMA32, but decreased it in HC474 cells by approximately 30% within 15 min.

The effect of TSO at 37° was next analysed in more detail. Figure 4A shows that in the presence of 100 μM TSO—a concentration not toxic for the cells—the GSH pool of the mutant line dropped to an undetectable level in less than 2 hr indicating that all the cells were depleted. This result is in agreement with the homogeneous distribution of μGST observed in Fig. 1. In contrast, the GSH pool of GMA32 was decreased by approximately only 40% by this treatment. Figure 4B shows the dose-dependent effect of a 3 hr TSO treatment on the GSH level in the two cell lines.

Long term effect of TSO on GSH pool

We also observed that, in contrast to short term cultures, prolonged growth of 10^5 GMA32 or HC474 cells for 1 or 2 days in the presence of TSO alone did result in an increase of their GSH pool three to five times above the steady state level. For instance, in one experiment, GSH pools of 7.5 and 11 nmol/10^6 cells, respectively, were found in GMA32 and HC474 cells grown 24 hr in 100 μM TSO while in parallel control cultures without TSO, the corresponding pools were 1.8 and 3.8 nmol/10^6 cells. Figure 5 shows that after a 6 hr TSO exposure, GSH depletion in HC474 began to be overcome and the GSH pool increased. A comparable increase with remarkably similar kinetics was observed with GMA32 (Fig. 5). The reversal of GSH depletion was not due to TSO exhaustion, as shown by a physiological assay of the TSO remaining in the cultures. Five centimetre Petri dishes containing 5 mL of culture medium supplemented with 100 μM TSO were seeded with either 2×10^5, 5×10^5 or 2×10^6 GMA32 or HC474 cells and, 24 hr later, the culture medium from each dish was tested for its ability to induce GSH depletion in HC474 cells. Medium conditioned by growing GMA32 cells preserved its ability to induce complete GSH depletion in HC474, whatever the initial input of cells. Medium from plates seeded with 2×10^5 HC474 cells also retained its ability to induce GSH depletion. On the contrary, medium conditioned by growing an initial input of 5×10^5 HC474 cells only reduced the GSH level by 50% in the HC474 test cells, a result comparable to that obtained by exposing cells to 10 μM fresh TSO. Medium from plates seeded with 2×10^6 HC474 cells did not induce GSH depletion in HC474, indicating complete TSO exhaustion (data not shown). These experiments show that there is a short period of time during which HC474 cells can be specifically depleted of GSH by TSO and that the GSH level rebounds after prolonged periods of treatment.

Alteration of nitrogen mustard resistance by TSO

Since GSH operates in the detoxification of melphalan and chlorambucil, we expected that TSO-induced GSH depletion would increase the toxicity of these drugs, but not that of BCNU, cisplatine or doxorubicin in HC474 selectively. This hypothesis was directly tested by exposing GMA32 and HC474 cells to 100 μM TSO for 3 hr before adding the drugs to the medium. Figure 6 shows that untreated HC474 cells are only slightly more resistant than GMA32 cells to BCNU, cisplatine, doxorubicin and melphalan. The ID_{50} value for chlorambucil was approximately 50% higher for HC474 than for GMA32 cells, whereas the ID_{50} values for the other drugs were similar for both lines. But at the highest drug concentrations tested, HC474 appeared to be

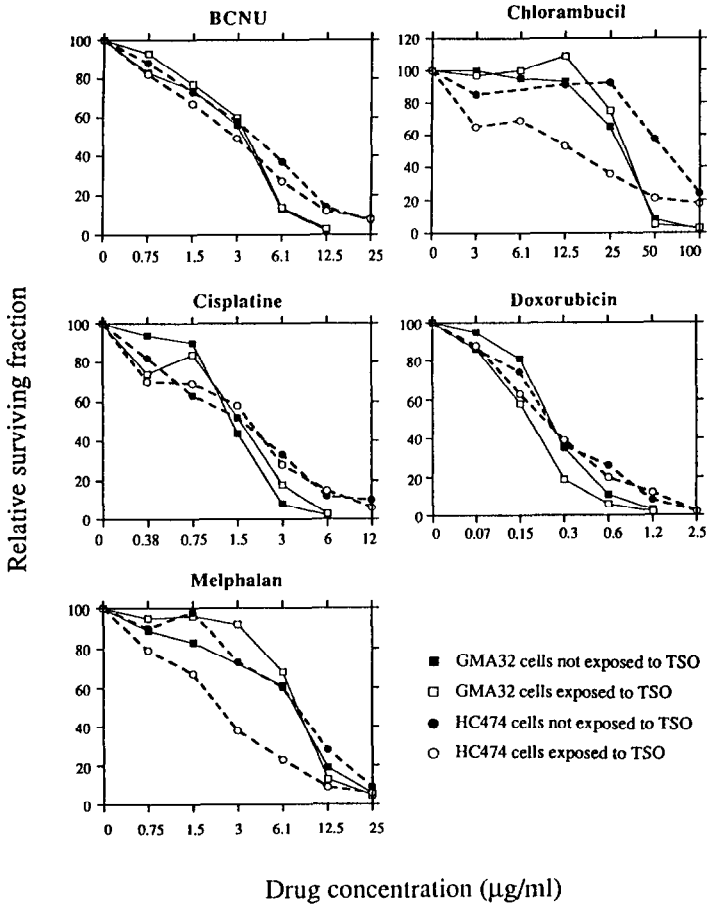

Fig. 6. Effect of TSO at 37° on the susceptibility of GMA32 and HC474 cells to anticancer drugs. After a 3 hr exposure of the cells to 100 μM TSO, the drugs were added and kept for 2 hr before being removed. The surviving cell fraction was determined 3 days later by the MTT assay.

more resistant than GMA32, since the ID_{90} of HC474 was approximately twice that of GMA32 cells. Figure 6 also shows that the susceptibility of GMA32 or HC474 cells to BCNU, cisplatine and doxorubicin was unaltered by the pretreatment of cells with TSO. In contrast, an important effect of TSO pretreatment was evident for HC474 exposed to either chlorambucil or melphalan: the ID_{50} of these drugs was decreased 4-fold in TSO-treated HC474 cells. These results indicate that a manipulation of the GSH pool can induce cells overexpressing GST to be more susceptible to anticancer drugs.

Comparative effect of TSO and BSO on nitrogen mustard susceptibility

The relative efficiency of TSO and BSO in increasing the susceptibility of HC474 cells to chlorambucil and melphalan was compared in the same experiment: the drug susceptibility of HC474 cells was verified after exposure to 100 μM BSO for 16 hr or 100 μM TSO for 3 hr. Figure 7 shows that HC474 cells exposed to TSO are slightly more resistant to both drugs than cells exposed to BSO.

Alteration of nitrogen mustard susceptibility of cells with a moderate level of μGST

The experiments reported above demonstrated the possibility of increasing the toxicity of nitrogen mustards in cells with a high level of μGST overexpression. This study was extended to HC4 cells that express only a 2-fold increment of GST activity as shown with CDNB as a substrate: HC4 cells were exposed to 100 μM TSO for 2 hr before being exposed to melphalan or chlorambucil for 2 hr. Figure 8 shows that in this cell line, exposing cells to TSO increased the efficiency of these two anticancer drugs to an extent comparable to that obtained by exposure to BSO.

DISCUSSION

The aim of this work was to explore the possibility of exploiting the overexpression of a GST gene isozyme, a frequent feature of tumor cells, to improve not only the efficiency but also the selectivity of anticancer drugs against these cells. The rationale was to use GST overexpression to depress the GSH

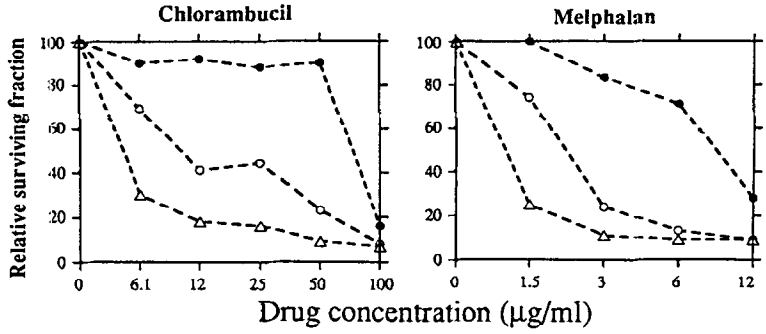

Fig. 7. Comparison of drug susceptibility of HC474 cells after GSH depletion induced by either BSO or TSO. The drugs were added and kept for 2 hr to HC474 cells previously exposed to either 100 μM TSO for 3 hr or 100 μM BSO for 16 hr. The relative surviving fraction was determined 3 days later by the MTT assay. (●) Untreated HC 474 cells, (○) HC474 cells exposed to TSO, (△) HC474 cells exposed to BSO.

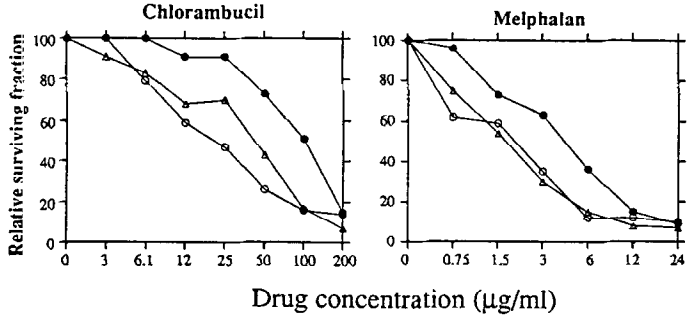

Fig. 8. Comparison of drug susceptibility of HC4 cells after GSH depletion induced by either BSO or TSO. The drugs were added and kept for 2 hr to HC4 cells previously exposed to either 100 μM TSO for 3 hr or 100 μM BSO for 16 hr. The relative surviving fraction was determined 3 days later by the MTT assay. (●) Untreated HC4 cells, (○) HC4 cells exposed to BSO, (△) HC4 cells exposed to TSO.

pool and, as a consequence, to improve the toxicity of drugs which are detoxified via a GSH dependent pathway.

We first observed that GSH starvation imposed on GMA32 fibroblasts by exposure to BSO did increase the toxicity of the alkylating agents chlorambucil and melphalan, but not that of BCNU, cisplatine or doxorubicin. This property seems to be in contradiction with several observations made of other experimental systems: *in vitro* denitrosation of BCNU by pure rat μGST has been established [35], but different isozymes operate with different catalytic efficiencies and GMA32 cells may not express an efficient isozyme. This interpretation is in agreement with the observation that HC474 cells did not resist BCNU. Cisplatine has been shown to react with GSH [36], and tumor cell susceptibility to this drug has been correlated with a low GSH pool [37, 38]. The exact molecular mechanism involved in the modulation of cisplatine toxicity by GSH is not fully understood: GSH can protect cells by quenching DNA platinum mono-adducts, but in addition the GS-platinum complex *per se* may be toxic [36]. Altering the GSH pool will alter the balance between these opposite effects of GSH with unpredictable consequences on cisplatine susceptibility. Some products of doxorubicin metabolism are substrates for GSTs [39]. In fact, evidence linking GSH level or the expression of a specific GST to the susceptibility to these drugs remains conflictual. In contrast, GSH operates in the detoxification of chlorambucil and melphalan. This observation is in complete agreement with both biochemical and genetical evidence that these drugs are detoxified by αGSTs [20, 40]. We have shown that this enzyme is expressed in our fibroblasts and that depleting GSH is likely to turn off this detoxification pathway.

Depleting GSH by any other method was expected to have a similar effect and inducing this depletion selectively in GST overexpressing cells was expected to target the toxicity of these agents to these cells. In our experimental system, this was easily obtained by exposing cells to substrates either preferential or specific to the overexpressed GST isozyme. TSO was used in most experiments, since in man this substrate is known to be absolutely specific for the product of the *GSTM1* gene [41]. In hamster, such

a specificity of TSO has not been established but the possibility of direct competition between this substrate and the drug for the same detoxification pathway is very unlikely since exposing GMA32 cells to TSO did not alter the pattern of drug susceptibility of these cells (Fig. 6). In contrast, subjecting HC474 cells to the same treatment greatly increased their susceptibility to chlorambucil and melphalan to an extent comparable to that obtained in the presence of BSO. This indicated that GSH depletion by itself is sufficient to increase drug toxicity. An important point of this study was to be sure that μGST overexpression mediates TSO-induced GSH depletion in HC474 cells: these mutant cells overexpress μGST by gene amplification and other genes involved in GSH metabolism could be overexpressed in this system. In particular, this line contains twice as much GSH as GMA32 cells. An increased GSH pool has also been observed in CHO cells resistant to nitrogen mustard through αGST gene amplification [20]. These mutant lines may contain an additional defect responsible for the pool expansion: the resistant CHO line was reported to express an increased γGT activity. This defect, which may account for GSH accumulation in that line [42] was not observed in HC474 cells (not shown). An overexpression of γGCS or GSH reductase activity was also ruled out by an *in vitro* assay (data not shown). Alternatively, GSH pool expansion may be a consequence of GST overexpression, since it has been shown that some GSH conjugates release feedback inhibition of γGCS and activate GSH synthesis [43]. GST overexpressing cells may contain an increased pool of various conjugates. Such an interpretation also account for the GSH pool expansion observed after prolonged TSO exposure.

We demonstrate here the possibility of increasing *in vitro* the susceptibility of GST overexpressing cells to alkylating anticancer drugs. The gain in toxicity of these drugs obtained after TSO treatment was lower than that obtained with BSO. The cause of this slight difference has not been analysed, but may simply reside in less efficient GSH depletion in cells exposed to TSO compared to cells exposed to BSO: a low residual GSH pool (<3%) would escape detection. In addition, after withdrawal of the depleting agent, GSH biosynthesis resumes earlier in TSO treated cells than in cells exposed to BSO, allowing a faster detoxification of the intracellular pool of alkylating agent. In the experiments reported here, the advantage of using TSO over BSO as depleting agent was the selectivity of GSH depletion for GST overexpressing cells. The same advantage should exist *in vivo* and the important question to be evaluated is the possibility of achieving significant GSH depletion *in vivo* by this procedure. The development of fluorescent GSH probes should help to quickly identify the adequate depleting substrates by flow cytometry analysis of small tumor samples.

In this work, we did not analyse the effect of directly inhibiting GST activity by using ethacrynic acid, for example. Previous studies showed that this inhibitor increases cell susceptibility to chlorambucil and melphalan through inhibition of αGST activity [29]: inhibiting this activity is expected to increase cell susceptibility to these drugs to a comparable extent in GMA32 and HC474 cells, since both cell lines express a comparable level of αGST.

The results reported here encourage attempts to develop methods for counter-selecting cells containing specialized drug metabolizing enzyme activities. In heterogeneous tissues, a differential response to such manipulations of the GSH pool may directly exploit the specific enzyme profile resulting from the differentiation status of the cells. A well-known example is the GSH depletion specifically induced in hepatocytes by paracetamol [44].

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