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Dependence of Aldehyde Dehydrogenase-mediated Oxazaphosphorine Resistance on Soluble Thiols

IMPORTANCE OF THIOL INTERACTIONS WITH THE SECONDARY METABOLITE ACROLEIN

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ABSTRACT. Acrolein is a highly reactive and cytotoxic by-product released during activation of oxazaphosphorine (OAP) anticancer alkylating agents. Previously, we demonstrated that transfected human aldehyde dehydrogenase (ALDH, EC 1.2.1.3) isozymes (class 1 or 3) protect V79/SD1 cells from mafosfamide (MAF) cytotoxicity, but protection from 4-hydroperoxy-cyclophosphamide (4-hpCPA) was weaker. Acrolein, an ALDH inhibitor, may be detoxified by conjugation with the nucleophilic thiol 2-mercaptoethanesulfonate (MESNA), which is released from MAF but not from 4-hpCPA. We examined the effect of acrolein or acrolein/thiol conjugates on ALDH activity *in vitro*. We found that both ALDH isozymes were inhibited by acrolein, with IC_{50} values of 35 and 144 μM for ALDH-1 or ALDH-3, respectively. Both isozymes were partially protected by NAD^+ cofactor, being at least five-fold more sensitive to acrolein if added before cofactor. In contrast, thiol conjugates of acrolein did not inhibit ALDH-3 activity, but were substrates only for ALDH-1. Further, acrolein was shown to be oxidized by ALDH-3, but not by ALDH-1. The effect of acrolein on ALDH-mediated resistance to OAP agents in intact cells was also examined. In control cells (without ALDH expression), acrolein and 4-hpCPA rapidly depleted intracellular GSH levels, whereas the effect of MAF was much less. Depletion of GSH by preincubation of V79/SD1 cells with a low concentration of acrolein (2 μM) before MAF exposure caused a two-fold reduction in ALDH-mediated resistance. Conversely, protection from 4-hpCPA cytotoxicity was enhanced by the addition of thiols (GSH, 2-mercaptoethanesulfonate, or *N*-acetylcysteine) during the drug exposure. These results suggest 1) that thiol content is an important determinant of the OAP resistance conferred by ALDH isoenzymes; and 2) a new mechanism whereby thiol modulation could increase the therapeutic index of OAP chemotherapy. BIOCHEM PHARMACOL **56**;1:31–39, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. mafosfamide; 4-hydroperoxycyclophosphamide; aldehyde dehydrogenase; glutathione; drug resistance; acrolein

The OAP‡ anticancer alkylating agents, and especially the prototypic drug CPA, have proven highly effective against hematopoietic malignancies, as well as some solid tumors such as breast cancer [1, 2]. Studies on cellular factors impinging on OAP sensitivity have employed analogs of CPA that are activated hydrolytically, thus bypassing the cytochrome P450-dependent ring hydroxylation required for the prototypic parent drug CPA [3]. This is useful experimentally because the P450 isozymes required for activation of CPA are often poorly expressed in tumor cell lines. These spontaneously activated analogs include MAF, which releases the thiol-containing moiety MESNA upon hydrolysis, and also 4-hydroperoxy-cyclophosphamide (4-hpCPA) or 4-hydroperoxyifosfamide (4-hpIF), both of which release H_2O_2 upon hydrolytic activation [3]. Resistance to MAF has been demonstrated in cells selected for resistance to CPA or 4-hpCPA, which were found to overexpress either class 1 [4–6] or class 3 [7–11] ALDH (EC 1.2.1.3). We previously generated ALDH-transfected cell lines that stably express ALDH isozymes and used these as genetic models to demonstrate a direct role for both class 1 and class 3 ALDH isoforms in conferring OAP-specific drug resistance [12,13]. Interestingly, the ALDH-mediated resistance conferred against MAF was 6- to 7-fold greater than the resistance conferred to 4-hpCPA with either isozyme.

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‡ *Abbreviations:* ALDH, aldehyde dehydrogenase; ALDO, aldophosphamide; L-BSO, L-buthionine-*S*,*R*-sulfoximine; CPA, cyclophosphamide; 4-hpCPA, 4-hydroperoxy-cyclophosphamide; 4-OH-CPA, 4-hydroxychclophosphamide; DMEM, Dulbecco's Minimal Essential Medium; MAF, mafosfamide; MESNA, 2-mercaptoethane sulfonate sodium; OAP, oxazaphosphorine; and PM, phosphoramide mustard.

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Both MAF and 4-hpCPA are hydrolytically activated OAP analogs that have been used clinically for purging autologous bone marrow prior to transplantation [14]. Both compounds undergo spontaneous hydrolysis to yield 4-OH-CPA, but they differ in the group released from the 4-carbon atom during hydrolysis. MAF activation releases MESNA, a low-molecular-weight sulfhydryl-containing agent that is used clinically to prevent OAP-induced hemorrhagic cystitis [15]. In contrast, 4-hpCPA activation releases hydrogen peroxide, which can impair cellular function by direct oxidative reactions or via partial reduction by ferrous ion to yield the highly reactive hydroxyl radical [16]. Thus, the differential protection by ALDH against cytotoxicity of MAF and 4-hpCPA could be due to the disparate effects of these two leaving groups.

It is well established that intracellular thiol levels can affect cellular sensitivity to anticancer DNA alkylating agents. Studies utilizing L-BSO have demonstrated that depletion of intracellular GSH levels sensitizes cells to various agents including 4-hpCPA [17, 18]. Conversely, compounds such as glutathione monoethyl ester [19, 20], 2-oxothiazolidine-4-carboxylate (OTZ) [21–24], or γ-glutamyl cysteine [25] have been effective at moderately increasing GSH levels and effecting a decrease in cellular sensitivity to alkylating agents.

Acrolein is a highly reactive α-β unsaturated aldehyde that is formed along with PM, the active antitumor DNA cross-linking component of OAPs, as a result of β-cleavage of ALDO. Generally considered an unwanted toxic by-product, acrolein has been shown to react rapidly with a large number of compounds by nucleophilic addition at the ethylenic carbon-3 atom. Acrolein-mediated cellular effects are numerous and include GSH depletion [26], formation of nucleotide adducts [27], generation of free radicals [28], DNA strand breakage [29], and mutagenicity in V79 cells [30]. Effects of acrolein that are particularly relevant to our studies include reactivity toward thiol groups and inhibition of ALDH-1 enzyme activity [31, 32]. Previous studies have not specifically addressed the role of GSH or other thiols in ALDH-mediated resistance to 4-hpCPA or MAF.

In the present studies, we have examined the effect of acrolein on ALDH-mediated OAP-specific resistance. The ALDH-transfected model cell lines developed for the drug resistance studies allowed comparison of the sensitivity of class 1 or class 3 ALDH to inhibition by acrolein. Further, we have examined the effects of varying thiol levels on the efficacy of the ALDH resistance mechanism. The results of these studies provide strong evidence that thiol levels are an important cellular factor that affects the degree of OAP resistance afforded by expression of ALDH isozymes. Our results suggest that expression of ALDH activity and maintenance of thiol levels may represent distinct but interactive cellular resistance mechanisms that reduce the therapeutic effectiveness of OAP anticancer agents with synergistic cooperativity.

MATERIALS AND METHODS

Materials

The aminoglycoside antibiotic hygromycin B was obtained from Calbiochem. MAF and 4-hpCPA were provided by Dr. P. Hilgard and Dr. J. Pohl of Asta Medica Inc. Acrolein, GSH, MESNA, *N*-acetylcysteine, and all other reagents were reagent grade or higher and were obtained from the Sigma Chemical Co. or Fisher Scientific.

Cell Culture

Chinese hamster lung fibroblast cells stably transfected with cytochrome P450 2B1 (V79/SD1) were provided by Dr. Johannes Doehmer [33]. Cells were grown at 37° in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum (Life Technologies). Cells were stably transfected previously with ΔpCEP4Δ/ALDH expression vectors (10 μg) by a calcium phosphate precipitation procedure [12, 13, 34] and selected for resistance to hygromycin B (0.7 mg/mL), conferred by the Hyg resistance cassette on the same plasmid. Another population of V79/SD1 cells was similarly transfected with the empty vector and selected for hygromycin resistance to serve as a transfected control cell line. This cell line, designated SD1/Hyg-1, had very low ALDH activity (equal to the parental SD1 cell population). The highest ALDH activity clones were utilized for these experiments, and included hALDH1-28 (expressing class 1 ALDH) and hALDH3-26 (expressing class 3 ALDH) [12,13].

ALDH Enzymatic Activity Analysis

Cells were lysed by brief sonication at 4° in 50 mM of Tris–HCl, pH 7.4, with 5 mM of EDTA, and lysates were centrifuged at 14,000 *g* for 5 min at 4°. Supernatants containing cytosolic protein were then utilized for determining enzyme activity. Protein was assayed in cytosols by the BCA (bicinchoninic acid) method (Pierce). Enzymatic reactions were performed with aliquots of crude cytosol in a 1-mL vol. as described previously [35]. Class 1 ALDH activity was determined with propionaldehyde (4 mM) as substrate and NAD^+ (0.1 or 1 mM) as cofactor. Class 3 ALDH was measured with benzaldehyde (1 mM) as substrate and NAD^+ (0.1 or 1.0 mM) or $NADP^+$ (1 mM) as cofactor, essentially by the method described by Manthey and Sladek [36]. A difference was that 2-mercaptoethanol was omitted from the reaction mixture since it reacts readily with acrolein [37]. One mU of activity was defined as the amount of activity that oxidized 1 nanomole of substrate per minute at 25°. For assays that included acrolein, it was generally added at the indicated concentrations to reaction mixtures after the pyridine nucleotide cofactor and immediately before the addition of aldehyde substrate and analysis. An exception was the inhibition assays shown in the right column of Table 1, where acrolein was added prior to the NAD^+ to assess whether the

TABLE 1. Inhibition of ALDH by acrolein in the presence or absence of 0.1 mM of NAD^+ cofactor

ALDH isozyme	Acrolein inhibition IC_{50} (μM)	
	NAD^+ added first	Acrolein added first
ALDH-1	34.5 ± 12.3	4.0 ± 2.2
ALDH-3	144 ± 22	28.4 ± 4.8

Crude cytosolic extracts of hALDH3-26 or hALDH1-28 cells were assayed spectrophotometrically using standard substrates propionaldehyde (ALDH-1) or benzaldehyde (ALDH-3), as described in Materials and Methods. A range of acrolein concentrations was added either before or after the addition of 0.1 mM of NAD^+, and then aldehyde substrate was added last. The contribution of acrolein oxidation by ALDH-3 to the measured overall rate of NADH production was less than 2% of the benzaldehyde oxidizing activity at the low lysate concentrations used. Results are the means ± SD of 4–8 assays.

presence of cofactor affected the degree of inhibition. The lower concentration of NAD^+ (0.1 mM) was tested in order to mimic the approximate concentration of NAD^+ in V79 cells [38]. To correct for any nonspecific binding of acrolein by protein, the total protein concentration was kept identical for hALDH1-28 and hALDH3-26 enzyme assays.

Cytotoxicity Assay

Cells were subcultured without hygromycin selection into 25-cm^2 flasks 24 hr prior to drug treatment. On the day of drug treatment, cells were trypsinized, resuspended in fresh medium and counted. Drug incubations were performed in 16 × 100 mm sterile polypropylene tubes (Sarstedt) in physiological saline drug exposure medium (0.9% NaCl, pH 7.4, + 10% fetal bovine serum). Cells (5×10^4 cells/mL) in 5 mL of drug exposure medium were incubated with increasing drug concentrations for 30 min at 37°. Cells were then chilled on ice for 5 min, and pelleted by low-speed centrifugation. Cells were resuspended in warm medium and diluted to 1×10^4 or 1×10^3 cells/mL. Aliquots (1 mL) of cells were added to 60 mm^2 plates containing 3 mL of medium (1×10^3 cells/plate for all concentrations, plus 1×10^4 cells/plate for the two highest drug concentrations). After 6–8 days, the medium was removed, and the colonies were fixed, stained, and counted. Relative survival was expressed as the number of colonies (≥50 cells) formed in the presence of the drug compared with the number of colonies formed in control wells containing no drug, as a percentage of untreated cells. For studies on thiol modulation of ALDH effects, cells were treated as described above except that during the drug incubation the indicated sulfhydryl compounds were included in the exposure medium at a concentration of 1 mM.

GSH Assay

Drug treatments prior to GSH assay were performed as described above for cytotoxicity except that the total number of cells was 3×10^6 in 5 mL of drug exposure medium. Following treatment, cells were placed on ice, pelleted by low-speed centrifugation, washed with PBS/5 mM of EDTA, and either used immediately for GSH assay or stored at −20°. Intracellular GSH content was assayed by the glutathione disulfide reductase method [39]. The assay buffer (0.1 M of KPO_4, 1 mM of EDTA, pH 7.5) included NADPH (0.4 mM), glutathione disulfide reductase (0.8 units), and 5,5′-dithiobis(2-nitrobenzoate) (0.44 mg/mL). Samples of 1×10^6 cells were lysed in 2% sulfosalicylic acid on ice for 5 min, and centrifuged at 12,000 *g* for 10 min at 4°. Aliquots of the supernatant were assayed by determining the change in absorbance at 412 nm over a 2-min reaction. A standard curve for each assay was used to calculate the nanomoles of GSH per reaction and was normalized to nanomoles GSH per 10^6 cells.

RESULTS

We have utilized transfected V79/SD1 cell lines expressing high levels of class 1 or class 3 ALDH to explore the hypothesis that the greater resistance conferred by ALDH to MAF than to 4-hpCPA is due to differences in the availability of nonprotein thiols to conjugate with acrolein, an ALDH inhibitor. The ALDH specific activity and drug sensitivity for each cell line have been characterized and for these studies were the same as previously reported [12,13].

Acrolein Inhibition of ALDH-1 and ALDH-3

Acrolein has been reported to inhibit class 1 ALDH [32]. This finding led us to examine whether acrolein could inhibit class 3 ALDH as well and to compare the relative sensitivities of the two isoforms to acrolein inhibition. For the purposes of comparison of acrolein inhibition of ALDH activity, 2-mercaptoethanol was deleted from the reaction mixture since it reacted with acrolein and blocked ALDH inhibition (data not shown). Also, total protein levels in each reaction were kept equal by the addition of cytosol from empty vector-transfected control cells (which have no detectable ALDH expression), to prevent any bias due to non-specific acrolein binding to total protein.

The ALDH-1 isozyme was four-fold more sensitive to acrolein inhibition (IC_{50} = 35 μM) than the ALDH-3 isozyme (IC_{50} = 144 μM) when tested at the approximate intracellular (0.1 mM) concentration of the NAD^+ cofactor [38] (Table 1). Sensitivity of both isozymes was reduced substantially by the prior addition of NAD^+, as reflected in the eight-fold (ALDH-1) and five-fold (ALDH-3) lower IC_{50} values for inhibition when acrolein was added before NAD^+ (Table 1). When the NAD^+ was increased to saturating concentration (1 mM), both isozymes were even less sensitive to acrolein inhibition, and ALDH-3, in particular, was highly protected even at 400 μM of acrolein (Fig. 1). Because ALDH-3 (but not ALDH-1) can also utilize $NADP^+$, the inhibition was also tested with the alternate cofactor. In contrast to the strong protection by NAD^+, addition of 1 mM of $NADP^+$ was far less protective even than 0.1 mM of NAD^+ for ALDH-3, even though

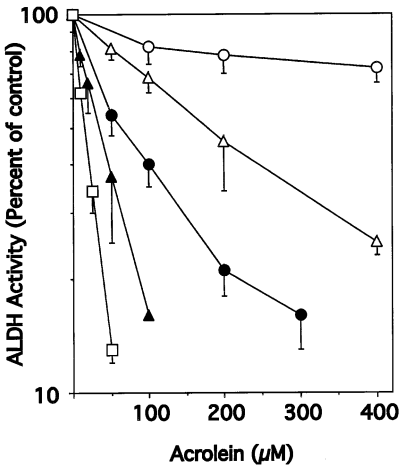

FIG. 1. Acrolein inhibition of ALDH activity in crude lysates with variable NAD^+ or $NADP^+$ additions. The indicated concentrations of acrolein were added to assay mixtures containing lysate and nucleotide cofactor, and the aldehyde substrate was added and assayed immediately thereafter. Closed symbols: inhibition of hALDH-1 in the presence of 0.1 (▲) or 1.0 (●) mM of NAD^+. Open symbols: inhibition of hALDH-3 in the presence of 0.1 (△) or 1.0 (○) mM of NAD^+, or 1.0 mM of $NADP^+$ (□). Total protein was made equal in each assay set by the addition of lysate from SD1/Hyg-1 control cells, and conditions for assay of ALDH-1 or ALDH-3 activity were identical except for substrates, as described in Materials and Methods. Typical total activities were 1.5 to 2.5 mU of ALDH-1 with propionaldehyde as substrate, or 10–20 mU of ALDH-3 with benzaldehyde as substrate. Each point on the graph represents the mean of 3–6 determinations, and error bars represent the standard deviation.

these two conditions reflect similar degrees of binding saturation (about twice their K_m concentrations). Because $NADP^+$ is generally present at lower concentrations than NAD^+ in cells, it appears unlikely that $NADP^+$ binding would have much effect on ALDH-3 sensitivity to acrolein in these cells in comparison to the NAD^+ effect.

Metabolism of Acrolein and its Thiol Conjugates by ALDH

Acrolein was shown previously to be an inhibitor, but not a substrate for ALDH-1 or ALDH-2 isozymes in the rat. However, the thioether conjugates at the β-carbon atom of acrolein of both GSH and mercaptoethanol were found to be substrates [40]. The reactivity of class 3 ALDH toward these substrates has not been examined specifically. We utilized cytosolic extracts of ALDH-1- or ALDH-3-transfected cell lines as a source of ALDH in enzyme assay mixtures containing either acrolein or acrolein-thiol conjugates (Table 2). Acrolein was found to be metabolized significantly by human ALDH-3, but not by ALDH-1 (Table 2). As previously shown for the rat class 1 isozymes, acrolein–GSH and acrolein–MESNA conjugates were substrates for human ALDH-1. Thus, in spite of the bulky thiol conjugate at the β-carbon atom, this isozyme continues to recognize the aldehyde function at the opposite end of the propionaldehyde moiety. The acrolein–GSH conjugate has been reported previously to be a class 1 ALDH substrate [41], but the MESNA conjugate has not been shown previously to be metabolized by ALDH-1. In contrast, with ALDH-3 no appreciable ALDH-3 enzyme activity was seen with either thiol conjugate (Table 2). However, the strong inhibition of ALDH-3 by acrolein was abolished completely by reaction with excess thiols (data not shown). These results indicate that thiols strongly protect class 3 ALDH but create alternative substrates for the class 1 isoform.

TABLE 2. Cellular specific activity for oxidation of acrolein or its conjugates with GSH or MESNA

	Cytosolic ALDH activity (nmol/min/mg)	
Substrate	**hALDH1-28**	**hALDH3-26**
Acrolein		
0.1 mM	<1	5.1 ± 0.4
0.5 mM	<1	18.2 ± 1.6
Acrolein-SG		
0.1 mM	2.2 ± 0.3	<1
Acrolein–MESNA		
0.1 mM	9.7 ± 0.3	<1

Crude cytosolic extracts prepared from hALDH1-28 or hALDH3-26 cells were assayed spectrophotometrically using either acrolein or its GSH (-SG) or MESNA conjugates as substrates at the indicated concentrations, as described in Materials and Methods. Substrates were added last after the addition of 1.0 mM of NAD^+ and 0.1 to 0.5 mg of cytosolic protein. Results are the means ± SD of 3–6 assays.

Depletion of Intracellular GSH by OAP Analogs or Acrolein

We compared the degree of GSH depletion following acute exposure of V79 SD1/Hyg-1 control cells to MAF or 4-hpCPA, or acrolein itself. The initial GSH level in the SD1/Hyg-1 cells was relatively low (1–2 nmol/10^6 cells), which is consistent with values previously reported in these cells [23]. Because MAF releases MESNA, which is expected to react with released acrolein and neutralize its GSH depletion effects, we suspected that depletion of GSH by MAF would be less significant than that caused by 4-hpCPA. Indeed, GSH depletion by MAF was only 40% at 100 μM, while the same concentration of 4-hpCPA completely exhausted cellular GSH (Fig. 2). Acrolein, as previously reported, was an extremely potent GSH depletor ($IC_{50} < 1$ μM), with complete ablation of cellular GSH at only 5 μM (Fig. 2). The results confirm that acrolein is a potent depletor of GSH levels in V79 cells in the low micromolar range, and that 4-hpCPA was also an effective GSH depletor in the cytotoxic range, while MAF reduced GSH by less than half. Thus, although intermediate in its ability to deplete GSH, 4-hpCPA was nonetheless clearly much more effective than MAF, presumably reflecting both the absence of the thiol MESNA released from MAF, and GSH depletion caused both by released acrolein and by the H_2O_2 released from 4-hpCPA.

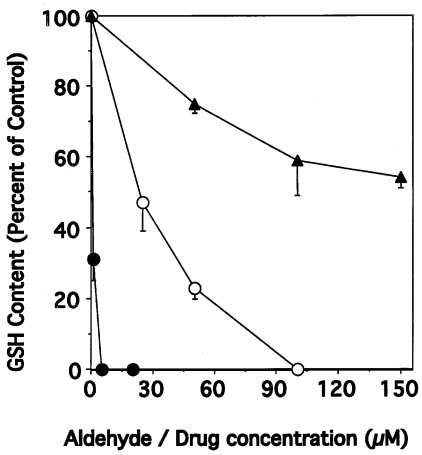

FIG. 2. Depletion of cellular thiols by treatment of SD1/Hyg-1 cells with acrolein, 4-hpCPA, or MAF. Cells (3×10^6) were treated for 30 min at 37° in physiological saline solution with acrolein (●), or the acrolein-generating OAP agents 4-hpCPA (○) or MAF (▲), as described in Materials and Methods. Following a wash and lysis in 2% sulfosalicylic acid, total GSH levels were determined by an enzymatic cycling method using a standard curve of known GSH amounts. The control level of GSH in V79/SD1 cells was 1–2 nmol/10^6 cells (data not shown). Each point represents the mean of at least 3 determinations, and error bars represent the standard deviation.

Acrolein Effects on ALDH-Mediated MAF Resistance

We reasoned that if differences in thiol availability were responsible for the greater resistance to MAF than 4-hpCPA, then thiol depletion should reduce the fold-resistance to MAF conferred by ALDH expression. Because acrolein is an irreversible inhibitor of ALDH and a potent GSH-depleting agent, and is generated by both OAP drugs, we tested whether prior exposure to a low acrolein concentration could sensitize hALDH3-26 or hALDH1-28 cells to OAP cytotoxicity. Cells were preincubated with 2 μM of acrolein for 30 min prior to a 30-min MAF exposure for cytotoxicity assay. This acrolein concentration reduced viability by 10% (IC_{10}) and lowered cellular GSH levels by more than 80% in control cells (Fig. 2). Acrolein preincubation significantly sensitized both control and transfected cells to MAF toxicity, but the effect was proportionately greater in the ALDH-expressing transfected cells, and thus the relative fold-resistance conferred by ALDH was decreased by nearly 60% for either isozyme (Table 3). This result is consistent with irreversible inhibition of ALDH activity by acrolein prior to drug exposure, and suggests that the MESNA released from MAF after the acrolein preincubation does not effectively reactivate ALDH activity.

Increase in ALDH-Mediated Resistance to 4-hpCPA by Thiol Addition during Drug Exposure

Elevation of thiol levels during drug exposure may protect ALDH from acrolein inhibition. The presence of thiols during the 4-hpCPA incubation may also minimize cell surface damage by H_2O_2 or acrolein released in the medium. Inclusion of GSH, MESNA, or *N*-acetylcysteine at 1 mM in the medium during drug exposure (Table 4) increased the IC_{90} in both the control and the ALDH-transfected lines. However, the effect was greater in the transgenic cell lines expressing ALDH, resulting in a significant enhancement of ALDH-mediated 4-hpCPA resistance. This synergistic effect was greatest in cells expressing class 3 ALDH ($P < 0.01$). Each thiol compound tested conferred this protective effect equally well, suggesting that general thiol effects such as neutralization of acrolein or H_2O_2 and consequent preservation of cellular GSH levels are responsible for synergy with ALDH.

DISCUSSION

Our previous studies showed that expression of ALDH-1 or ALDH-3 in V79 cells by stable transfection resulted in resistance to MAF that is much greater (6- to 7-fold) than to 4-hpCPA despite the fact that both are hydrolytically activated to yield the same common intermediate [12, 13]. This is consistent with the work of others who reported lower levels of resistance to 4-hpCPA in cells resistant to OAP-alkylating agents due to increased ALDH expression [8, 9, 42]. The key structural difference between these

TABLE 3. Reduction in ALDH-mediated resistance by pretreatment with the GSH depletor and ALDH inhibitor acrolein

Cell line	MAF* IC_{90} (μM)	MAF + acrolein† IC_{90} (μM)	Fractional fold-resistance
SD1/Hyg-1	31 ± 2 (1.0)	20 ± 6‡ (1.0)	
hALDH3-26	383 ± 76§ (12.4)	110 ± 9§‖ (5.5)	0.44 (44%)
hALDH1-28	640 ± 98§ (20.6)	171 ± 43§¶ (8.6)	0.42 (42%)

Cells were pretreated for 30 min with 2 μM of acrolein, a concentration that was only slightly toxic (clonogenic survival vs untreated cells of 91 ± 4%) but depletes GSH by more than 80%. The MAF fold-resistance values (relative to the SD1/Hyg-1 control) in the clonogenic survival assay are shown in parentheses. The IC_{90} values are the means ± SD of 3 experiments. The fold-resistance was reduced by slightly more than half by the acrolein pretreatment.

*MAF: mafosfamide (30-min drug exposure).

†Acrolein was preincubated with cells for 30 min prior to drug addition.

‡Significantly different from control SD1/Hyg-1 without acrolein ($P < 0.05$).

§Significantly different from SD1/Hyg-1 control ($P < 0.002$).

‖Significantly different relative to their respective untreated (without acrolein) control values ($P < 0.005$).

TABLE 4. Enhancement of ALDH-dependent resistance to 4-hpCPA by addition of thiols to the drug exposure medium

Thiol added	SD1/Hyg-1 IC_{90} (μM)	hALDH1-28 IC_{90} (μM)	hALDH3-26 IC_{90} (μM)
None	25 ± 3 (1.0)	54 ± 6 (2.2)	67 ± 5 (2.7)
GSH	59 ± 10 (1.0)	207 ± 46 (3.5)	276 ± 32 (4.7)
MESNA	54 ± 1 (1.0)	196 ± 24 (3.6)	256 ± 12 (4.7)
N-Acetylcysteine	53 ± 9 (1.0)	163 ± 5 (3.1)	270 ± 23 (5.1)

Thiol (1 mM) was present in the drug exposure medium only during the 30-min 4-hpCPA incubation. The IC_{90} values from the clonogenic survival assay are given as the means ± SD of at least 3 determinations. Fold-resistance values relative to the SD1/Hyg-1 control are shown in parentheses. Increases in IC_{90} relative to SD1/Hyg-1 or to control without thiols were all significant ($P < 0.01$).

analogs is that hydrolytic activation of MAF results in the release of MESNA, whereas 4-hpCPA is hydrolytically activated with concomitant release of H_2O_2 instead. Thus, while MAF activation is accompanied by thiol enhancement, 4-hpCPA activation generates a thiol-depleting moiety. This is consistent with our results showing that exposure of V79 cells to MAF only weakly depleted GSH in comparison with much stronger depletion by 4-hpCPA and especially acrolein.

Sulfhydryl agents, and in particular GSH, are known to protect cells from the cytotoxicity of various anticancer agents [43, 44]. Resistance to OAP cytotoxicity has been ascribed to increased cellular GSH in many instances, both *in vitro* and *in vivo* [45, 46]. Elevated GSH has been found in numerous cell lines selected for resistance to alkylating agents or drugs, such as doxorubicin, that undergo redox-cycling to produce free radicals [47]. A close correlation was shown between sensitivity to 4-hpCPA and GSH content or fractional depletion by drug treatment in a wide range of cell lines with varying GSH levels [48]. GSH has been shown to be conjugated with 4-OH-CPA to form a 4-thio metabolite, a reaction that may be enhanced by glutathione *S*-transferases [49]. GSH also reacts with cyclic aziridinium ions formed from PM [50]. Alternatively, GSH may conjugate with the ring-opened ALDO metabolite at the 4-carbon, a reaction that has been proposed to favor rapid recyclization of ALDO to the same 4-glutathionyl-CPA thioether derivative [51].

Another important consideration concerns the potent thiol depletor acrolein, which is released in (1:1) stoichiometric amounts with the DNA cross-linking agent PM from both OAP agents when their common activated intermediate, ALDO, is cleaved by β-elimination. Acrolein is rapidly and spontaneously conjugated at the β-carbon with free sulfhydryl groups, including those in GSH or MESNA [37]. Because acrolein reacts so rapidly with GSH, most cellular acrolein may ultimately be converted to GSH conjugates, and in the case of MAF-treated cells also to a MESNA conjugate, so long as these thiols are available in excess. When cellular thiols are depleted, acrolein is more likely to conjugate with protein thiols that may be essential for function or enzymatic activity, such as the essential cysteine 302 residue in the active site of ALDH-1 [52]. Thiol depletion by acrolein may be attenuated by release of the thiol MESNA during MAF activation, a conjecture supported by an earlier study which demonstrated that treatment of cells with BSO did not enhance the sensitivity of a human tumor cell line to MAF [53]. In the current study, our results showed directly that MAF was a much less effective thiol depletor than 4-hpCPA in V79 control cells, while acrolein was far more potent than either parent compound.

A further issue to be considered with regard to acrolein metabolism concerns the toxicity and metabolic fate of the aldehyde group, which can also react with thiols as well as other nucleophilic groups even after nucleophilic additions at the β-carbon atom. We showed that acrolein is a potent inhibitor, but not a substrate of human class 1 ALDH, as has been found previously with the rat isozyme [40]. In contrast, acrolein oxidation was catalyzed by human ALDH-3, an observation that to our knowledge has not been reported previously. The nature and kinetics of this catalysis are currently under investigation. Because the presumptive product acrylate is toxic and may also deplete cellular thiols, the relative importance of this aspect of OAP metabolism on ALDH-mediated drug resistance remains to be determined.

Turning to the related issue of oxidation of the aldehyde group in the thiol conjugates, we found that ALDH-1, but not ALDH-3, could oxidize the aldehyde functional group of both the GSH and MESNA conjugates of acrolein. Thus, ALDH-3 can only oxidize acrolein directly, while ALDH-1 can only oxidize its thiol conjugates. The inability of ALDH-1 to oxidize acrolein, and its greater sensitivity to inactivation by acrolein, may be due to susceptibility of ALDH-1 to alkylation and inactivation of critical cysteine residues such as the active site Cys-302 by acrolein. This was suggested in a previous report in which the rat ALDH-3 isozyme was much less sensitive than ALDH-2 to alkylation of cysteine residues and consequent inhibition by another α,β-unsaturated aldehyde, 4-hydroxynonenal [54]. The inability of ALDH-3 to oxidize thiol adducts of acrolein may be due to steric hindrance by the thiol adduct or to the altered bond angle or decreased reactivity of the aldehyde group of the adduct.

Presently it is uncertain whether oxidation of ALDO by ALDH is limited primarily by enzyme turnover rate or by availability of substrate or other cellular cofactors *in vivo*,

since rates of ALDO oxidation by purified ALDH may not reflect the rates in intact cells or crude cytosol [8, 13]. Our previous results with transfected cell lines expressing a range of ALDH activities indicated that resistance increased linearly with ALDH-1 expression, whereas it reached a plateau with ALDH-3 expression. This demonstrated that the resistance conferred by ALDH-3 was limited by factors other than enzyme expression level [12,13]. These factors might include substrate supply, or availability of cofactors such as $NADP^+$ or GSH, for example. Alternatively, the limitation in ALDH-3 mediated resistance could be related to inhibition by the substrate/inhibitor acrolein and possibly also feedback inhibition by the ALDO oxidation product carboxyphosphamide at the high MAF levels required to kill the most resistant cells with the highest ALDH-3 expression. Our present results indicate that acrolein is a competing substrate for ALDH-3, in addition to causing irreversible inhibition, particularly in the absence of NAD^+. On the other hand, while acrolein was not a substrate for ALDH-1, its activity with 100 μM of acrolein–GSH conjugate was of a similar magnitude, and ALDH-1 was even more sensitive to irreversible inhibition by acrolein.

The ability of GSH to scavenge acrolein and prevent inhibition of ALDH-mediated oxazaphosphorine resistance is a potentially important role for thiol modulation of OAP sensitivity. Because V79 cells have intrinsically low GSH content (~2 nmol/10^6 cells, data not shown), these cells may be more acutely sensitive to acrolein-mediated effects than cell lines with higher intrinsic GSH such as A549 cells [24]. The effect of acrolein on ALDH-mediated resistance was tested by preincubation with a low concentration of acrolein that was highly effective at GSH depletion, but with minimal direct toxicity. Subsequent drug challenge after washing to remove acrolein revealed that all cells were sensitized to MAF, but the effect was greater in the ALDH-expressing cells than in controls, with the result that the ALDH-mediated fold-resistance was reduced by more than half. This could be explained by irreversible inhibition of ALDH by the acrolein pretreatment, or, in the case of ALDH-1, by reversible competitive inhibition by the acrolein–GSH adduct, which is also a substrate for this isozyme. Alternatively, GSH depletion during the pretreatment with acrolein could result in greater sensitivity to inhibition of ALDH by acrolein released during MAF exposure, because in the absence of GSH the acrolein would probably not be completely detoxified by the MESNA generated from MAF in equimolar amounts.

The reduction by acrolein of ALDH efficacy in conferring resistance to MAF suggested that thiol enhancement might conversely increase the ALDH-mediated resistance to 4-hpCPA, which does not release any potentially supportive thiols but rather releases H_2O_2, a thiol depletor. A related experiment was performed to determine whether enhancement of thiols during drug exposure could protect ALDH and allow higher levels of resistance to 4-hpCPA. As expected, addition of thiols increased the resistance of control cells to 4-hpCPA, but the increased resistance observed in both ALDH-expressing lines with added thiols was even greater than that in control SD1/Hyg-1 cells, resulting in a further two-fold enhancement of ALDH-mediated fold-resistance. These effects are likely due to reduction of H_2O_2 and conjugation of acrolein released in the medium, but may also be due to uptake of *N*-acetylcysteine or MESNA. Although GSH generally is not permeable in most cell types, GSH degradation products may be taken up and utilized as precursors for GSH synthesis. Thus, overall we have shown approximately a four-fold range of difference in the ALDH-dependent resistance to OAP cytotoxicity under conditions of thiol depletion versus thiol addition. This is consistent with our hypothesis that a supportive interaction can occur between cellular thiols and ALDH.

Elevated class 1 or class 3 ALDH expression has allowed analysis of the ability of GSH to protect specific ALDH isoforms from inactivation or inhibition by acrolein and examination of whether thiol enhancement would increase ALDH-mediated resistance to OAP agents. The potential synergistic effect of cellular thiols appears to be greater with class 3 than class 1 ALDH expression, as indicated by the greater enhancement of ALDH-3-mediated resistance by thiol addition. One reason for this may be that acrolein–thiol conjugates apparently act as substrates for oxidation by ALDH-1, and hence may continue to interfere with ALDH-1 as competitive inhibitors of ALDO oxidation. In contrast, conjugation of acrolein with thiols eliminates a competing substrate as well as blocking an irreversible inhibitor.

These studies suggest that thiol depletion may affect CPA efficacy by altering the ability of ALDH isozymes to detoxify ALDO, in addition to the known actions of GSH in conjugation with PM and acrolein. There is potential for therapeutic manipulation of thiol levels during OAP treatment, and both MESNA and GSH have been used clinically to protect against bladder toxicity during CPA infusion. Selective targeting of tissues with a glutathione delivery system or selective depletion of glutathione would be expected to modulate cell sensitivity to OAP agents [17]. Our results indicate that selective depletion of tumor cell GSH might be a useful approach for partial reversal of the ALDH-mediated OAP resistance in tumor cells. Conversely, selective enhancement of thiols in normal hematopoietic stem cells might augment their protection by ALDH-1 expression, which appears to be a major factor attenuating the marrow toxicity of CPA therapy. The interactions we have shown between ALDH and GSH indicate that the feasibility of enhancing the tumoricidal efficacy of CPA by thiol modulation will be greater when ALDH isozymes are expressed in tumor cells. An important corollary conclusion is that efforts to deplete thiols must be selective for tumor tissues in order to avoid compromising the role of ALDH-1 in the preservation of hematopoietic stem cells (55).

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