Protection by Transfected Rat or Human Class 3 Aldehyde Dehydrogenases against the Cytotoxic Effects of Oxazaphosphorine Alkylating Agents in Hamster V79 Cell Lines

DEMONSTRATION OF ALDOPHOSPHAMIDE METABOLISM BY THE HUMAN CYTOSOLIC CLASS 3 ISOZYME*

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Expression of class 3 aldehyde dehydrogenase (ALDH-3) has been associated with acquired or inherent resistance to oxazaphosphorine (OAP) antineoplastic alkylating agents (e.g. cyclophosphamide). We previously demonstrated that expression of transfected rat ALDH-3 can confer OAP-specific resistance in human MCF-7 cells (Bunting, K. D., Lindahl, R., and Townsend, A. J. (1994) J. Biol. Chem. 269, 23197-23203). However, the aldophosphamide intermediate inactivated by human class 1 ALDH (hALDH-1) has not proven to be a good substrate for the purified hALDH-3. We have examined the ability of transfected human or rat ALDH-3 to confer OAP resistance in V79/SD1 cells. Clones expressing elevated human (386–5938 milliunits/mg) or rat (4–597 milliunits/mg), benzaldehyde/NADP (substrate) ALDH-3 activity were 1.3- to 12-fold resistant to mafosfamide relative to control cells (<1 milliunit/mg). Resisance was correlated with hALDH-3 activity, and was reversed by pretreatment with the ALDH inhibitor diethylaminobenzaldehyde. Transfectants were cross-resistant to 4-hydroperoxycyclophosphamide and 4-hydroperoxycyclofosfamide but not to phosphoramide mustard, ifosfamide mustard, melphalan, or acrolein. DNA interstrand cross-links were reduced commensurately with the fold resistance to mafosfamide in the highest activity clone. A key finding was the detection of a metabolite, most likely carboxyphosphamide, that is formed only by cytosols from cells expressing either class 3 or class 1 ALDH.

Increased expression of either class 1 or class 3 aldehyde dehydrogenase (ALDH) isoforms has been found in tumor cells surviving cytotoxic selection for OAP resistance (1–3). High de novo expression of one or both has also been associated with intrinsic OAP resistance in tumor cell lines (4), and transient induction of the class 3 ALDH isozyme with methylcholanthrene (5) or with phenolic antioxidants (6) coincided with the development of resistance in a human breast cancer cell line. Irreversible enzymatic oxidation of aldophosphamide (ALDO), an aldehyde-containing activated intermediate of CPA, to the inactive metabolite carboxyphosphamide (7) has been shown to be catalyzed by purified yeast (8), murine (9, 10), or human (11–13) ALDH-1 in vitro. In contrast, the role of class 3 ALDH in OAP resistance is still unclear due to the lack (or low level) of ALDO oxidation by purified human class 3 ALDH (hALDH-3) in vitro (14, 15). In particular, the class 3 ALDH normally expressed in human stomach exhibited poor activity with ALDO as substrate (15, 16). However, a low activity for ALDO oxidation was reported with human class 3 ALDH purified from OAP-selected or methylcholanthrene-induced tumor cells (4, 14).

Although purified human ALDH-3 had weak catalysis with ALDO as a substrate in enzyme studies, we have previously shown that MCF-7 cells induced to express rat class 3 tumor-associated ALDH (tALDH-3) by stable transfection of a plasmid expression vector were resistant to OAP analogs (17). The resistance was OAP-specific and closely correlated with tALDH-3 activity, and it was reversible by pretreatment with the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Since the rat and human class 3 ALDH share significant sequence homology (81%) and similar substrate preferences, and because expression of a human class 3 ALDH has been strongly implicated as a causative factor in OAP-resistant cell lines, it was important to determine whether expression of the human cytosolic ALD-3 would also be sufficient to confer resistance.

We have utilized transfection to produce genetically modified clonal derivative cell lines that should differ only in their levels of expression of ALDH activity. Using this transgenic model system, we were able to assess the capacity and activity dependence of this resistance mechanism over a very broad range of ALDH expression and to compare the rat and human class 3 ALDH with the human class 1 ALDH. The results indicated that the rat and human class 3 ALDH confer comparable levels of OAP-specific resistance at similar expression levels, but the absolute resistance with the class 3 isozymes was less than that conferred in this model system by the transfected human class 1 ALDH (28). The resistance was correlated with class 3 ALDH activity, but the relationship was hyperbolic rather than the linear correlation observed with class 1 ALDH-mediated resistance.
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resistance. Importantly, the degree of protection from cytotoxicity was shown to be quantitatively similar to the reduction in levels of DNA interstrand cross-links. Protection against both cytotoxicity and DNA cross-links was abolished by preincubation with the ALDH competitive inhibitor DEAB. Finally, we were able to directly demonstrate metabolism of labeled activated cyclophosphamide by cytotoxic extract from transfected lines expressing hALDH-3 or hALDH-1, but not by extract from nonexpressing control cells.

EXPERIMENTAL PROCEDURES

Materials—Sources of chemical and pharmacologic materials are described in our companion study that reports resistance conferred by hALDH-1 (28). The chloroethyl-3-H-labeled CPA was custom-synthesized by Amersham Corp. and generously provided by Dr. James P. Kehr (University of Texas-Austin) for these studies.

Vector Construction—The cDNA for human class 3 ALDH was amplified by polymerase chain reaction from human stomach cDNA (Clontech), under the following cycle parameters: 45-s denaturation at 94 °C, 30-s annealing at 42 °C, and 150-s extension at 72 °C for 35 cycles followed by a 10-min incubation at 72 °C to allow the polymerase to fully extend to the end of the sequence (to ensure that engineered XhoI restriction sites were intact). A 43-base pair oligonucleotide forward primer (5'-CACGCTCAAGCAGGAAAGCGCCACTGATGGACAGTCGAG-3') was synthesized to contain an XhoI site in the 5'-end, and a 27-base pair oligonucleotide reverse primer (5'-GTGCCGGCATGCCTTGCTGCAG-3'), synthesized to contain an XhoI site in the 5'-end and a 150-bp HindIII fragment, was used to amplify an approximately 1.4-kilobase pair cDNA product containing the complete coding region of the class 3 ALDH. The product did not amplify an approximately 1.4-kilobase pair HindIII fragment following restriction enzyme digestion (data not shown).

The rat class 3 ALDH cDNA was excised from the pMTP-3H expression vector previously used to transfect MCF-7 cells (17) by digestion with EcoRI and BamHI, and then gel purified and ligated into an EcoRI and BamHI digested and dephosphorylated pGEM-Z (−) plasmid (Promega). The rat class 3 cDNA was then released with XhoI and BamHI, gel-purified, and directionally ligated into an XhoI and BamHI-digested and dephosphorylated ΔCEPΔ4 mammalian expression vector, a derivative of thepcEPI4 vector (Invitrogen) that was modified to prevent episomal replication and favor host integration (28).

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Culture and Transfection of V79 (SD1) Cells—Chinese hamster lung fibroblast cells (V79/SD1), generously provided by Dr. Johannes Doehner (21) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a 5% CO2 atmosphere. Cells were transfected, selected for resistance to hygromycin B (0.7 mg/ml), and maintained as described in our companion study (28). The empty vector-transfected cell line (designated SD1/Hyg-1), had very low ALDH activity (equal to parental V79/SD1 cells).

Analysis of ALDH Expression—The transfection efficiency with the ΔCEPΔ4 expression vector in V79 cells was high, resulting in a large number of resistant colonies. Forty-eight clones that survived hygromycin selection were expanded and processed for protein and activity assays as described in our companion report (28). Aliquots of crude cytosol, with benzaldehyde (1 mM) as substrate and NADP+ (1 mM) as cofactor were utilized to determine enzyme activity by a modification (17) of the assay described by Manthey and Sladek (18). One milliliter of the enzyme was defined as the amount of activity that oxidized 1 nmol of substrate/min at 25 °C.

Western blot analysis, and densitometric analysis of a parallel Coomassie-stained SDS-polyacrylamide gel electrophoresis gel were performed essentially as described in our companion study (28). Rabbit anti-rat class 3 ALDH antisera (generously provided by Dr. Ronald Lindahl, University of South Dakota) that was cross-reactive with human ALDH-3 was used as primary antibody to probe the blots at 1:3000 dilution.

Cytoxicity and Alkaline Elution Assay—The protocol for the clonogenic survival assay was modified from that of Sreerama and Sladek (3), with slight modifications as described in our companion study (28). Cells were processed for alkaline elution and analyzed for rad equivalents of DNA interstrand cross-links as described in our companion study (28).

Metabolite Analysis—A washed rat liver S9 fraction (MOLTOX, Inc.) prepared from rats treated with phenobarbital and 5,6-benzoflavone to induce cytochrome P-450 expression was used to activate chloroethyl-3-H-labeled CPA to the 4-hydroxy intermediate that tautomerizes to yield ALDO, the putative ALDH substrate. The microsomes were first pelleted from the S9 fraction at 105,000 × g for 1 h at 4 °C and then resuspended in half the original volume of buffer (10 mM KPO4, pH 7.4, 150 mM KCl, 10 μM dithiothreitol, and 0.1 mM EDTA, total volume 250 μl) by brief sonication on ice. This wash step was essential to remove cytosolic protein including endogenous ALDH activity presumed to be present in the S9 fraction, in order to prevent artificial oxidation of ALDO by the activation premix itself. A 100-μl aliquot of resuspended microsomes was incubated with 20 μCl of labeled CPA and 1.0 mM NADPH in a final volume of 2.5 ml buffer (100 mM KPO4, pH 7.4, 120 mM KCl, and 5 mM MgCl2) for 10 min at 25 °C. Aliquots (500 μl) of this activation premix were adjusted to contain 1 × ALDH assay buffer, 5 mM mercaptoethanol, 100 μl of cell cytosol (12,000 × g supernatant, approximately 5 mg/ml), and either 1 mM NAD+ (for class 1 and control extracts) or 1 mM NADP+ (for class 3 extracts) in a final volume of 1.0 ml. The incubation was continued for an additional hour at 25 °C. Protein was precipitated by addition of an equal volume of acetone, and the 12,000 × g supernatant of this precipitate was evaporated to dryness in a warmed SpeedVac (Savant, Inc.). The residues were redissolved in 10 μl of methanol, spotted onto aluminum-backed silica TLC plates (60 F254, 20 × 20 cm, E. Merck, Inc., which had been preluted twice with methanol), dried, and chromatographed in a mixture of butanol-water (20:3). The TLC plate was dried in an oven at 160 °C, and then analyzed on a PhosphorImager, regions in each lane corresponding to the carboxyphosphamide band were scraped from the dried plate, and total counts/min were determined by scintillation counting.

RESULTS

Transfection of Rat or Human Class 3 ALDH Expression Constructs—Class 3 ALDH-expressing cell lines were generated by calcium phosphate transfection of rat or human class 3 ALDH expression vector constructs into V79 cells. The rat class 3 ALDH cDNA used previously to transfet MCF-7 cells (17) was incorporated in these studies with the V79 recipient cells in an effort to obtain higher levels of expression with the modified vector, and to determine whether rat ALDH-3 could serve as a surrogate model for human drug resistance. While both rat and human class 3 ALDH were efficiently expressed in most hygromycin-resistant transfecant lines, the absolute range of activities, and absolute protein expression was higher using the human ALDH-3 vector. Three each of the rat class 3 and human class 3 ALDH transfectants were chosen to span the range of activity obtained, thus providing a wide range of expression as well as a rigorous way to directly compare protection by rat versus human class 3 ALDH at similar ALDH expression. Doubling times (approximately 12 h) in class 3 ALDH transfectants and in control lines were similar.

Transfected cell lines exhibited elevated ALDH activity relative to SD1 parental and SD1/Hyg-1 (empty vector-transfected) control cell lines (<1 mU/mg) (Table 1). Rat class 3 ALDH transfectants (designated tALDH-X) showed increased activity ranging from 4 to 597 milliunits/mg and human class 3 ALDH transfectants (designated hALDH-X) showed increased activity ranging from 386 to 5938 milliunits/mg (Table 1). Characterization of Transfected Cells—Transfected cell lines were assayed to determine relative levels of class 3 ALDH DNA, RNA, and protein by Southern, Northern, and Western blotting, respectively. Southern blot analysis revealed that the hALDH-3 transfecant lines, which had higher ALDH activities, contained more total ALDH-3 cDNA than did the
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Table I

| Cell line | ALDH activity benzaldehyde/NADPH | IC50 | Resistance |
|-----------|-------------------------------|------|------------|
|           | milliunits/mg | μM   | foldd      |
| V79/SD1   | <1               | 29 ± 9 | 0.94       |
| SD1/Hyg-1 | <1               | 31 ± 2* | 1.0        |
| tALDH-8   | 4 ± 2            | 41 ± 5† | 1.3        |
| tALDH-9   | 47 ± 14          | 66 ± 13† | 2.1        |
| hALDH-3-31 | 597 ± 86       | 137 ± 15§ | 4.4        |
| hALDH-3-35 | 386 ± 79       | 94 ± 65§ | 3.0        |
| hALDH-3-26 | 989 ± 127      | 193 ± 11§ | 6.2        |
| hALDH-3-26 | 5938 ± 894     | 383 ± 76§ | 12.4       |

* Not significant; p > 0.05 relative to SD1 parental.
† Significant; 0.01 > p > 0.005 relative to SD1/Hyg-1 control cell line.
‡ Significant; p > 0.001 relative to SD1/Hyg-1 control cell line.
§ Significant; p < 0.001 relative to SD1/Hyg-1 control cell line.

The highest activity rat and human class 3 ALDH transfect cell lines were chosen to determine sensitivity to a range of OAP and non-OAP drugs (Table I). A lower level of resistance was seen to 4-hydroperoxy CPA analogs (4-hydroperoxy CPA, 2.7-fold; 4-hydroperoxyifosamide, 5.1-fold) in hALDH-3-26 cells. No resistance was seen in either tALDH-3 or hALDH-3-26 to non-oxazaphosphorines phosphoramide mustard, ifosfamide mustard, melphalan, or acrolein. These data indicate that resistance conferred by class 3 ALDH expression is OAP-specific.

Western Blot Analysis—The Western blot analysis revealed a broad range of expression of rat or human class 1 ALDH expression (28), no direct correlation of CDNA content with ALDH activity was seen within each group. This may be due to variable incorporation into transcriptionally active or inactive sites (22).

For sensitivity to oxazaphosphorines and non-oxazaphosphorines alkylating agents, Mafosfamide cytotoxicity experiments indicated that class 3 ALDH can provide a potent protective effect, as illustrated by the rightward shift in the dose-response curves corresponding with the relative levels of ALDH activity (Fig. 2A). The IC50 values were determined for V79/SD1 parental and SD1/Hyg-1 (empty vector-transfected) cell lines and were found to be 29 and 31 μM, respectively. Rat class 3 ALDH-transfected cell lines showed increasing resistance to MAF with IC50 values of 41 μM for tALDH-8, 66 μM for tALDH-9, and 137 μM for tALDH-3 (Table I). Human class 3 ALDH-transfected cell lines showed even higher MAF IC50 values, commensurate with their higher ALDH-3 expression: 94 μM for hALDH-3-31, 193 μM for hALDH-3-35, and 383 μM for hALDH-3-26 (Table I and Fig. 2A). The highest level of MAF fold resistance (relative to the SD1/Hyg-1 transfected control cell line) was calculated to be 4.4-fold in tALDH-3 cells and 12.4-fold in hALDH-3-26 cells (Table I). In contrast to the linear correlation between expression of class 1 ALDH and resistance, the composite plot of rat and human class 3 ALDH activity versus MAF IC50 in these cells (Fig. 2B) exhibited a nonlinear relationship.

Inhibitor Studies—The ALDH inhibitor DEAB was utilized to determine whether the protective effects could be reversed. This potent inhibitor has previously been shown to be a substrate for class 3 ALDH enzymes (Km = 6 μM; data not shown); hence, for inhibition studies we utilized 25 μM DEAB. The results indicated that the MAF sensitivity of either tALDH-3 or hALDH-3-26 cells in the presence of DEAB was indistinguishable from that in SD1/Hyg-1 cells (Table III). Further, control SD1/Hyg-1 cells, which have very low ALDH activity, were not sensitized to MAF by DEAB treatment, and DEAB alone showed no cytotoxicity in control cells at concentrations as high as 500 μM (data not shown). Importantly, DEAB did not potentiate cytotoxicity to the non-OAP compound PM (Table III). These results support the conclusion that catalytic activity, and not merely protein binding, of ALDH-3 is required to confer oxazaphosphorine-specific resistance to cells which are normally very sensitive to OAP drugs.

Alkaline Elution Studies—A significant protection (13-fold) against formation of DNA interstrand cross-links was seen in hALDH-3-26 cells relative to SD1/Hyg-1 control cells (Fig. 3).
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Fig. 2. A, sensitivity to MAF cytotoxicity of V79/SD1 cells expressing transfected human ALDH-3. Cells were treated with mafosfamide for 30 min as described under "Experimental Procedures." Clonogenic survival was determined as the number of colonies in drug-treated plates divided by the number of colonies in control untreated plates. The symbols denote: (●) control V79/SD1 parental and (●), SD1/Hyg-1 (empty vector-transfected) cell lines; (●) hALDH-3-31; (△) hALDH-3-35; and (▲) hALDH-3-26. B, correlation between enzyme activity and resistance to mafosfamide in V79/SD1 cells expressing transfected rat or human class 3 ALDH. A plot of mafosfamide activity (milliunits/mg) versus mafosfamide IC<sub>90</sub> (μM) indicates that resistance correlates linearly with enzyme activity at levels above about 2000 milliunits/mg. Above this level further increases in ALDH-3 activity have a progressively decreasing effect on resistance to mafosfamide. Symbols are: (●) control cell lines; (●) rat class 3 hALDH-transfected cell lines; (●) human class 3 ALDH-transfected cell lines.

Strongly suggesting that expression of hALDH-3 lowered formation of phosphoramide mustard. Cross-link formation was restored to the level observed in control cells when hALDH-3-26 cells were pretreated with 75 μM DEAB for 10 min prior to and during a 30-min incubation with 100 μM mafosfamide (data not shown).

Metabolite Analysis—Catalysis of oxidation of ALDO to carboxyphosphamide by human ALDH-3 preparations purified from stomach tissue has to date been undetectable or extremely weak. However, the OAP-specific resistance, the reduction in DNA cross-links, and the reversibility of both by the ALDH inhibitor DEAB all point toward a function for hALDH-3 in direct catalytic inactivation of ALDO. Thus, we wished to determine whether the hALDH-3 expressed in the cell lysate of hALDH-3-26 cells could catalyze this reaction. A rat micromolar activation system was used to oxidize [chlorehyl-3H]CPA to the labeled 4-hydroxy-CPA metabolite, which in turn taumorizes to form labeled ALDO, the putative ALDH substrate. This activation system was mixed with the hALDH-3-26 lysate, and the labeled reaction products were separated by TLC. A band was formed in the class 3 ALDH-containing reaction that was also present in the class 1 ALDH-containing reaction, but not in either the reaction with control lysate or no lysate (Fig. 4, arrow). The calculated R<sub>f</sub> value for this band (0.29) corresponded well with the R<sub>f</sub> value for carboxyphosphamide (0.26) previously reported with a similar TLC analysis method (23).

Regions including the band were scraped from each lane, and the silica powder was mixed with scintillation fluid and counted. The reaction with the hALDH-3-26 cytosol (hALDH-3) yielded 7385 cpm, compared with 12372 cpm with the hALDH1–28 cytosol (hALDH-1). The SD1/Hyg-1 (control cytosol, no ALDH) and reagent blank (no cytosol) lanes yielded 2914 and 3012 cpm, respectively. Thus, the net catalysis by hALDH3 (4373 cpm) was approximately half of that by hALDH1 (9360 cpm) after subtraction of the blank lane value, under the conditions of this coupled assay. This metabolite analysis provides strong evidence that oxidation of ALDO to carboxyphosphamide is catalyzed by the human class 3 ALDH, albeit at a lower rate than with the class 1 isozyme.

**Discussion**

An important role for the class 1 aldehyde dehydrogenase isozyme in resistance to the anticancer drug cyclophosphamide has been well supported by both cellular expression and enzymology studies. Elevation of either class 1 or class 3 ALDH have been found in OAP-selected resistant cell lines (1–3). The ALDH-1-mediated resistance was accompanied by increased formation of carboxyphosphamide (1), the inactivated product of ALDO oxidation of aldoxophosphamide, and decreased DNA cross-linking was also found (24). The role of ALDH-1 in OAP resistance has also been directly supported by enzymologic studies that demonstrated favorable kinetics for ALDO oxidation by the purified enzyme (9, 10, 13, 16). The validity and high capacity of resistance conferred by hALDH-1 has been firmly established in our companion report (28) in which we demonstrated that expression of transfected class 1 ALDH indeed confers high level OAP-specific resistance.

The ability of the class 3 ALDH to confer OAP resistance has been less clear than that of the class 1 isozyme. Several studies indicated association of increased hALDH-3 expression with OAP resistance (3, 4, 6, 14), and we have previously shown that expression of rat class 3 ALDH conferred OAP-specific resistance in MCF-7 cells (17). Although most purified human class 3 ALDH isozymes have consistently exhibited low or absent
activity for ALDO oxidation (3, 4, 6, 16), evidence has been presented that class 3 ALDH purified from human tumor cells exhibited a measurable, albeit low ALDO oxidation activity (4, 5, 15). Since class 3 ALDH isozymes. The control lines used included both the original parent cell lines and empty vector-transfected and hygromycin-selected clonal lines to be sure that selection for antibiotic resistance did not affect OAP sensitivity.

The results of these studies clearly demonstrate that elevated expression of human or rat class 3 ALDH can confer relatively high-level OAP-specific resistance in V79 cells. The capacity of increased ALDH-3 activity to confer increasing levels of MAF resistance is limited, however, approaching an asymptotic limit with diminishing increments in resistance above approximately 2000 milliunits/mg activity. While the mechanism is not yet fully understood, reversal of resistance by the competitive inhibitor DEAB strongly suggests that catalysis of ALDO oxidation is the mechanism of resistance. The OAP specificity of human ALDH-3-mediated resistance and reversibility with ALDH inhibitors is consistent with results of analogous studies using high ALDH-3-expressing drug-selected (3, 15) or ALDH-3-transfected (17) cell lines, and those results also supported direct oxidation of ALDO as a likely mechanism of resistance. Indeed, the specificity of class 3 ALDH-mediated resistance only for OAP analogs that can give rise to an aldehyde-containing intermediate has provided the best evidence to date that catalytic oxidation of ALDO to carboxyphosphamide is the most likely mechanism of resistance.

The analysis by TLC of metabolites formed by hALDH-3 (26) in the samples containing added hALDH-1 or hALDH-3.

activity for ALDO oxidation (3, 4, 6, 16), evidence has been presented that class 3 ALDH purified from human tumor cells exhibited a measurable, albeit low ALDO oxidation activity (4, 5, 15). Since class 3 ALDH is expressed de novo in rat hepatoma cells (25, 26), and is elevated in some human primary breast carcinoma samples (27) and carcinoma cell lines (2), the intriguing possibility exists that ALDH-3 expression may constitute a tumor-associated intrinsic resistance mechanism in a subset of malignancies.

A limitation of the above-mentioned correlation studies is the use of drug selection to obtain OAP-resistant cell lines, an approach that can result in cells that differ from drug sensitive parental cells in other respects in addition to increased expression of class 3 ALDH. Similarly, while studies with inducers or inhibitors have shown a correlation between ALDH-3 expression and OAP-specific resistance, such agents are often pleiotropic and can affect the expression or efficacy of resistance mechanisms other than, or in addition to ALDH. With these considerations in mind, we have attempted to address the role of ALDH isozymes in OAP resistance by utilizing transfected cell lines that should differ only in the level of expression of ALDH isozymes. The control lines used included both the original parent cell lines and empty vector-transfected and hygromycin-selected clonal lines to be sure that selection for antibiotic resistance did not affect OAP sensitivity.

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The analysis by TLC of metabolites formed by hALDH-3 from activated labeled CPA has provided new and compelling evidence in support of direct catalysis of ALDO oxidation as the primary mechanism of OAP-specific resistance conferred by this isozyme. A metabolite corresponding to the expected Rf value for carboxyphosphamide (26) was detected in reaction mixtures that contained cytosols derived from either the class 1 or class 3 ALDH-expressing cells (but not with control cell cytosol). The relative amount of this metabolite produced by
cytosol containing hALDH-3 was nearly half of that produced by cytosol containing hALDH-1, which is consistent with the higher level of resistance conferred by expression of transfected hALDH-1 (21-fold versus 12-fold for hALDH-3).

Although the human cytosolic ALDH-3 appears to catalyze ALDO oxidation, it remains to be explained why the measured activity with this substrate was absent with enzyme preparations purified from human stomach (15, 16). One possible reason for the lack of activity toward ALDO with purified stomach ALDH could be a selective modification during purification that abrogates hALDH-3 activity with ALDO but leaves intact activity with the standard assay substrate benzaldehyde. This could be due to chemical modification, conformational changes, or loss of activity modulators during purification. We have found that hALDH-3 activity is much more labile than hALDH-1 activity in crude cytosol, with most of the benzaldehyde/NADP⁺ activity lost within 10 min at 37 °C (data not shown).

A second potential reason for the poor ALDO activity with purified hALDH-3 may relate to the extreme sensitivity of the enzyme to inhibition by acrolein, a secondary product of the spontaneous β-elimination of PM from ALDO. The hALDH-3 isozyme may be relatively well protected in intact cells, where acrolein would likely react rapidly and nonspecifically with many competing nucleophiles such as glutathione and other non-protein or protein sulfhydryls. However, in a purified enzyme preparation the ALDH would be the sole target protein and hence would potentially be much more vulnerable to inhibition and/or covalent modification by acrolein. Consistent with this possibility, we have found that the human class 3 ALDH was also inherently severalfold more sensitive to acrolein inhibition than the human class 1 isoform, when measured with a constant amount of cytosolic protein in the assay mixture.²

The greater sensitivity of the hALDH-3 to inhibition by acrolein may also play a role in the nonlinear relationship between the degree of resistance and the level of expression of this isozyme. Whereas resistance increased linearly with hALDH-1 expression in transfected V79/SD1 cells (28), the relationship was asymptotic in cells expressing transfected hALDH-3, and thus indicates that some factor is limiting for protection by this isozyme. The greater sensitivity to acrolein inhibition is one possible reason, since this by-product would be present in increasing amounts at the higher IC₅₀ concentrations of OAPs required to kill the higher expressing clones. Since glutathione is protective against acrolein, progressive depletion of this thiol pool at higher MAF concentrations might also result in a nonlinear resistance response by increasing the susceptibility of hALDH-3 to inhibition by acrolein. Another possible reason for the nonlinearity of resistance could relate to the sensitivity of hALDH-3 to inhibition by other OAP metabolites at high drug concentrations, including carboxyphosphamide, the product of ALDH oxidation of ALDO.

We have shown that both the hALDH-1 (28) and hALDH-3 isoforms are potentially major determinants of cellular sensitivity to cyclophosphamide during chemotherapy treatment. The class 3 isoform represents a potentially tumor-specific target for adjuvant chemotherapy treatment due to its increased expression in certain types of neoplasia. However, targeting by the use of an inhibitor would need to be selective for class 3 and not class 1 ALDH, since the relatively modest marrow toxicity of CPA appears to be at least partly due to the high levels of hALDH-1 in hematopoietic progenitor cells. An alternative gene therapy approach, that would take advantage of the OAP resistance conferred by class 1 or class 3 ALDH expression, could be to use expression vectors to engender constitutive ALDH expression in marrow stem cells. While ALDH-1 activity normally decreases with differentiation, stable expression of high hALDH-1 activity could enhance the ability of cells to survive CPA chemotherapy throughout the hematopoietic cascade, rather than only in stem cells. Alternatively, the resistance could be augmented in both stem cells and their downstream progeny by transfection of class 3 ALDH, which is normally low in hematopoietic cells. The studies described in this report and in our companion study (28) provide a rational basis for these strategies to take therapeutic advantage of the effect of ALDH expression on cellular sensitivity to oxazaphosphorine anticancer alkylating agents.

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REFERENCES

1. Hilton, J. (1984) Cancer Res. 44, 5156–5160
2. Koelling, T. M., Yeager, A. M., Hilton, J., Haynie, D. T., and Wiley, J. M. (1990) Blood 76, 1209–1213
3. Sreerama, L., and Sladek, N. E. (1993) Biochem. Pharmacol. 45, 2487–2505
4. Rekha, K. G., Sreerama, L., and Sladek, N. E. (1994) Biochem. Pharmacol. 48, 1943–1952
5. Sreerama, L., and Sladek, N. E. (1993) Adv. Exp. Med. Biol. 328, 99–113
6. Sreerama, L., Rekha, G. K., and Sladek, N. E. (1995) Biochem. Pharmacol. 49, 669–675
7. Sladek, N. E. (1988) Pharmacol. Ther. 37, 301–355
8. Hill, D. L., Laster, W. R. J., and Struck, R. F. (1972) Cancer Res. 32, 658–665
9. Russo, J. E., and Hilton, J. (1988) Cancer Res. 48, 2963–2968
10. Manthey, C. L., and Sladek, N. E. (1988) Biochem. Pharmacol. 37, 2781–2790
11. Sladek, N. E., Dockham, P. A., and Lee, M. O. (1991) Adv. Exp. Med. Biol. 284, 97–104
12. Lee, M. O., Manthey, C. L., and Sladek, N. E. (1991) Biochem. Pharmacol. 42, 1279–1285
13. Dockham, P. A., Lee, M. O., and Sladek, N. E. (1992) Biochem. Pharmacol. 43, 2453–2469
14. Sreerama, L., and Sladek, N. E. (1994) Cancer Res. 54, 2176–2185
15. Sreerama, L., and Sladek, N. E. (1994) Biochem. Pharmacol. 48, 617–620
16. Von, E. U., Meier, T. D., Agarwal, D. P., and Goedde, H. W. (1994) Cancer Lett. 76, 45–49
17. Bunting, K. D., Lindahl, R., and Townsend, A. J. (1994) J. Biol. Chem. 269, 23197–23201
18. Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870
19. Shine, J., and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1342–1346
20. Hsu, L. C., Chang, W.-C., Shibuya, A., and Yoshida, A. (1992) J. Biol. Chem. 267, 3030–3037
21. Dockham, J., Dogra, S., Friedman, T., Monier, S., Adesnik, M., Glatt, H., and Oesch, F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5769–5773
22. Townsend, A. J., Tu, C. P., and Cowan, K. H. (1992) Mol. Pharmacol. 41, 230–236
23. Hadiil, A.-H., and Idris, J. R. (1988) J. Chromatogr. 427, 121–130
24. Hilton, J. (1984) Biochem. Pharmacol. 33, 1867–1872
25. Lindahl, R., and Feinstein, R. N. (1976) Biochim. Biophys. Acta 452, 345–355
26. Lin, K. H., Leach, M. F., Winters, A. L., and Lindahl, R. (1986) In Vitro Cell. Dev. Biol. 22, 263–272
27. Sladek, N., and Sreerama, L. (1995) Proc. Am. Assoc. Cancer Res. 35, 325
28. Bunting, K. D., and Townsend, A. J. (1996) J. Biol. Chem. 271, 11884–11890