Increased levels of glutathione *S*-transferase π transcript as a mechanism of resistance to ethacrynic acid

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Subpopulations of HT 29 human colon carcinoma cells (HT/M and HT/S) were selected for resistance to the glutathione *S*-transferase (GST) inhibitor ethacrynic acid (EA). Both clones displayed a 2-fold resistance to the selection agent and required its constant presence for the maintenance of the resistant phenotype. Purification and characterization of GST isoforms showed similar profiles in the wild-type (WT) and EA-resistant clones, with microheterogeneous forms of the π isoenzyme detected in each case. Metabolism of EA *in vitro* in the presence of GSH and the isolated GST from each cell line was characterized by a biphasic disappearance of the parent drug; the initial rate at which each of these enzymes metabolized EA was similar. These enzymes also displayed similar K_m values for 1-chloro-2,4-dinitrobenzene. However, the amount of GST isolated per total cellular protein was 3.0-fold in HT/M and 1.6-fold in HT/S relative to WT in the continuous presence of EA. Under these conditions GST activity was increased by 2.3-fold in HT/M and 3.2-fold in HT/S as were GSH levels (2.7- and 4.1-fold for HT/M and HT/S respectively). When EA was removed, enzyme activity and GSH concentrations decreased to values similar to those of the WT. Slot-blot and Southern analyses of the DNA gave no evidence of GST- π -gene amplification or rearrangement. However, RNA analyses by both slot-blot and Northern studies indicate a 2.5–3.5-fold elevation in the GST π transcript in the EA-resistant population. Results from these studies indicate that: (1) maintenance of the EA-resistant phenotype requires constant presence of the agent; (2) the 2-fold resistance to EA can be quantitatively related to a 2–3-fold increase in GST activity and amount which appears to be the result of a 2.5–3.5-fold elevation in GST transcript; (3) EA, a Michael-reaction acceptor, can induce GST at the transcriptional level.

INTRODUCTION

The success of antineoplastic chemotherapy is severely limited by the phenomenon of drug resistance. Whether this resistance is natural or acquired, the outcome is ineffectiveness of therapy and regrowth of the tumour by resistant cells, resulting in a lesion which is refractory to subsequent chemotherapy. The biochemical alterations associated with the development and maintenance of drug resistance are numerous, and the cellular mechanisms underlying these modifications are multiple. Through their altered expression, the glutathione *S*-transferases (GSTs) are one family of enzymes which have been implicated as causative agents in the expression of the drug-resistant phenotype [1,2]. These enzymes are recognized for their detoxification of compounds (including various anticancer drugs) either through catalytic conjugation of substrates with glutathione (GSH) or their selenium-independent peroxidase activity [3]. In early work linking GSTs to resistance [4,5] a comparison was made of a Walker 256 rat mammary carcinoma cell line sensitive to the alkylator chlorambucil and a subpopulation which was made resistant to this agent. The resistant cells expressed elevated levels of a Yc GST subunit, suggesting the GST α family as having a role in the detoxification of alkylating agents. Subsequently, numerous studies have linked the overexpression of certain GST isoforms to a characteristic resistant phenotype. Further evidence supporting the link between GST α and the detoxification of alkylators has been provided by results from transfection studies [6,7] as well as data from enzyme reactions *in vitro* [8,9]. Additionally, GST π (an isoform linked to malignant transformation [10] and frequently overexpressed [11] in tumour tissue) may have a role in the detoxification of lipid hydro-

peroxides [12,13]. Although these GST isoforms have overlapping substrate specificities, correlations between a specific GST isozyme and the detoxification of a compound (i.e. resistance) are evident.

While it may be possible to overcome some types of resistance through the use of multiple or elevated levels of cytotoxic agents, the results most often are inadequate. Recently it has become popular to approach this problem by the modulation of the cellular target presumed responsible for resistance; the ultimate goal of this mode of therapy is an increased therapeutic index and circumvention of resistance. In previous work we [14] and others [15,16] have demonstrated successful examples of this approach involving the phenoxyacetic acid derivative ethacrynic acid (EA) in combination with various chemotherapeutic agents. EA is a GST substrate [17] that can also bind covalently to certain GST isoforms [18]. Our studies showed pretreatment of HT 29 human colon carcinoma cells with EA at non-toxic levels inhibited GST activity by approximately 50%, with a concomitant 4-fold sensitization of these cells to chlorambucil. A similar study in mice [19] produced results which corroborated the potentiation of alkylating agent efficacy by EA. Although the competitive inhibition of GST by EA may play a primary role in the increased efficacy of alkylating agents in these studies, of some concern is the possibility that chronic exposure to EA may produce cellular alterations which diminish the potentiation of cytotoxicity initially afforded by the drug. EA can be structurally classified as a Michael-reaction acceptor, of which its α , β -unsaturated ketone moiety constitutes the electrophilic property typical of this class of compounds. Talalay *et al.* [20] have shown that the induction of GST activity as well as substrate activity requires the presence of an electro-

Abbreviations used: GST, glutathione *S*-transferase; EA, ethacrynic acid; EMS, ethyl methanesulphonate; HT/M, EA-resistant cells selected in the presence of EMS and EA; HT/S, EA-resistant cells selected in the presence of EA; CDNB, 1-chloro-2,4-dinitrobenzene; BSO, DL-buthionine-S,R-sulphoximine; WT, wild-type; PBS, phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/8 mM-sodium phosphate, pH 7.4); TEA, triethylamine/phosphoric acid [0.0025% (v/v) each]; IC₃₀ (IC₅₀, IC₉₀), concentration causing 30% (50%, 90%) inhibition of growth.

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philic Michael acceptor. Thus EA can induce its own metabolism. In the present paper we describe properties of cells which have acquired resistance to EA. Our data quantitatively correlate elevated GST transcript, increased GST activity/amount and EA resistance, suggesting the induction of GST expression plays a prominent role in the expression of the EA-resistant phenotype and confirming the regulation of GST expression by Michael-reaction acceptors.

MATERIALS AND METHODS

Materials

A cDNA clone which codes for a human anionic GST was generously provided by Dr. I. Listowsky, Albert Einstein College of Medicine, Bronx, NY, U.S.A. Restriction endonucleases were from Amersham International, and [α - 32 P]dCTP (6000 Ci/mmol) was from New England Nuclear.

Cell culture

The human colon carcinoma HT 29 cell line (HT 29) was grown as a monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 2 mM-L-glutamine, 50 units of penicillin/ml, 50 μ g of streptomycin/ml and 10% heat-inactivated fetal-calf serum (complete MEM). All cultures were maintained at 37 °C in a humidified CO₂/air (1:19) atmosphere. Cells were determined to be mycoplasma-free on a quarterly basis. The HT/M cell line was formed by mutagenization of a double subclone of the HT 29 parental cells with ethyl methane-sulphonate (EMS; 340 μ g/ml, 24 h). After recovery, single cells were plated and grown in the presence of 72 μ M-EA. Resistant clones were selected and cultured with continuous exposure to the same concentration of this agent. Another subpopulation of HT 29 cells resistant to EA (HT/S) was formed by a single-step selection against 72 μ M-EA, which were then maintained by continuous exposure to the agent.

Cytotoxicity assay

Cells in the exponential phase of growth were trypsin-treated and replated in 25 cm² plastic culture flasks (750 cells/flask; 10 ml total volume) in the presence of the drug to be tested. Plating efficiency was 41% for wild type (WT), 47% for HT/M, and 61% for HT/S. Drugs were dissolved to give a 20 $\times$ stock concentration [EA in ethanol/water, 1:1, v/v]. At 10 days after plating, the media was aspirated from the flasks; colonies were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) (5 min). After removal of the fixative, colonies were stained for 5 min with 0.1% Crystal Violet and washed with water (4–5 times) until background stain was negligible. Colonies were counted on a Bio-Tran III Automatic Count/Area Totalizer (New Brunswick Scientific Co., Edison, NJ, U.S.A.). Survival was determined by standardizing vehicle-treated flasks to 100% and expressing drug-treated determinations as a percentage of this value. All data points represent the mean $\pm$ S.E.M. of triplicate determinations.

Enzyme purification

GST was purified by affinity h.p.l.c. according to the method of LaCreta *et al.* [21].

GSH determination

Cells (5–10 million) were sonicated on ice and proteins were precipitated with 5-sulphosalicylic acid at a final concentration of 3%. Intracellular GSH levels were determined spectrophotometrically in the 10000 g supernatant as previously described [22].

GST activity determination

For all cases GST activity was evaluated spectrophotometrically, utilizing 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [23]. Determination of the kinetic constants for CDBN followed established methodology [24]. Each reaction was carried out in 1.0 ml of phosphate buffer (0.1 M, pH 6.5, 25 °C) containing 1.0 mM-GSH. CDBN concentrations were varied such that 0.02 ml of substrate in 100% ethanol was added to the mixture. The enzyme (0.25 μ g) was added last, and the true rates were determined after subtraction of background. The K_m and V_{max} values were calculated from Lineweaver–Burk plots.

Electrophoresis

Proteins were analysed by one-dimensional SDS/PAGE [25] using 12% (w/v) polyacrylamide containing 0.1% SDS. Separated proteins were revealed by silver staining [26]. Agarose isoelectric focusing (i.e.f.) was carried out on 3% (w/v) agarose cast on GelBond (Pharmacia, Piscataway, NJ, U.S.A.) in a pH range of 4–6.5. Proteins were separated at 15 W and 1500 V for 3 h and then revealed by Coomassie Blue stain. For Western blotting [27], proteins were transferred to nitrocellulose and incubated with polyclonal antibodies raised against various forms of GST (Medlabs, Dublin, Ireland). Immunoreactive proteins were revealed with 4-chloro-1-naphthol (Bio-Rad, Richmond, CA, U.S.A.) as substrate.

Determination of EA metabolism

Reaction mixtures (2.2 ml final vol.) containing 72 μ M-EA in PBS were equilibrated to 37 °C. GSH (216 μ M final concn.) and GST (0.035 μ g/ μ l) were added to initiate the reaction. Samples containing EA were prepared for h.p.l.c. analysis by the following extraction procedure. Aliquots (100 μ l) were removed at selected times into PBS to give a total volume of 1 ml. The mixture was acidified with 1 ml of 1 M-HCl, followed by the addition of 5 ml of ethyl acetate. EA was extracted into ethyl acetate by rocking the mixture on a nutator (Becton, Dickinson and Co., Parsippany, NJ, U.S.A.) for 5 min at room temperature. The organic and aqueous layers were separated by centrifugation (1000 g) for 10 min at room temperature. Exactly 4 ml of the ethyl acetate layer was removed and evaporated to dryness under a stream of N₂ at room temperature. The residue was reconstituted in 0.5 ml of 30% (v/v) acetonitrile in triethylamine/phosphoric acid [0.0025% (v/v) each] (TEA). The h.p.l.c. injector system was a Rheodyne model 7025 injection valve equipped with a 0.2 ml sample loop.

The chromatographic system consisted of a Hewlett–Packard (Palo Alto, CA, U.S.A.) 1090L liquid chromatograph equipped with a diode array detector. Chromatography was performed on a Hewlett–Packard reversed-phase C₁₈ analytical column (Hypersil ODS; 5 μ m; 100 mm $\times$ 4.6 mm internal diameter). The column effluent was monitored at 280 nm. The isocratic mobile phase of acetonitrile/TEA (2:3, v/v) was delivered at a flow rate of 1.5 ml/min. Quantification of EA was performed daily by generating a standard curve (concentration versus peak height; 0.002–1.00 mg of EA/ml). Standard curves were weighted by 1/peak height. EA-disappearance curves were fitted to a bi-exponential equation using the NONLIN 84 computer program and a weighting factor 1/concentration. Rate constants were obtained as output, and half-lives calculated from 0.693/rate constant.

Nucleic acid studies

High-molecular-mass DNA was extracted by standard procedures [28], and total cellular RNA was isolated by the method of Chomczynski & Sacchi [29]. Slot-blot analysis involved

application of increasing amounts of the nucleic acid to a nylon membrane (Zeta-Probe; Bio-Rad, Richmond, CA, U.S.A.). For Southern analysis, DNA was digested with either *Eco*RI or *Hind*III endonuclease, size-fractionated on a 1%-agarose gel, and transferred to the Zeta-Probe matrix. Northern analysis involved size fractionation of the RNA on a 1% agarose gel that contained 2.2 M-formaldehyde, followed by transfer to the Zeta-Probe membrane. In all cases the nucleic acids were hybridized to either a GST π or β -actin DNA probe that was 32 P-labelled by using a random-primed DNA labelling kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Membranes were washed using standard protocol stringency conditions recommended by the manufacturer. Quantification of the autoradiographs was by densitometric scanning of the appropriate signals.

RESULTS

Subpopulations of HT 29 cells resistant to EA were selected either with or without prior exposure to a mutagen. In the former case, exponentially growing WT cells were exposed to EMS [IC_{30} (concn. causing 30% inhibition of growth) $340 \mu\text{g/ml}$] and allowed to recover. The mutagen-treated culture was replated as single cells and grown in the presence of twice the IC_{90} of EA ($72 \mu\text{M}$) for 3 weeks. A clone was designated HT/M and determined to be approx. 2-fold resistant to EA (Fig. 1). In the case of the selection method involving EA alone ($72 \mu\text{M}$; 4 weeks), the chosen clone, HT/S, was also 2-fold resistant to EA (Fig. 1). Removal of EA from the culture conditions for 1 growth cycle (3–5 days) afforded complete reversal of resistance. A cross-resistance pattern of the selected clones against various anticancer agents was then developed from colony-forming assays (results not shown). The most significant cross-resistance was against the nitrogen mustard melphalan; the HT/M clone displayed a 3.7-fold resistance to this agent, whereas HT/S cells were 2.0-fold resistant relative to WT. Isolation and Western-blot analysis of membrane proteins from parental and resistant cells showed no evidence of P-glycoprotein, indicating the multidrug-resistant phenotype was not an issue in the expression of EA resistance (results not shown).

To compare the GST isozyme profile and expression in WT

Fig. 1. Determination of EA resistance

Individual cells (750) from WT (●), HT/M (○) and HT/S (□) populations were seeded in the presence of EA. Surviving colonies were stained and counted after 10 days of growth. The IC_{50} and the corresponding resistance (the ratio of $IC_{50}^{HT/M}$ or $IC_{50}^{HT/S}$ to IC_{50}^{WT}) are the mean values for triplicate experiments and are representative of continuous-drug-exposure conditions. The IC_{50} values (EA) were $26 \mu\text{M}$ for WT and $48 \mu\text{M}$ for HT/M and HT/S cells. In both cases the selected clones were approx. 2-fold resistant to EA.

Fig. 2. Analysis of GST isoenzymes

(a), Panel (i), GST from WT, HT/M, and HT/S cells was isolated by GSH-affinity h.p.l.c. Equivalent amounts of protein ($5 \mu\text{g}$) were analysed by SDS/PAGE and silver-stained. In each of the three cell lines, one GST isoenzyme at 23 kDa was detected. Removal of high-molecular-mass non-GST proteins was afforded by repurification of the preparation by additional GSH affinity chromatography. (a), Panel (ii), in a parallel analysis of the GST isolations seen in (i), equivalent levels of the % of GST per total protein) were analysed by SDS/PAGE, transferred to nitrocellulose, and sequentially immunoreacted with polyclonal antibodies raised against various forms of GST. The antibody reaction sequence was as follows: 1, rabbit anti-(rat liver GST Ya); 2, rabbit anti-(rat liver GST Yb); 3, rabbit anti-(human placental GST π). Only GST π was detected in all three protein purifications. (b) Agarose i.e.f. of GST preparations. Isolated GST ($7.5 \mu\text{g}$) from WT, HT/M, and HT/S cells was separated by agarose i.e.f. over the pH range of 4–6.5. Duplicate gels were either stained with Coomassie Blue (i) or proteins were transferred to nitrocellulose and immunoreacted with a polyclonal antibody specific for GST π (ii). Three forms of GST π were detected in each of the cell types. The pI values were determined to be 4.27, 4.34 and 4.44.

and resistant cells, these proteins were isolated by affinity h.p.l.c. Fig. 2(a) (top) shows silver-staining one-dimensional SDS/PAGE of these isolates, in which equal amounts of protein were compared. Only one band is evident in the 20–30 kDa range, suggesting only one GST isozyme is expressed in all samples. However, the amount of the GST isolated per total initial amount of protein (i.e. cellular homogenate) differs between these populations. In HT/M total GST is 3.0-fold higher than in

Table 1. Data summary of Lineweaver-Burk analyses evaluating the affinity of WT, HT/M and HT/S GST for CDNB

In all cases GSH (1.0 mM) and enzyme (0.25 μ g) concentrations were held constant while the amount of CDNB was varied. Double-reciprocal plots were generated from best-fit determinations of spectrophotometric assay data. The K_m and V_{max} values were determined from the x - and y -intercepts, respectively. All data points are means for triplicate experiments; errors were less than 5% of the calculated values.

GST source	K_m^{CDNB} (mM)	V_{max}^{CDNB} (μ mol/min per ml)
WT	1.59	0.23
HT/M	1.18	0.22
HT/S	1.25	0.20

Fig. 3. Comparison of WT and EA-resistant GSH levels (a) and GST activities (b) in the presence or absence of EA

Exponentially growing cells were harvested, sonicated (15 s) and centrifuged at 10000 g for 10 min at 0 °C. All spectrophotometric measurements were made using the supernatants. The values represented graphically are indicative of HT/M (▨) and HT/S (▤) data normalized to WT (■) for comparison and are means $\pm$ S.E.M. for three individual experiments done in triplicate. Baseline GST activities were 66.1 ± 4.3 , 155 ± 22 and 212 ± 19 nmol/min per mg for WT, HT/M, and HT/S respectively. The basal GSH levels were 3.46 ± 0.3 , 9.32 ± 0.7 and 14.3 ± 1.5 nmol/mg for WT, HT/M and HT/S respectively.

WT, whereas HT/S is 1.6-fold greater. This can be seen on a Western blot in which equivalent levels of the percentage of GST per total protein were compared (Fig. 2a, ii) and enzyme is further identified by its reactivity with a rabbit anti-(human GST π) polyclonal antibody. Increased amounts of the π isoform are apparent in the HT/M isolate, whereas the α and μ forms of GST are not expressed (as determined by absence of reactivity with polyclonal antibodies against GST α and μ). Our laboratory has

Fig. 4. Slot-blot analyses of total cellular RNA (top) and high-molecular-mass DNA (bottom) from EA-sensitive and resistant cells

RNA (2.5, 5.0, 7.5 and 10.0 μ g) or DNA (0.5, 0.75, 1.0 and 2.5 μ g) was applied to the Zeta-Probe matrix under low vacuum. In either case EA represents nucleic acid from HT/M cells. These membranes were probed with a 32 P-labelled cDNA for human GST π as described in the text. Loading error was corrected for by comparison with autoradiograph signals after hybridization with β -actin.

reported [30] multiple forms of this HT 29 π isoform which mainly differ in their pI values. Agarose i.e.f. was utilized to determine whether this type of microheterogeneity occurred in the EA-resistant populations (Fig. 2b, i and ii). In all three clones there are three distinct forms of GST π with pI values of 4.27, 4.34 and 4.44. There appear to be no obvious differences in the respective levels of each of these forms.

The catalytic properties of the purified GST from WT, HT/M, and HT/S cells were compared using either EA or CDNB as substrate. In the case of EA, the metabolism of this agent *in vitro* was monitored by h.p.l.c. Levels of EA remaining over time were determined by comparison to a standard curve generated from known concentrations of the drug. The disappearance of EA in the presence of isolated GST from the WT and EA-resistant cells was biphasic (results not shown). However, the initial rates of disappearance and the corresponding half-lives of EA with each isolated GST were not significantly different as determined by weighted non-linear regression analysis (k_1 values: 0.398 min^{-1} , 0.464 min^{-1} and 0.327 min^{-1} for WT, HT/M and HT/S respectively). The affinity of GST from WT, HT/M, and HT/S clones for the substrate CDNB was determined spectrophotometrically. GSH levels and GST amounts were held constant while the CDNB concentration was varied. The K_m and V_{max} values derived from Lineweaver-Burk analyses of these data are given in Table 1. The affinities for CDNB of all the GSTs were similar, and the V_{max} values were identical ($0.20 \mu\text{mol/min per ml}$).

The determination of GSH levels and GST activities in the WT, HT/M, and HT/S cells was made through standard spectrophotometric assays. As with all of these studies, the resistant populations were maintained in the presence of 72 μ M-EA. However, before each assay a portion of these cultures was seeded and grown in the absence of EA (3 days) to evaluate the effects of short-term removal of the selection agent. A comparison of GSH levels (top) and GST activities (bottom) either in the presence or absence of EA is depicted in Fig. 3. Constant exposure to EA produces elevated GSH levels and increased GST activities in the HT/M and HT/S populations relative to WT. GSH levels are 2.7-fold and 4.1-fold elevated while GST activities are 2.3 and 3.2-fold increased (HT/M and HT/S respectively). However, if the selection pressure is removed for a short time (3 days), the levels of GSH and the GST activity decline to levels similar to (HT/M) or below (HT/S) those of the WT.

Nucleic acids from WT and HT/M cells were isolated and compared for the expression and amount of the GST π gene and transcript. Fig. 4 depicts slot-blot analysis of RNA (top) and DNA (bottom) utilizing a cDNA clone for GST π . Although

Fig. 5. Northern (a) and Southern (b) analyses of nucleic acids from EA-sensitive and -resistant cells

In the case of Northern analysis, RNA was size-fractionated and transferred to the Zeta-Probe matrix. Size was determined through comparison with 28 S, 18 S and β -actin transcripts. Lanes 1 and 3 represent duplicate samples of 20 μ g of WT RNA, whereas lanes 2 and 4 are duplicate samples of 20 μ g of HT/M RNA. A 1.2 kb transcript is detectable in both the WT and HT/M preparations, but is 3–4-fold overexpressed in the latter (as compared with β -actin transcript levels). Southern analyses involved digestion of 12.5 μ g of high-molecular-mass DNA by *Eco*RI (lanes 1 and 2 represent WT and HT/M respectively) or *Hind*III (lanes 3 and 4 represent WT and HT/M respectively). DNA size (kb) was determined through comparison with known standards and is indicated to the left and right of the autoradiograph. These membranes were probed with a 32 P-labelled cDNA for human GST π as described in the text. Loading error was corrected for by comparison with autoradiograph signals after hybridization with a β -actin probe.

there is no evidence of GST- π -gene amplification in the resistant cells, the RNA data (top) indicate there is 2.5–3.5-fold more GST π transcript in the EA-resistant cells relative to WT. These results were confirmed by the Northern (a) and Southern (b) analyses shown in Fig. 5. There is a 3–4-fold elevation in a 1.2 kb GST π transcript (lanes 2 and 4) in HT/M cells. Digests of high-molecular-mass DNA indicate there is no obvious rearrangement of this gene.

DISCUSSION

EA-resistant cells were selected from a double subclone of the HT 29 human colon carcinoma cells in the presence (HT/M) and absence (HT/S) of a mutagen. The 2-fold resistance to EA and the biochemical properties presumed responsible for the expression of this phenotype in both HT/M and HT/S cells were unstable (one passage in culture) in the absence of the selection pressure. In each cell line microheterogeneous forms of GST π were the only GST subunits expressed. The expression of a single GST class may be a reflection of the relatively homogeneous population used in these experiments, since the WT cells are the result of a double subcloning of HT 29 cells. Before this selection,

the HT 29 population expressed low levels of GST μ (results not shown). Perhaps this change in GST isoform expression after subcloning may also be influenced by the differentiation status of these clones [31] or culture conditions [32]. A further characterization of the isolated GST from WT, HT/M and HT/S cells suggests these enzymes are similar in their ability to use a particular substrate. An analysis of the metabolism of EA *in vitro* in the presence of GSH and the GST from each cell line showed a biphasic disappearance of EA in all cases, for which a number of possible explanations exist. Most likely, the presumed EA–GSH conjugate product acts as an inhibitor of its own formation [33], causing a change in the reaction rate as the product concentration increases. Alternatively, reports have indicated that EA (or an intermediate) can covalently bind to GST [18]; the effect of this binding on catalytic function, however, is questionable [33]. It is also possible that GSH depletion may affect EA metabolism. A comparison of the k_1 values is most indicative of the differences between the GSTs with respect to EA metabolism, since substrate depletion and product inhibition are minimal at the earlier times. As indicated in these studies which monitored substrate disappearance, these enzymes appear to metabolize EA at similar rates. To compare further the catalytic capacity of these enzymes, initial-velocity studies with CDNB as the varied-concentration substrate at fixed GSH concentrations were carried out. The K_m and V_{max} values were determined from linear double-reciprocal plots. The K_m^{CDNB} values reported here are similar to those previously reported for human placental GST π [34,35]. The HT/M and HT/S enzymes had slightly greater affinities for CDNB as compared to the WT GST, but these differences do not appear significant enough to be a major factor in the expression of the resistant phenotype. They may reflect an interaction (either covalent or non-covalent) of EA with GST.

Although the GST isozyme profile did not change with the development of EA resistance, the amount of GST protein was elevated in this phenotype. The GST activities also paralleled this increase in the HT/M and HT/S cells, both of which could be quantitatively correlated with EA resistance. From these data it appears that alterations in thiol metabolism are central to the expression of EA resistance. The *Eco*RI and *Hind*III digests of the DNA from sensitive and resistant cells produced GST π restriction fragments similar to those previously described [36,37], indicating that gene amplification and rearrangements are not relevant. Alternatively, RNA slot-blot analysis showed an increase in the amount of GST π transcript produced in the HT/M population relative to WT. These data were corroborated by Northern analysis, which produced a similar elevation in a 1.2 kb transcript. Quantitatively, the 2-fold resistance to EA appears to be related to a 2–3-fold elevation in GST activity/amount which is likely the result of a 2.5–3.5-fold increase in GST transcript.

Although alterations in GST expression and function appear central in the selection of EA resistance, elevated GSH levels remain an issue. In an attempt to discern the relative importance of this alteration in the resistant population, buthionine sulfoximine (BSO) was utilized to deplete GSH through the inhibition of γ -glutamylcysteine synthase (results not shown). In the case of WT cells, BSO depleted GSH levels to about 20% of control without altering GST activity or resistance to EA, suggesting GSH is not the primary determinant in the protection of these cells to EA. The response to BSO in the EA-resistant cells differed from that of the WT population. Exposure to BSO caused HT/M and HT/S GSH levels to decrease to 10% of control with a concomitant decline in GST activity (80 and 60% of control; HT/M and HT/S respectively) and a sensitization to EA. The multiple changes which occur upon BSO treatment do not allow a clear evaluation of the precise role of GSH in these

drug-resistant populations. Furthermore, the cross-resistance of the HT/M and HT/S cells to melphalan was the most pronounced for all the agents tested. GST α has been the isoform implicated in the detoxification of alkylators [8,9], but this family of isozymes is not expressed in either the WT or EA-resistant cells. Increased spontaneous melphalan-GSH conjugation would be an expected corollary of elevated GSH levels [38], resulting in the observed melphalan resistance.

The goal of this work was to assess the development and stability of EA resistance and define the properties responsible for this resistant phenotype. Pharmacologically these effects can be described as an unstable resistance to EA accompanied by cross-resistance to alkylators. Biochemically, increased GST expression and activity, as a result of elevated amounts of GST transcript can be quantitatively correlated to the etiology of the EA-resistant phenotype. Previously, the stable increased expression of GST π by transfection techniques has afforded resistance to EA [39,40]; the data reported here indicate that EA alone can be an inducer of this isozyme, also resulting in EA resistance. Further elucidation of the underlying molecular mechanisms responsible for the biochemical effects of EA may aid understanding of the complex role of thiol metabolism and enzyme regulation in drug resistance.

This work was supported by National Institutes of Health grants CA43830, CA06927 and RR05539, a Bristol Myers drug-resistance grant, and an appropriation from the Commonwealth of Pennsylvania. We thank Donna Bunch for the preparation of this manuscript.

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Received 16 April 1991/2 July 1991; accepted 18 July 1991