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IDENTIFICATION OF GLUTATHIONE *S*-TRANSFERASE AS A DETERMINANT OF 4-HYDROPEROXYCYCLOPHOSPHAMIDE RESISTANCE IN HUMAN BREAST CANCER CELLS

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Abstract—Aldehyde dehydrogenase (ALDH) is well known for its involvement in the resistance of tumor cells to cyclophosphamide (CPA) and its activated derivatives, such as 4-hydroperoxy-CPA (4HC). The role of other drug-metabolizing enzymes such as glutathione *S*-transferase (GST) in CPA resistance is, however, less certain. In the present study of a human breast cancer cell line (MCF-7) exhibiting about 6-fold resistance to 4HC (MCF/HC), cellular levels of glutathione (GSH) were increased 1.4-fold, while cytosolic GST and ALDH activities were increased 2.7- and 7.2-fold, respectively, relative to the MCF-7 parental line. No significant changes in glutathione peroxidase and NADPH cytochrome P450 reductase activity, and no increase in microsomal GST and GSTπ mRNAs were found in the resistant cells. Treatment with the ALDH substrate octanal sensitized the cells to the cytotoxic effects of 4HC to a modest extent in both MCF-7 and MCF/HC cells [dose modification factor (DMF) of 1.4 and 1.6, respectively]. Depletion of GSH by treatment with the GSH synthesis inhibitor buthionine sulfoximine (BSO) enhanced the cytotoxic effect of 4HC to a similar extent in both cell lines. By contrast, ethacrynic acid, which inhibited GST activity by $> 85\%$ in MCF-7 and MCF/HC cell extracts without depletion of GSH, sensitized the resistant but not the parental cells to 4HC cytotoxicity, indicating the importance of GST as a determinant of 4HC resistance in these cells. This conclusion is supported by the observation that in MCF/HC cells, ethacrynic acid in combination with BSO increased the DMF 3-fold higher than did BSO or EA alone, while in the parental MCF-7 cells ethacrynic acid with BSO had no significant chemosensitization effect over BSO alone. These studies establish that in addition to ALDH, GST overexpression can contribute to acquired resistance of tumor cells to 4HC and, furthermore, suggest that modulators that target the GSH/GST system could be useful in overcoming CPA resistance in the clinic.

Key words: cyclophosphamide resistance; glutathione *S*-transferase; aldehyde dehydrogenase; ethacrynic acid

Oxazaphosphorines are widely used antineoplastic and immunosuppressive agents that are characterized by a relatively high oncologic specificity [1–3]. The oxazaphosphorine CPA† is a prodrug that is activated by liver cytochrome P450 (CYP) enzymes by a metabolic pathway that ultimately yields the alkylating agent phosphoramide mustard. The specific P450 catalysts of CPA activation have been identified as members of the CYP2B and CYP2C subfamilies in both the rat liver model [4] and human liver [5], while CYP3A enzymes have been shown to play an important role in the activation of the isomeric drug ifosphamide [5, 6]. 4-Hydroxy-CPA, the primary metabolite formed by these enzymes, equilibrates with the ring-opened aldophosphamide, which spontaneously decomposes to yield phosphoramide mustard and acrolein in equimolar amounts [2]. Phosphoramide mustard possesses DNA-alkylating activity and is generally considered to be the therapeutically significant, cytotoxic metabolite of CPA [2]. 4-Hydroxy-CPA can also be generated from the activated CPA analogs mafosfamide and 4HC under physiological conditions by non-enzymatic routes [2]. Since 4HC and mafosfamide are more stable but pharmacologically equivalent to 4-hydroxy-CPA, the former two drugs have been employed as activated CPA equivalents for various *in vitro* and preclinical investigations and applications [2].

The development of resistance of tumor cells to CPA, like that of many other anticancer drugs, is associated with multiple biochemical changes, including changes in drug metabolism in the drug-

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† Abbreviations: CPA, cyclophosphamide; 4HC, 4-hydroperoxy-cyclophosphamide; GST, glutathione *S*-transferase; GSTms, microsomal GST; GSH, glutathione; ALDH, aldehyde dehydrogenase; EA, ethacrynic acid; BSO, buthionine sulfoximine; IC_{50} or IC_{90}, drug concentration required to inhibit by 50 or 90% cell growth as colony formation activity; DMF, dose modification factor, defined as IC_{50} or IC_{90} in the absence of modulator divided by IC_{50} or IC_{90} in the presence of modulator; and MTT, 3-(4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide.

resistant cells. Of particular importance in the case of CPA is the metabolic fate of aldophosphamide, which can be inactivated by oxidation to carboxyphosphamide in a reaction catalyzed by ALDH. Indeed, overexpression of a cytosolic isoform ALDH (class-1) is associated with CPA resistance in L1210 cells [7, 8], while a class-3 ALDH is overexpressed approximately 100-fold in human breast adenocarcinoma cells exhibiting over 30-fold oxazaphosphorine-specific resistance [9–11]. The functional role of ALDH in CPA resistance has been further established by the reversal of CPA resistance by known ALDH inhibitors [12, 13] and by studies in which MCF-7 cells transfected with a cDNA encoding a rat class-3 ALDH isoenzyme acquire resistance to mafosfamide [14]. However, in most cases, the oxazaphosphorine resistance phenotype cannot be fully explained by ALDH overexpression [15]. In fact, in some 4HC-resistant cells there are no apparent changes in ALDH activity at all [16], indicating that other mechanisms of resistance are operative.

GSH/GST represents an important cellular drug-metabolizing system that has been demonstrated to be involved in cellular resistance to a number of anticancer drugs (see reviews [17, 18]). In particular, the CPA metabolite aldophosphamide can be inactivated by conjugation with cellular GSH to form a hemithioacetal [19]. GSH can also participate in conjugation reactions with either acrolein or phosphoramide mustard, leading to the formation of non-toxic derivatives [2, 19]. Alterations in any of these GSH-dependent detoxifying enzymes or metabolic steps could, in principle, lead to changes in intracellular phosphoramide mustard levels and, consequently, changes in cytotoxicity. While increases in cellular GSH levels and/or GST activity have been reported in several CPA- or 4HC-resistant cell lines [16, 20, 21], the functional significance of these changes has not been established, and consequently, the pharmacologic contribution of GSH/GST elevation to CPA resistance remains unknown. Therefore, the present studies on 4HC resistance in the human breast cancer cell line MCF-7 were carried out to evaluate the role of the GSH/GST system in CPA resistance. Our findings lead us to conclude that in addition to ALDH, elevation of GST activity serves as a determinant for the oxazaphosphorine resistance phenotype in this breast cancer cell line.

MATERIALS AND METHODS

Chemicals and cell lines

4HC powder was purchased from Nova Pharmaceutical (Baltimore, MD) and dissolved in cell culture medium immediately before use. Mafosfamide was a gift from Dr. J. Pohl, ASTA Pharma (Bielefeld, Germany), and was dissolved in medium just before use. Chlorodinitrobenzene (CDNB) and 5'-sulfosalicylic acid were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dulbecco's modified Eagle's medium (D-MEM) powder, penicillin/streptomycin liquid and trypsin/EDTA solution were purchased from GIBCO (Grand Island, NY). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). MCF-7 is a human adenocarcinoma cell line, and its 4HC-resistant subline, MCF/HC, was developed by stepwise escalation of 4HC concentrations in culture medium [22]. Both cell lines were provided by Dr. Beverly Teicher, Dana-Farber Cancer Institute, and were grown as monolayers at 37°, in 95% air and 5% CO_2, in D-MEM supplemented with antibiotics and 10% fetal bovine serum and were passaged weekly. The drug-resistant MCF/HC cells were maintained in culture without further selection with 4HC. The resistance phenotype was found to remain stable during the course of these studies. MCF/HC cells proliferated at a 1.22-fold slower rate than the drug-sensitive MCF-7 cells.

Cytotoxicity assays

Colony formation assay. Colony formation was assayed as described earlier [23]. Briefly, exponentially growing cells were pretreated in monolayer for 24 hr with 50 μM BSO and/or 50 μM EA for 15 min. For the combination study of 4HC and octanal, cells in monolayer were exposed to 4HC and octanal simultaneously for 1 hr. Thereafter, 4HC was added to the medium and then incubated for a further 1 hr. Cells were then collected by trypsinization and seeded for colony formation at three densities in duplicate per treatment, using a 6-well Falcon plate.

MTT cytotoxicity assay. Cells were plated in 96-well plates (Falcon) at a density of 1×10^4 cells/0.1 mL/well. Twenty-four hours later, 0.1 mL of fresh medium with or without drug was added to each well. The cells were incubated with drug for 2 hr, following which the cells were allowed to grow in drug-free medium for a further 48 hr. Thereafter, cells were incubated for 4 hr at 37° in medium containing 0.5 mg/mL of MTT. After removal of the bulk of the medium, the formazan crystals remaining behind were dissolved in acid–isopropanol and read on a Titertek Multiskan plate reader at 560 nm [24]. The IC_{50} values were read from individual survival curves for each treatment in quadruplicate, and the results of at least three separate experiments were analyzed by Student's *t*-test.

Glutathione assay

Total cellular GSH [reduced GSH + oxidized GSH, i.e. GSSG] was determined spectrophotometrically by an enzymatic recycling method [25]. For studies of the effects of drug treatment on cellular GSH, GSH measurements were carried out using aliquots of cells treated exactly under the conditions of the colony formation assay, with BSO ± EA ± 4HC, as given in Fig. 1. Briefly, cells were collected and washed once with cold phosphate-buffered saline at 208 *g* for 10 min at 4°. Cell pellets were resuspended in 9 vol. of ice-cold double-distilled water at a concentration of *ca.* 2×10^6 cells/mL with vigorous shaking, to which 1 vol. of 30% 5-sulfosalicylic acid was added to give a final concentration of 3%. The mixture was then incubated on ice for 10 min, followed by centrifugation (1876 *g* for 10 min at 4°). The supernatant was then assayed for GSH content [23].

Enzyme determination

GST activity in the cytosol was determined according to Habig *et al.* [26] using 1 mM CDNB as substrate and 1 mM GSH as co-substrate in a 1-mL reaction containing 100–400 μg protein in 0.1 M phosphate buffer (pH 6.5) at 30°. Cells were collected as described above. Following centrifugation, cell pellets were resuspended in 0.1 M phosphate buffer, pH 6.5, and then were sonicated with a Fisher Sonic Dismembrator (model 300) for 2 × 10 sec on ice at an instrument setting of 35%. The sonicate was then centrifuged at 11,750 *g*, 4° for 10 min in an Eppendorf microcentrifuge, and the supernatant was assayed for GST activity. The inherent inhibitory effect of EA on the catalytic activity of MCF-7 and MCF/HC cytosolic GST activity was examined in a separate experiment by addition of EA directly to cell extracts [23]. For ALDH assays, cells in 0.1 M K-PO_4 buffer (pH 7.4) were sonicated (3 × 10 sec) and centrifuged at 105,000 *g*, for 60 min at 4°. The supernatant fraction was then assayed for ALDH activity [27]. Reaction mixtures (1 mL, pH 8.2) contained 32 mM tetrasodium pyrophosphate, 1 mM EDTA, 5 mM GSH, 4 mM NADP, and 4 mM benzaldehyde substrate. Reactions were initiated by adding the enzyme fraction, and the reaction was monitored at 340 nm for 2.5 min at 37°. Cytosolic GSH peroxidase towards both hydrogen peroxide and cumene hydroperoxide was measured as previously described [28]. Microsomal protein was obtained by ultracentrifugation and assayed at 550 nm for cytochrome P450 reductase activity [29]. Protein content was measured by the method of Bradford.

Northern blot

Total RNA was isolated from tumor cells (2–6 × 10^7 cells) using a standard guanidinium-thiocyanate-phenol-chloroform extraction method, with modifications [30]. RNA was fractioned by electrophoresis under denaturing conditions in 1% agarose gels containing 40 mM 3-(*N*-morpholino)propanesulfonic acid, 10 mM sodium acetate (pH 7.0), 0.66 M formaldehyde, and 1 mM EDTA (10 μg RNA/lane). The consistency of RNA loading was confirmed by ethidium bromide staining. The RNA was then transferred to nylon membranes (GeneScreen, Du Pont-New England Nuclear) by capillary transfer in 10× standard saline citrate and then fixed to the filters by UV cross-linking. Gene-specific probes for individual GST mRNAs [31] were synthesized, deblocked, purified and 5′-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase as detailed elsewhere [30]. The oligonucleotide sequences corresponding to microsomal GST (GSTms probe ON-37), GSTπ (Yp probe ON-41) and GSTμ (Ybs probe ON-40), as well as the conditions used for prehybridization and hybridization of each oligonucleotide, have been described in detail elsewhere [31]. After high stringency washings, the blots were air dried and exposed to Kodak XAR-5 film with two intensifying screens at −80° for 5–6 days.

RESULTS

We first characterized the biochemical changes that occurred in the 4HC-resistant cell line MCF/HC relative to the parental MCF-7 cell line (Table 1). The most significant changes included a 7.2-fold increase in ALDH activity, a 2.7-fold increase in GST activity and a 1.4-fold increase in cellular GSH content in the resistant cells ($P < 0.05$, compared with MCF-7 cells). The increase in GSH levels in the resistant cells was even more marked (2.3-fold) when expressed on the basis of cell number (see Table 4, below). No changes in GSH peroxidase or P450 reductase activity were apparent in the resistant cell line. The present finding in 4HC-resistant cells of increases in three metabolic parameters that potentially could contribute to oxazaphosphorine metabolism (ALDH, GST, and GSH) contrasts with earlier reports in other oxazaphosphorine-resistant cells of increases in only one or two of these parameters, i.e. increases in ALDH activity [8, 9], increases in GST activity [20, 21] or elevated cellular GSH levels [16, 20, 32]. Considering that multiple selection steps were used to select for 4HC resistance in this cell line [22], it is not surprising to find multiple biochemical alterations in MCF/HC cells.

Since multiple biochemical changes in drug-resistant cells do not necessarily indicate multiple determinants for the resistance phenotype [33], we carried out functional studies to ascertain which of the three parameters elevated in MCF/HC cells is causally related to 4HC resistance. Previous studies in MCF-7 cells treated with the GSH synthesis inhibitor BSO and the GST inhibitor EA have shown that cellular GSH is an important determinant for the sensitivity of these cells to the alkylating agents melphalan, 4HC and cisplatin, while basal expression of GST does not play a major role in drug cytotoxicity [23]. By contrast, in the present study of the 4HC-resistant subline MCF/HC, inhibition of GST activity by treatment with EA resulted in a significant enhancement of 4HC cytotoxicity with a DMF of 2.0 (Table 2). Furthermore, the combination of GSH synthesis inhibition with GST activity inhibition (BSO + EA treatment) increased the extent of resistance-reversal substantially ($P < 0.05$, compared with that of either BSO + 4HC or EA + 4HC), giving a DMF of 5.9. Overall, the combination of BSO + EA decreased the IC_{90} of 4HC from 133 to 22.5 μM, a concentration that is indistinguishable from the IC_{90} measured for parent MCF-7 cells treated with 4HC alone (19.9 μM) (Table 2, Fig. 1). In control experiments, BSO and EA were both shown to be relatively nontoxic under the experimental conditions used (see Fig. 1 legend). Since EA treatment did not alter significantly the cytotoxicity of 4HC or BSO + 4HC in the MCF-7 parental cells (Table 2, MCF-7 column, line 2 vs 1, and line 3 vs 4), these findings, taken in conjunction with the increased level of GST activity in the resistant cells, strongly suggest that the increased GST activity plays a major role in 4HC resistance in this line. The significant sensitization to 4HC induced by BSO pretreatment in both cell lines, on the other hand, indicates that GSH plays a role in the basal sensitivity of these cells to this alkylating agent.

ALDH is well known for its involvement in CPA metabolism and resistance [2] and is overexpressed

Table 1. Biochemical characterization of 4HC resistance in the human breast cancer cell line MCF/HC*

	MCF-7	MCF/HC	Fold-increase	Statistical comparison†
ALDH (nmol/min/mg)	1.05 ± 0.26 (3)	7.6 ± 1.7 (3)	7.2	$P < 0.05$
GST (nmol/min/mg)	2.78 ± 1.18 (4)	7.5 ± 2.0 (4)	2.7	$P < 0.05$
GSH (nmol/mg protein)	186 ± 28 (4)	267 ± 43 (4)	1.4	$P < 0.05$
GSH peroxidase (nmol/min/mg)				
H_2O_2	2.3 ± 1.3 (3)	2.3 ± 2.0 (4)	1.0	$P > 0.05$
Cumene hydroperoxide	2.3 ± 2.3 (3)	2.4 ± 2.4 (4)	1.0	$P > 0.05$
P450 reductase (nmol/min/mg)	35.5 ± 9.4 (3)	27.5 ± 8.2 (3)	0.8	$P > 0.05$
Protein ($\mu g/10^6$ cells)	150 ± 34 (8)	245 ± 88 (7)	1.6	$P < 0.05$

* Results (means ± SD) are based on at least 3–8 independent experiments (values in parentheses) carried out on different days using cells at different passage numbers.

† Differences between the two cell lines were analyzed by Student's *t*-test.

Table 2. Significance of the GSH/GST system in 4HC resistance in MCF/HC cells

	IC_{90} (μM)			
Treatment*	MCF-7†	DMF‡ (MCF-7)	MCF/HC	DMF‡ (MCF-HC)
4HC	19.9 ± 5.0	1.0	133 ± 13	1.0
4HC + EA	15.9 ± 3.8	1.3	68 ± 7§	2.0
4HC + BSO	9.0 ± 2.3§	2.2	70 ± 24§	1.9
4HC + BSO + EA	7.6 ± 0.8§‖	2.6	22.5 ± 2.5§‖¶	5.9

* Cells in the exponential phase were pretreated with or without 50 μM BSO for 24 hr and/or 50 μM EA for 15 min. Thereafter, they were treated with 4HC for 1 hr in the continued presence or absence of BSO and/or EA. Cells were then collected and seeded for colony formation assay (see Materials and Methods). Results are means (± SD) of three (MCF-7) and four (MCF/HC) independent experiments.

† Results in MCF-7 cells are from our earlier publication [23].

‡ Dose modification factor relative to 4HC treatment alone.

§ $P < 0.05$ compared with 4HC alone.

‖ $P < 0.05$ compared with the corresponding 4HC + EA treatment group.

¶ $P < 0.05$ compared with the corresponding 4HC + BSO treatment group.

by 7-fold when measured using benzaldehyde as substrate in MCF/HC cells (Table 1). Accordingly, we carried out experiments to evaluate whether this overexpression of ALDH contributes to the 4HC resistance phenotype. An MTT-based cytotoxicity assay [24] was used to evaluate a series of ALDH alternative substrates/ALDH inhibitors for their effectiveness at reversing the resistance of MCF/HC cells to 4HC. Five ALDH inhibitors were tested (2-hr incubation with 4HC): octanal, 250 and 500 μM; benzaldehyde, 0.4 and 4 mM; cyanamide, 100 and 500 μM; disulfiram, 10 and 50 μM; and 4-pyridine carboxaldehyde, 100 and 500 μM [9]. Of these, octanal was the most active with respect to resistance reversal (IC_{50} values; Table 3 and data not shown). The effectiveness of octanal was confirmed using a colony formation assay with a 1-hr drug exposure protocol (Table 3). Octanal sensitized both cell lines to 4HC; however, this sensitization was only significant in the case of the 4HC-resistant cells (DMF for octanal in MCF/HC cells = 1.6 to 1.9; Table 3). Similar results were obtained when another activated CPA analog, mafosfamide [2], was used for this modulation study. MCF/HC cells exhibited 5.2-fold resistance to mafosfamide (IC_{50}: 210 μM vs 1100 μM in MCF/HC cells), and octanal increased the cytotoxicity of mafosfamide by 1.8- and 3.0-fold (DMF) in the sensitive and resistant cells, respectively, as determined using the MTT cytotoxicity assay (1-hr drug exposure) (data not shown). These studies, using different activated CPA analogs and different cytotoxicity assays, demonstrated that the preferential sensitizing effect of octanal to MCF/HC as compared with MCF-7 cells is not limited to

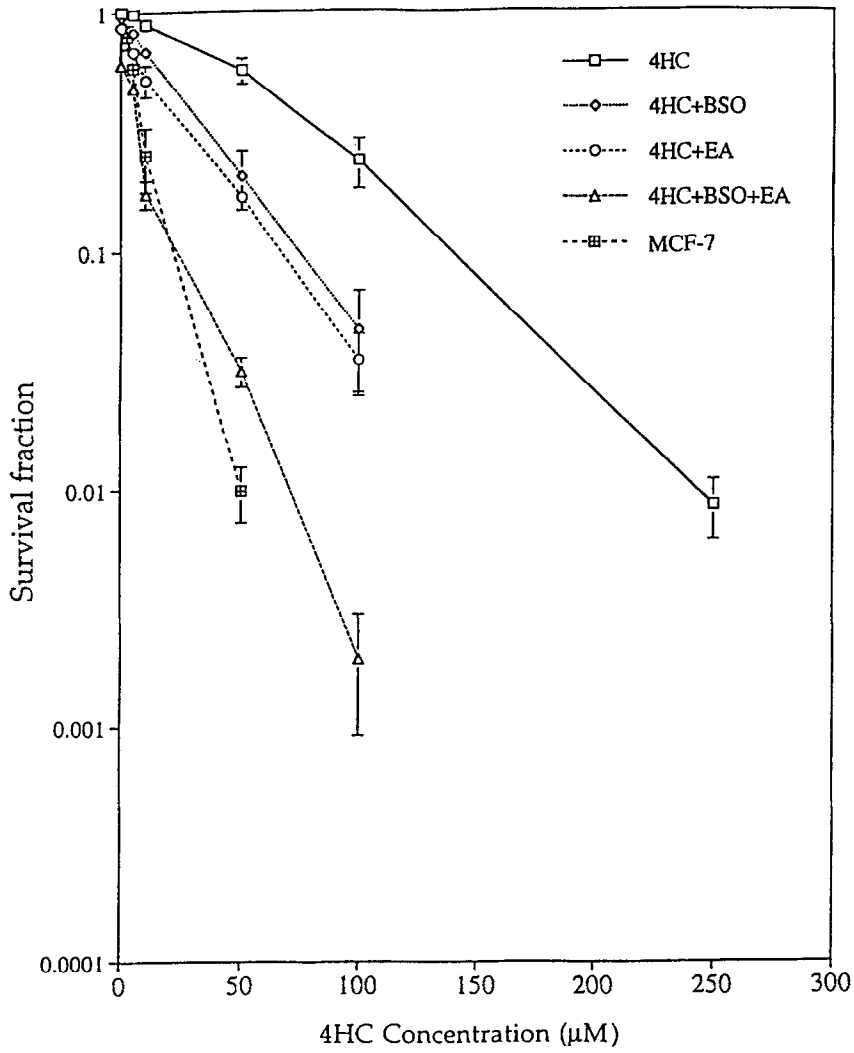

Fig. 1. Reversal of 4HC resistance by EA and/or BSO in MCF/HC cells. Exponentially growing cells were pretreated with 50 μM BSO for 24 hr and/or with 50 μM EA for 15 min, followed by treatment with 4HC for 1 hr in the continued presence or absence of BSO and/or EA, as indicated. The cells were then trypsinized and seeded for colony formation at three densities per treatment in duplicate. Results are the means of three (MCF-7) and four (MCF/HC) separate experiments (± SEM). Data are expressed relative to the surviving fraction determined in the absence of 4HC. In control studies, BSO and EA alone, in the absence of 4HC, had little or no cytotoxic effect (surviving fraction = 1.00 ± 0.19 and 0.95 ± 0.06 for BSO, and 0.98 ± 0.03 and 0.86 ± 0.07 for EA, in MCF-7 and MCF/HC cells, respectively), whereas the BSO + EA combination was moderately cytotoxic (surviving fraction = 0.81 ± 0.06 and 0.61 ± 0.06 in MCF-7 and MCF/HC cells, respectively). After subtraction of the toxicity due to BSO + EA in MCF/HC cells by taking the surviving fraction of BSO + EA as 100% for the triple combination, the normalized IC_{90} (33.3 ± 3.6) in BSO + EA + 4HC was still statistically lower than that of either EA + 4HC ($P < 0.001$) or BSO + 4HC ($P < 0.01$).

4-HC, indicating that the elevated ALDH activity present in these cells makes a partial contribution to the 4HC resistance phenotype of this line.

4HC can deplete cellular GSH by a mechanism that involved conjugation of GSH with 4HC and/or its metabolites. The cytotoxicity of 4HC can be enhanced in tumor cells that have reduced GSH levels due to the resultant increase in net formation of cytotoxic 4HC metabolites [19, 34–37]. It was, therefore, of interest to analyze the impact of BSO and EA treatment on the cellular levels of GSH following 4HC treatment of MCF-7 as compared with MCF/HC cells. Although cellular GSH was 1.4-fold higher per protein (Table 1) and 2.3-fold higher per cell in the resistant line (Table 4), BSO treatment resulted in a similar percentage of GSH depletion in both cell lines (72–75% decrease) (Table 4). EA treatment resulted in 1.4- and 1.13-fold

Table 3. Enhancement of 4HC cytotoxicity by octanal in MCF-7 and MCF/HC cells

	IC_{50}* (μM)			IC_{90}† (μM)		
	4HC	4HC + Octanal	DMF	4HC	4HC + Octanal	DMF
MCF-7	59 ± 8	42 ± 16	1.4	22.4 ± 0.6	16.0 ± 0	1.4
MCF/HC	157 ± 17	82 ± 21	1.9	100 ± 11	63.0 ± 5.6	1.6

* Cells in 96-well plates were treated with 4HC in the presence or absence of 500 μM octanal for 2 hr. After removal of drug-containing medium, cell proliferation was determined using an MTT-based cytotoxicity assay (48-hr incubation) as detailed in Materials and Methods. Results are mean values (± SD) based on 4 separate experiments, each in quadruplicate. The difference in IC_{50} values between the 4HC and 4HC + octanal treatment groups was statistically significant in the MCF/HC cells ($P < 0.05$, Student's t-test), but not in the MCF-7 cells ($P > 0.05$). Octanal alone was nontoxic with surviving fractions of 0.93 and 1.02 for MCF-7 and MCF/HC cells, respectively. For determination of IC_{50} with the MTT assay, the results have been normalized by taking the absorption of octanal alone as 100% (octanal control) for the combination.

† Cells in log-phase were treated with 4HC + 250 μM octanal for 1 hr, followed by trypsinization and seeding for the colony formation assay using the same methods as in Table 2. Values are means (± SD) of two separate experiments with each group consisting of 6 wells (3 densities in duplicate). The surviving fractions for octanal alone were 1.03 and 1.14 for the sensitive and resistant cells, respectively.

Table 4. Effects of BSO with or without EA treatment on cellular GSH in MCF-7 and MCF/HC cells

	MCF-7		MCF/HC	
	GSH (nmol/10^6 cells)	% of Control	GSH (nmol/10^6 cells)	% of Control
Control	27.9 ± 1.5	100	65.3 ± 5.3	100
BSO	7.0 ± 0.5	25	18.3 ± 1.3	28
EA	39.0 ± 7.8	140	73.6 ± 6.6	113
BSO + EA	3.0 ± 1.1	11	10.4 ± 2.5	16
4HC	10.6 ± 3.0	38	44.8 ± 5.1	69
4HC + BSO	0.25 ± 0.14	1	7.1 ± 3.0	11
4HC + EA	21.7 ± 7.1	78	49.6 ± 5.5	76
4HC + BSO + EA	0.47 ± 0.08	2	2.2 ± 0.3	3

Cells in log-phase were pretreated with or without 50 μM BSO for 24 hr and/or 50 μM EA for 15 min, followed by coincubation with 100 μM 4HC for 1 hr. Cells were then collected and prepared for GSH assays as described in Materials and Methods. Results are means (± SEM) of three (MCF-7) or four (MCF/HC) independent determinations.

increases in cellular GSH in MCF-7 and MCF/HC cells, respectively, a phenomenon that could correspond to a cellular-adaptive response to EA-dependent GSH consumption. Thus, the resistance-reversal effect of EA observed in Table 2 is not due to EA-stimulated GSH depletion, as was observed previously in chlorambucil-resistant cells [38]. By contrast, EA stimulated a further depletion of GSH in BSO-pretreated cells (57.7 and 43.4% further GSH depletion in MCF-7 and MCF/HC cells, respectively, relative to that of BSO alone) (Table 4, line 4 vs line 2). Thus, the GSH consumption effect of EA becomes apparent in these cells only under conditions of GSH synthesis inhibition. The combination of BSO with EA may, therefore, correspond to a useful strategy to achieve more complete GSH depletion through the simultaneous inhibition of GSH synthesis and stimulation of GSH consumption [39]. Treatment with 100 μM 4HC resulted in a similar decrease in absolute GSH levels in the sensitive and the resistant cells (GSH reduction:17.3 and 20.5 nmol/10^6 cells in MCF-7 and MCF/HC lines, respectively) (Table 4, line 5). Since the extent of GSH depletion by 4HC is directly related to 4HC concentration [23], these findings suggest that there may not be significant differences in the net intracellular accumulation of 4HC and its metabolites in the resistant as compared with the parental cells. The further enhancement by BSO and the partial reversal by EA of 4HC-mediated GSH (Table 4) depletion indicate that these three agents have independent effects on cellular GSH. The similar enhancement of 4HC cytotoxicity, as well as the similar percentage of GSH depletion by BSO in the two cell lines, suggests that the chemosensitizing effect of BSO may be related to

Table 5. Inhibitory effect of EA on GST activity in extracts from MCF-7 and MCF/HC cells*

EA (μM)	MCF-7 cells GST (nmol/min/mg)	MCF-7 cells % of Control	MCF/HC cells GST (nmol/min/mg)	MCF/HC cells % of Control
0	2.59 ± 0.25	100	8.73 ± 1.32	100
5	1.86 ± 0.37	72	5.51 ± 0.77	63
10	1.49 ± 0.27	58	4.59 ± 0.85	53
50	0.19 ± 0.10	7	2.29 ± 0.55	26
100	ND	ND	1.01 ± 0.52	12

* EA was added to cell extracts to give the final concentrations indicated, and GST activity was determined using chlorodinitrobenzene as substrate. Results are based on experiments using cell extracts from three separate experiments (means ± SEM). Final protein concentrations of the extracts were 285 ± 44 and 197 ± 78 μg/mL of the GST assay mixture for MCF-7 and MCF/HC cells, respectively. ND, not detectable.

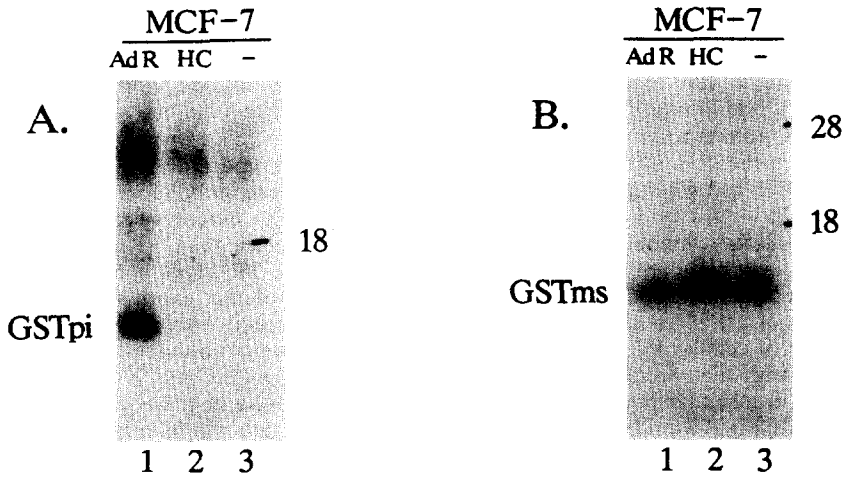

Fig. 2. Detection of GST mRNA expression by northern blot analysis. Ten micrograms of total RNA from MCF/AdR (lane 1), MCF/HC (lane 2) and MCF-7 cells (lane 3) were electrophoresed in 1% agarose gels, transferred to nylon membranes, and hybridized with gene-specific oligonucleotide probes for GSTπ mRNA (panel A) and GSTms (panel B), as shown. Markers for the 18S and 28S ribosomal RNAs are shown at the right.

the *percent* GSH depletion rather than the *actual* GSH levels of the cells.

EA is a well established GST substrate [26] that can, in part, reverse resistance of tumor cells to several anticancer drugs, including chlorambucil [38, 40], melphalan [41] and mitomycin C [42]. To investigate whether the resistance-reversing effect of EA seen in the present study is mediated by its inhibition of GST, extracts from both cell lines were assayed for EA inhibition of GST activity. As shown in Table 5, EA inhibited GST activity in a concentration-dependent manner, and at concentrations of 5 and 10 μM, the percent inhibition was similar in extracts from MCF-7 and MCF/HC cells. However, at higher EA concentrations, MCF/HC GST activity was partially resistant to EA, such that ~12% residual activity was present at 100 μM EA (Table 5). This could reflect the increased expression of some EA-insensitive GST isozymes, such as GSTπ [43] or GSTms [44] in the resistant cell line.

GSTπ can inactivate the 4HC metabolite acrolein more efficiently that GSTμ or GSTα [45]. GSTπ overexpression has been demonstrated to be associated with about a 10-fold 4HC resistance phenotype in a human melanoma line (G3361/4HC) [21]. GSTms protein, a trimeric protein with a subunit molecular weight of 14 kDa, which is structurally and biochemically distinct from the cytosolic GSTs (dimers of ~22–25 kDa) but shares certain functional properties [44], is also reported to catalyze conjugation of GSH with CPA and its active metabolites [46, 47]. To clarify whether these GST isozymes contribute to the increased GST activity of the resistant cells, GST mRNAs were examined by northern blot analysis using gene-specific GST oligonucleotide probes [31]. As shown in Fig. 2, GSTms mRNA was present at a similar level in parental and in 4HC-resistant MCF-7 cells (Fig. 2), whereas GSTπ mRNA was detected only in a positive control [MCF/AdR cells (Fig. 2), which greatly overexpress this GST enzyme [48]]. Attempts to detect GSTα or GSTμ mRNAs were unsuccessful, suggesting that these GSTs may be expressed at very

low levels or not at all in these cell lines (data not shown).

DISCUSSION

Although GST activity and/or cellular GSH levels have been observed to increase in several CPA- and 4HC-resistant cell lines [20, 21, 49], the possible functional significance of GSH/GST overexpression in oxazaphosphorine resistance has not been examined previously. While successful modulation of the resistance phenotype by targeting GST and/or GSH using EA or BSO as modulators has been described in cell lines made resistant to chlorambucil [38, 40] and mitomycin C [42], our studies establish that such a resistance mechanism can also apply to activated CPA. Moreover, in contrast to studies with other alkylating agent-resistant lines, where EA treatment often sensitizes both the drug-resistant and the parental drug-sensitive cell lines [38, 41], in the present study EA exhibited a significant chemosensitizing effect only in the drug-resistant MCF/HC cells. This preferential chemosensitization by EA of MCF/HC as compared with MCF-7 cells was seen both when the cells were pretreated with BSO to deplete GSH and when the cells were not pretreated (Table 2; lines 4 vs 3 and lines 2 vs 1). This observation, together with the 2.7-fold increase in GST activity in these cells, strongly suggests that GST plays a determinant role in the resistance phenotype. By contrast, although there was a significant increase of cellular GSH in the resistant cells as compared with the parental cell line, GSH depletion resulted in a similar potentiation of 4HC cytotoxicity in both MCF-7 and MCF/4HC cells. Thus, GSH plays an important role in cellular 4HC metabolism and in the expression of 4HC cytotoxicity in *both* the sensitive and the resistant cells.

Several CPA metabolites can be metabolized and thereby inactivated by conjugation to GSH [19]. However, it is not clear whether these conjugations require enzymatic involvement. In cell-free systems, GSTms can catalyze the conjugation of GSH with CPA and its metabolites [45–47, 50]. In our present studies in intact MCF-7 parental cells, however, this conjugation may primarily occur nonenzymatically, i.e. independent of GST, as suggested by the lack of an enhancing effect of EA on 4HC cytotoxicity, either in the presence or absence of BSO (Fig. 1 and Table 2). By contrast, in the drug-resistant MCF/HC cells, GST clearly plays an important role in 4HC metabolism. The partial but significant 4HC resistance-reversing effect of EA in these cells indicates that a least a portion of GSH conjugation with 4HC and its active metabolites is catalyzed by the overexpressed GST. Moreover, the more substantial reversal of 4HC resistance by EA in combination with BSO in the resistant cells (Table 2 and Fig. 1) suggests that 4HC detoxification in MCF/HC cells requires the joint participation of GSH and GST. Therefore, the development of 4HC resistance in MCF-7 cells is accompanied by induction of the GSH/GST system and by a change of cellular 4HC metabolism from a largely GST-independent process to a process that is both GST-independent and GST-dependent. These changes would enable the resistant cells to inactivate intracellular 4HC more efficiently through GST catalyzed drug-GSH conjugation, which may be therefore causally related to the decreased sensitivity of the resistant cells to 4HC following repeated exposure to this agent. It remains to be established whether there is an increase in the rate of formation of GSH-conjugate in the resistant cells.

Overexpression of GST has been demonstrated to be functionally important in the development of tumor cell resistance to other alkylating anticancer agents such as melphalan and chlorambucil [38, 40, 41, 43]. Interestingly, the ~3-fold increase in GST activity in MCF/HC cells is associated with partial cross-resistance to nitrogen mustard and thio-TEPA, but no cross-resistance to melphalan and mitomycin C is apparent [22]. This suggests that the particular GST enzyme form(s) overexpressed in the resistant cells may be preferentially active with 4HC and does not catalyze the conjugation of GSH to melphalan and mitomycin C or their active metabolites. Northern blot analysis, however, failed to reveal any significant change in the expression of GSTπ or GSTms, two GST enzymes that may be associated with 4HC resistance [21, 46, 47, 50]. Therefore, the 2.7-fold increase in GST activity in MCF/HC cells may be due to overexpression of some other GST isoenzymes, such as a GST related to a novel 29 kDa microsomal GST [51] or perhaps a GSTα form [40]. Attempts to detect expression of GSTμ or GSTα forms by northern blot analysis of MCF-7 or MCF/HC RNA were unsuccessful, suggesting that the corresponding GST mRNAs are not expressed at significant levels in these cells. Alternatively, it is conceivable that the increase in GST activation in MCF/HC cells may result from an elevation of one or more GST proteins through protein stabilization or other mechanisms without an effect on GST mRNAs, in which case the changes in expression of individual GSTs would not be detected by these northern blot methods. Additional probes and more sensitive analytical methods will be required to identify the specific GST isozymes that are elevated in MCF/HC cells and to elucidate the underlying mechanism for this elevation.

Cytosolic ALDH represents an important oxazaphosphorine-specific drug-metabolizing enzyme system [2, 15] and is the first enzyme shown to be specifically involved in CPA resistance [8]. In the present studies, 4HC resistance was found to be associated with a 7.2-fold increase in ALDH activity. Moreover, partial resistance-reversal was conferred by the ALDH inhibitor octanal, suggesting a partial contribution of ALDH to 4HC resistance in MCF/HC cells. Further attempts to examine whether application of ALDH inhibitors in combination with GSH/GST inhibitors leads to full reversal of 4HC resistance were unsuccessful due to the toxicities associated with octanal + EA in combination. Alternative strategies, for instance the combination of GSH depletion with anti-sense RNA approaches aimed at selectively blocking specific GST and/or ALDH enzyme expression, could be useful in this regard. The *partial* contribution of the ALDH overexpression to the 4HC resistance phenotype observed in MCH/HC cells in the present study

contrasts with the more striking, dominant role of ALDH overexpression in CPA or 4HC resistance apparent from studies of several other drug-resistant cell lines. This could reflect the comparatively low level of ALDH overexpression in our cell line (7.2-fold ALDH overexpression vs 100 to 300-fold in other CPA-resistant lines: L1210/OAP, P388/CLA and MCF/OXA) [8, 15] and/or possible differences with respect to ALDH isoenzyme expression patterns in these cells. The precise profile of ALDH isoenzyme expression in MCF/HC cells remains to be identified.

Recently, a large overexpression of cytosolic ALDH activity (134-fold increase relative to MCF-7 cells) was reported to be associated with a substantial (>40-fold) resistance to mafosfamide in MCF/OAP cells [9]. In this case the overexpressed ALDH has been identified as a type 2 ALDH-3, a form distinct from the type 1 ALDH-3 found in parental MCF-7 cells, on the basis of the distinctive physical and catalytic properties of these enzymes [52]. While the MCF/HC subline used in the present study and the MCF/OAP subline used in the studies of Sladek and co-workers [9–11] were both derived from an original cell line developed by Frei and associates [22], the two resistant cell lines appear distinct. This is indicated by the 5.2-fold resistance to mafosfamide associated with a 7.2-fold increase in cytosolic ALDH activity in our MCF/HC cells, as compared with a >40-fold resistance to mafosfamide associated with a 134-fold elevation in ALDH activity in MCF/OAP cells. Conceivably, the two cell lines may correspond to different stages of resistance induction, perhaps as a result of continued resistance development that occurred over time due to longer term culturing in the presence of activated oxazaphosphorines. Interestingly, the 6.7-fold resistance to 4HC exhibited by our MCF/HC cells appears to be closer to the 9-fold resistance to 4HC originally reported by Frei *et al.* [22]. Independent of the precise relationship between the MCF/HC and MCF/OAP cells, however, it is clear from the present study that, in addition to ALDH overexpression, changes in GST expression can functionally contribute to the oxazaphosphorine resistance phenotype.

Taken together, our studies establish that overexpression of GST and ALDH both contribute to the resistance of MCF/HC cells to 4HC. Mechanistically, increased GST and ALDH activity may be coordinately up-regulated by the repeated exposure of the MCF-7 cells to 4HC during resistance induction. The drug-metabolizing enzymes GST and ALDH are both encoded by genes that belong to the polycyclic aromatic hydrocarbon-responsive gene battery [53, 54]; activation of these genes involves receptor-mediated events that are initiated by the binding of aromatic hydrocarbons to the intracellular *Ah* receptor [55]. Further evidence supporting this possibility comes from the observation that several polycyclic aromatic hydrocarbon agonists of the *Ah* receptor, including 3-methylcholanthrene, benzo[*a*]-pyrene and 9,10-dimethyl-1,2-benzanthracene, can each induce both ALDH and GST in the parent MCF-7 cells [10]. It would be of interest to explore further whether other polycyclic aromatic hydrocarbon-responsive enzymes from the *Ah* gene battery are overexpressed in MCF/HC cells, and what functional role they may play in 4HC resistance.

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