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INTRINSIC CELLULAR RESISTANCE TO OXAZAPHOSPHORINES EXHIBITED BY A HUMAN COLON CARCINOMA CELL LINE EXPRESSING RELATIVELY LARGE AMOUNTS OF A CLASS-3 ALDEHYDE DEHYDROGENASE*

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Abstract—A cultured human colon carcinoma cell line, viz. colon C, exhibiting intrinsic cellular resistance to mafosfamide mediated by relatively elevated levels of a cytosolic class-3 aldehyde dehydrogenase was identified. Colon C cells were found to be much less sensitive/more resistant (about 10-fold as judged by LC_{90} values) to mafosfamide than were two other cultured human colon carcinoma cell lines, viz. RCA and HCT 116b, and, as compared to the barely detectable aldehyde dehydrogenase activity (NADP-dependent enzyme-catalyzed oxidation of benzaldehyde to benzoic acid) in RCA and HCT 116b cells, that in colon C cells was about 200-fold greater. The three cell lines were equisensitive to phosphoramide mustard. Aldehyde dehydrogenase activity was confined to the cytosol in colon C cells (as well as in the other two cell lines) and, on the basis of its physical, immunological and catalytic characteristics, the operative enzyme was judged to be a Type-1 ALDH-3 identical to the Type-1 ALDH-3 expressed in methylcholanthrene-treated human breast adenocarcinoma MCF-7/0 cells and very nearly identical to the Type-1 ALDH-3 expressed in human normal stomach mucosa. Class-1 and class-2 aldehyde dehydrogenases were not found in these cells. The relative insensitivity to mafosfamide on the part of colon C cells was not observed when exposure to mafosfamide was in the presence of benzaldehyde or 4-(diethylamino)benzaldehyde, each a relatively good substrate for ALDH-3, whereas it was retained when exposure to mafosfamide was in the presence of acetaldehyde, a relatively poor substrate for this enzyme. Sensitivity to mafosfamide on the part of HCT 116b and RCA cells, and to phosphoramide mustard on the part of all three cell lines, was unaffected when drug exposure was in the presence of any of the three aldehydes. Together with earlier reports from our laboratory, these observations demonstrate that intrinsic, as well as stable and transient acquired, resistance to oxazaphosphorines, such as mafosfamide and cyclophosphamide, can be mediated by relatively increased levels of cytosolic class-3 aldehyde dehydrogenases.

Key words: aldehyde dehydrogenase; cyclophosphamide; aldophosphamide; mafosfamide; oxazaphosphorines; drug resistance

Cyclophosphamide and other oxazaphosphorines, e.g. ifosfamide, 4-hydroperoxycyclophosphamide and mafosfamide, are widely used in the treatment of certain malignancies. However, as is the case with virtually all anticancer drugs, a major impediment to successful therapy with these agents is that the target tumor cell population, or subpopulation thereof, often already is, or soon becomes, relatively insensitive to them.

Using cultured human breast adenocarcinoma MCF-7/0 cells as a model, we have shown that acquired cellular resistance to the oxazaphosphorines can be due to elevated levels of cytosolic class-3 aldehyde dehydrogenases, ostensibly because the relevant enzymes catalyze the detoxification of these agents [1–3]. Thus, elevated levels of a cytosolic class-3 aldehyde dehydrogenase, viz. Type-1 ALDH-3‡§, accounted for the transient acquired resistance

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‡ Abbreviations: ALDH, human aldehyde dehydrogenase; LC_{90}, drug concentration required to effect a 90% cell-kill; pI, isoelectric point; and mIU, milli-International Unit of enzyme activity [nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case of glutathione *S*-transferase activity, nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity and nmol *p*-nitrophenol formed/min in the case of esterase activity].

§ Stomach mucosa ALDH-3 was the first human cytosolic class-3 aldehyde dehydrogenase to be characterized extensively [1] and thus is viewed as the prototypical human cytosolic class-3 aldehyde dehydrogenase. As used herein and in our earlier publications [1–3], the designations Type-1 and Type-2 ALDH-3 refer to human class-3 aldehyde dehydrogenases that exhibit physical properties identical, or very nearly so, to those exhibited by the prototypical enzyme, and to a relatively gross variant of the prototypical enzyme identified in MCF-7/OAP cells, respectively.

exhibited by MCF-7/0 cells grown in the presence of (1) Ah receptor ligands, e.g. polycyclic aromatic hydrocarbons such as methylcholanthrene, or (2) agents, e.g. phenolic antioxidants such as catechol*, that activate genes via antioxidant responsive elements (ARE) present in upstream regions therein, for a few days. Further, elevated levels of a Type-2 ALDH-3 accounted for the stable acquired resistance exhibited by an MCF-7/0 subline, termed MCF-7/OAP, generated by growing MCF-7/0 cells in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide for several months.

Yet to be demonstrated, however, is that intrinsic, as opposed to chemically-induced acquired, cellular resistance to the oxazaphosphorines can also be due to relatively greater levels of a cytosolic class-3 aldehyde dehydrogenase, whether Type-1 or Type-2. Since catalytic activity not unlike that effected by class-3 aldehyde dehydrogenases reportedly varies widely in cytosolic fractions prepared from primary, and especially metastatic, human colon adenocarcinomas [4], we chose to work with cultured human colon carcinoma cell lines in attempting to demonstrate that intrinsic cellular resistance to the oxazaphosphorines can indeed be due to relatively greater levels of a cytosolic class-3 aldehyde dehydrogenase. Herein, we report success in that regard. The operative enzyme in the model investigated was identified to be of the Type-1 variety.

MATERIALS AND METHODS

Mafosfamide and 4-hydroperoxycyclophosphamide were provided by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany. Phosphoramide mustard·cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 4-(Diethylamino)benzaldehyde was purchased from the Aldrich Chemical Co., Milwaukee, WI. All other chemicals and reagents were obtained from the sources reported in previous publications [1, 3].

The sample of human normal colon tissue was procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. It was obtained from a 71-year-old Caucasian female by biopsy, immediately frozen, and subsequently stored until used at −70°. The supplier certified the tissue to be non-pathological.

Human colon carcinoma cell lines, viz. colon C†, HCT 116b and RCA, [5, 6] were provided by Dr. M. G. Brattain, Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH. They were propagated as monolayer cultures at 37° in dishes/flasks containing the growth medium described below; the atmosphere of 5% CO_2 in air was fully humidified. Mean population doubling times were approximately 25 (colon C), 22 (HCT 116b) and 30 (RCA) hr. Mean plating efficiencies were approximately 60, 65 and 50%, respectively.

Cultured tumor cells in asynchronous exponential growth were harvested and checked for viability (usually greater than 95% as judged by trypan blue exclusion; preparations exhibiting less than 85% viability were discarded) as previously described [1]. This was the preparation used in the colony-forming assays. Cells were further handled in two different ways when enzyme activity in cell-free fractions was to be quantified or when ALDH-3 was to be purified. They were reharvested by low-speed centrifugation (500 *g* for 10 min), resuspended (1×10^7 cells/mL) in homogenization medium, and then used when enzyme activity in cell-free fractions was to be quantified. They were also reharvested by low-speed centrifugation when ALDH-3 was to be purified, but were stored at −20° as a pellet overlaid with homogenization medium until used. Enzyme activity was essentially unaffected by freezing and storage at −20°.

Homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4. Drug-exposure medium was horse serum (10%) in a phosphate-buffered saline-based solution, pH 7.4, prepared as previously described [7]. Growth medium was horse serum (10%) in Dulbecco's Modified Eagle's Medium supplemented with L-glutamine (2 mM), sodium bicarbonate (3.7 g/L), gentamicin (50 mg/L), Basal Medium Eagle amino acids solution (6 mL/L), Basal Medium Eagle vitamin solution (6 mL/L), and sodium pyruvate (1 mM).

Preparation of aldophosphamide from 4-hydroperoxycyclophosphamide, Lubrol®-treated whole homogenates, subcellular (105,000 *g* soluble and solubilized 105,000 *g* pellet) fractions and anti-Type-1 ALDH-3 IgY, and purification of human stomach mucosa Type-1 ALDH-3 and MCF-7/OAP Type-2 ALDH-3, were as previously described [1].

Drug exposure and colony-forming assay. Drug exposure and the colony-forming assay used to determine surviving fractions were as previously described [1]. Essentially, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and were exposed to mafosfamide, phosphoramide mustard and/or vehicle for 30 min at pH 7.4 and 37° after which they were harvested and cultured in drug-free growth medium for 15 days. When exposed to mafosfamide and/or phosphoramide mustard in the presence of benzaldehyde, acetaldehyde and/or 4-(diethylamino)benzaldehyde, the cells were preincubated with the respective aldehydes for 5 min prior to the addition of the drug to the drug-exposure medium. 4-(Diethylamino)benzaldehyde was initially dissolved in dimethyl sulfoxide, and this solution was then diluted with drug-exposure medium. The dimethyl sulfoxide concentration during drug exposure did not exceed 0.1%; this concentration of dimethyl sulfoxide did not affect cell proliferation. Colonies (≥50 cells) were then visualized with methylene blue dye and counted. The protocol described in a previous publication [3] was used when attempts were made to induce ALDH-3 and other enzymes with methylcholanthrene.

Enzyme and other assays. Aldehyde dehydrogenase, esterase, glutathione *S*-transferase and DT-

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† This cell line is also known as HCT C[5] and C [6].

diaphorase* activities, and glutathione and protein concentrations, were determined as previously described [1, 3] except when kinetic constants defining aldehyde dehydrogenase-catalyzed oxidation of 4-(diethylamino)benzaldehyde were determined. An isocratic reverse-phase HPLC/spectrophotometric method was used in the latter case because 4-(diethylamino)benzaldehyde strongly absorbs at 340 nm, thus complicating the accurate measurement of NAD(P)H formation in the direct spectrophotometric assay [1] ordinarily used to quantify aldehyde dehydrogenase-catalyzed oxidation of aldehydes. The reaction mixture was as before [1] except that it also contained up to 0.1% dimethyl sulfoxide. This was because 4-(diethylamino)benzaldehyde was initially dissolved in dimethyl sulfoxide. Catalytic activity of the enzyme was unaffected by this amount of dimethyl sulfoxide. Incubation was at 37° for 5 min. The reaction was stopped by placing the reaction mixture in an ice-water bath. Twenty microliters of the reaction mixture were injected directly into a 5 μm Ultrasphere ODS (4.6 mm × 25 cm) column (Beckman Instruments Inc., San Ramon, CA) fitted with a 5 μm Adsorbosphere C_{18} guard column (Alltech Associates, Inc., Deerfield, IL). The HPLC system (Beckman Instruments, Inc., Fullerton, CA) consisted of a model 110A pump, a model 210 sample injection valve with a 20-μL sample loop, and a model 160 fixed-wavelength detector equipped with an 8-μL flow cell. The mobile phase was an aqueous (HPLC grade water; EM Science, Gibbstown, NJ) solution of 0.1 M potassium phosphate, pH 6.0/4.5% methanol that was prepared fresh daily and that had been vacuum-filtered (0.45 μm; Millipore, Bedford, MA) and degassed. Elution was at room temperature. The flow rate was 2.0 mL/min. An HP 3390A electronic integrator (Hewlett-Packard, Avondale, PA) was used to monitor the column eluate at 340 nm with the detector attenuation set at 0.08 a.u.f.s. NADH and NADPH retention times were 8.4 and 4.0 min, respectively. NADPH was used as the internal standard when quantification was of NADH, and vice versa. NADH and NADPH concentrations were estimated with the aid of calibration curves generated by using known concentrations of the authentic compounds. The lower limit of detection of NADH or NADPH was 20 pmol. All experiments were done in duplicate.

Analytical non-denaturing PAGE, isoelectric focusing, immunoblot analysis and molecular weight determinations were also as previously described [1] except that commercially-available pre-cast polyacrylamide gradient (4–20%) gels, viz. mini-PROTEAN II ready gels, were used when the native molecular weight was determined by linear gradient gel electrophoresis.

Chromatographic purification of cytosolic class-3 aldehyde dehydrogenase. Cytosolic class-3 aldehyde dehydrogenase present in colon C cells was purified to apparent homogeneity by subjecting the soluble fraction (105,000 *g* supernatant) of these cells to successive DEAE-Sephacel, CM-Sepharose CL 6B and Reactive Blue 2-Sepharose CL 6B column chromatography at 4–6°, as previously described [1]. Purified enzyme was placed in 25 mM sodium phosphate buffer (pH 7.5) supplemented with 1 mM EDTA and 0.05% dithiothreitol, and aliquots of this preparation were stored at −20°.

Data analysis. Double-reciprocal plots of initial rates, each determined in duplicate, versus 5–10 substrate concentrations were used to estimate each of the K_m and V_{max} values. Computer-assisted weighted linear regression analysis [8] was used to fit lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight-line functions.

RESULTS

Aldehyde dehydrogenase activity in Lubrol®-treated whole homogenates obtained from three different human colon carcinoma cell lines, viz. colon C, HCT 116b and RCA, was quantified in initial experiments. As compared with the low levels found in HCT 116b and RCA cells, enzyme activity, specifically NADP-dependent enzyme-catalyzed oxidation of benzaldehyde, was about 200-fold higher in colon C cells (Table 1). As judged by isoelectric focusing and inhibitor studies, and the inability of the Lubrol®-treated whole homogenates to catalyze the oxidation of succinic semialdehyde, ALDH-1, ALDH-2 and succinic semialdehyde dehydrogenase, each a known catalyst of aldophosphamide oxidation [9], were not present in any of the three cell lines (data not presented). Subcellular distribution studies on colon C cells revealed that NADP- and NAD-dependent enzyme-catalyzed oxidation of benzaldehyde and acetaldehyde was confined to the cytosol, more accurately, to the soluble (105,000 *g* supernatant) fraction. The isoelectric focusing pattern and pI values exhibited by the aldehyde dehydrogenase present in a Luburol®-treated whole homogenate of colon C cells strongly suggested that it was a Type-1 ALDH-3; detectable levels of the enzyme were not found when Lubrol®-treated whole homogenates of HCT 116b and RCA cells were submitted to isoelectric focusing (Fig. 1). Validation of the foregoing suggestion was pursued in the next series of experiments. Purified enzyme was used for this purpose.

Purification of the colon C aldehyde dehydrogenase was achieved by subjecting the soluble (105,000 *g* supernatant) fraction of colon C cells to successive DEAE-Sephacel, CM-Sepharose CL 6B and Reactive Blue-2 Sepharose CL 6B affinity chromatography. As judged by isoelectric focusing (Fig. 2), non-denaturing linear gradient PAGE (not shown), and SDS–PAGE (Fig. 3), the enzyme had been purified to homogeneity. Yield, specific activity and fold-purification of the apparently pure enzyme were 47%, 32,379 mIU/mg protein and 233, respectively (Table 2). The specific activity of the purified colon C enzyme was identical to that of purified human stomach mucosa Type-1 ALDH-3 (32,951 mIU/mg protein) [1], as well as to that induced by methylcholanthrene in human breast adeno-

* Also known as NAD(P)H:quinone oxidoreductase.

Table 1. Aldehyde dehydrogenase activity in Lubrol®-treated whole homogenates of human colon carcinoma cell lines, viz. colon C, HCT 116b and RCA*

Substrate (mM)	Cofactor	ALDH activity (mIU/10^7 cells)		
		Colon C	HCT 116b	RCA
Benzaldehyde (4)	NAD	380 ± 20	1.2 ± 0.1	2.7 ± 0.2
	NADP	647 ± 18	2.1 ± 0.1	3.3 ± 0.1
4-Pyridinecarboxaldehyde (0.5)	NAD	310 ± 8	7.3 ± 0.6	4.0 ± 0.3
	NADP	584 ± 9	0.7 ± 0.2	0.6 ± 0.0
Octanal (0.1)	NAD	255 ± 8	10.6 ± 0.4	2.6 ± 0.2
	NADP	361 ± 11	8.2 ± 0.3	4.8 ± 0.5
Acetaldehyde (4)	NAD	77 ± 4	1.1 ± 0.1	4.5 ± 0.2
	NADP	96 ± 4	4.0 ± 0.2	1.6 ± 0.1
Aldophosphamide (0.16)	NAD	3.2 ± 0.2	0	0
	NADP	0	0	0

* Preparation of Lubrol®-treated whole homogenates and quantification of the aldehyde dehydrogenase activity in such homogenates prepared from 1.5×10^5 to 1×10^7 exponentially growing cells were as described in Materials and Methods. Concentrations of NAD and NADP were 1 and 4 mM, respectively. Values are the means ± SEM of duplicate determinations on each of three samples.

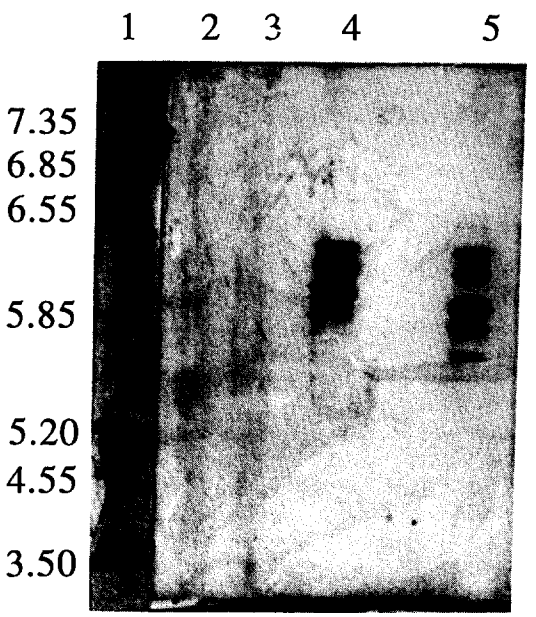

Fig. 1. Isoelectric focusing of aldehyde dehydrogenases present in the Lubrol®-treated whole homogenates of human colon carcinoma cell lines, viz. HCT 116b, RCA and colon C. Lubrol®-treated whole homogenates prepared from 1×10^7 (HCT 116b and RCA) or 2×10^5 (colon C) cells, an aliquot of purified human stomach mucosa Type-1 ALDH-3 sufficient to generate 10 nmol NADH/min as determined by spectrophotometric assay when benzaldehyde (4 mM) was used as substrate, and pI standards were subjected to isoelectric focusing as described in Materials and Methods. Lane 1 (pI standards) was stained for proteins with Coomassie Brilliant Blue R-250. Lanes 2 (HCT 116b), 3 (RCA), 4 (colon C) and 5 (purified human stomach mucosa Type-1 ALDH-3) were stained for aldehyde dehydrogenase activity; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

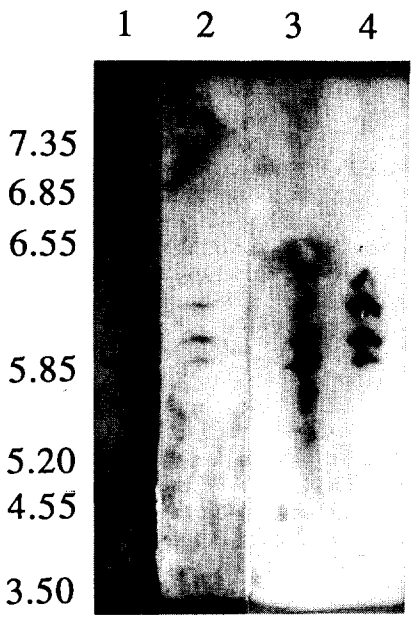

Fig. 2. Isoelectric focusing of the cytosolic class-3 aldehyde dehydrogenase purified from colon C cells. Purification of colon C cell line cytosolic class-3 aldehyde dehydrogenase was as described in Table 2 and Materials and Methods. Aliquots of purified colon C cytosolic class-3 aldehyde dehydrogenase and human stomach mucosa Type-1 ALDH-3 sufficient to generate 5.0 nmol NADH/min, as determined by spectrophotometric assay when benzaldehyde (4 mM) was the substrate, were placed on the gel and isoelectric focused as described in Materials and Methods. Lane 1 (pI standards) and Lane 2 (colon C enzyme) were stained for proteins with Coomassie Brilliant Blue R-250. Lanes 3 (stomach mucosa Type-1 ALDH-3) and 4 (colon C enzyme) were stained for aldehyde dehydrogenase activity as described in Materials and Methods; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

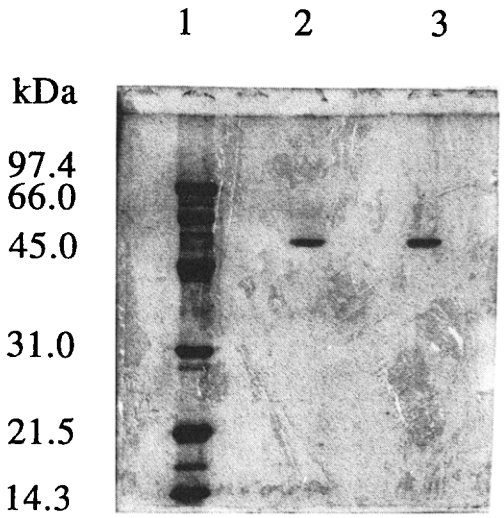

Fig. 3. Subunit molecular weight of the cytosolic class-3 aldehyde dehydrogenase purified from colon C cells as determined by SDS–PAGE. Purification of colon C cytosolic class-3 aldehyde dehydrogenase was as described in Table 2 and Materials and Methods. Electrophoresis of molecular weight markers (Lane 1), and of 5 μg each of purified colon C cytosolic class-3 aldehyde dehydrogenase (Lane 2) and human stomach mucosa Type-1 ALDH-3 (Lane 3), was as described in Materials and Methods. Molecular weight markers were: lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin monomer (66 kDa), and phosphorylase B (97.4 kDa). Proteins were visualized by staining with Coomassie Brilliant Blue R-250. A plot of logM_r versus mobility was used, as described in Materials and Methods, to estimate the subunit molecular weight of colon C cytosolic class-3 aldehyde dehydrogenase.

carcinoma MCF-7/0 cells (32,966 mIU/mg protein) [3]. The colon C enzyme exhibited several physical properties that were identical to those exhibited by human stomach mucosa and methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3s, [1, 3], and different from those exhibited by the MCF-7/OAP Type-2 ALDH-3 [1], viz. pI values (Fig. 2), relative native molecular weight (110 kDa as determined by non-denaturing linear gradient PAGE and 109.5 kDa as determined by Sephacryl S-200 gel permeation column chromatography; not shown), subunit molecular weight (54.5 kDa) determined by SDS–PAGE (Fig. 3), and recognition of both native and denatured enzyme by anti-stomach mucosa Type-1 ALDH-3 IgY (Fig. 4).

Characteristic of cytosolic class-3 aldehyde dehydrogenases, the purified colon C enzyme preferred, as judged by K_m values, long-chain aliphatic, and aromatic, aldehydes, viz. octanal, benzaldehyde, 4-(diethylamino)benzaldehyde and 4-pyridinecarboxaldehyde, over short-chain aliphatic aldehydes, viz. acetaldehyde, as substrates (Table 3). The K_m and V_{max} values that define catalysis of the oxidation of octanal, benzaldehyde, 4-pyridinecarboxaldehyde and acetaldehyde by colon C class-3 aldehyde dehydrogenase were very similar to those obtained with human stomach mucosa and methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3s (kinetic constants defining the catalysis of 4-(diethylamino)benzaldehyde oxidation by the latter two enzymes were not determined) [1, 3]. Like methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3 and MCF-7/OAP Type-2 ALDH-3 [1, 3], the colon C enzyme catalyzed the oxidation of aldophosphamide to carboxyphosphamide when NAD was used as the cofactor. In that regard, it differed from human stomach mucosa Type-1 ALDH-3 since the latter is almost undetectably able to do so [10]. As was the case with other cytosolic class-3 aldehyde dehydrogenases, the reaction did not proceed when NADP was used as the cofactor.

As was the case with the other human cytosolic class-3 Type-1 aldehyde dehydrogenases [1, 3], NAD and NADP could each serve as the cofactor for the colon C enzyme when the substrate was other than aldophosphamide, e.g. benzaldehyde (Tables 1 and 3), but, as judged by K_m values which were virtually identical to those obtained with human stomach mucosa and methylcholanthrene-induced Type-1 ALDH-3s [1, 3], NAD was much preferred (Table 4). As in the case of human stomach mucosa and methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3s [1, 3], cofactor "activation" was observed in the case of colon C class-3 aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde when the concentration of NAD exceeded 1 mM, and high

Table 2. Purification of a cytosolic class-3 aldehyde dehydrogenase from colon C cells*

Purification step	Total activity† (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg protein)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	4532	100	32.5	139	1
DEAE-Sephacel chromatography	3240	72	2.2	1473	11
CM-Sepharose CL 6B chromatography	2472	55	0.65	3803	27
Reactive Blue 2-Sepharose CL 6B chromatography	2137	47	0.066	32,379	233

* Purification was as described in Materials and Methods.

† Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify aldehyde dehydrogenase activity.

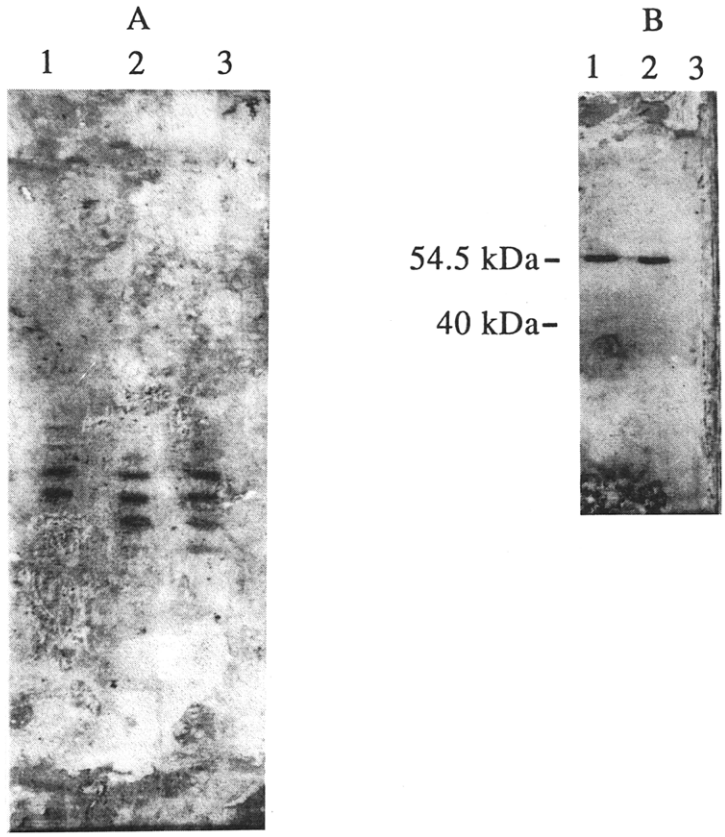

Fig. 4. Immunoblot visualization of native and denatured class-3 aldehyde dehydrogenase purified from colon C cells and subjected to isoelectric focusing (native) and SDS–PAGE (denatured). Purification of colon C cytosolic class-3 aldehyde dehydrogenase was as described in Table 2 and Materials and Methods. Anti-stomach mucosa ALDH-3 IgY was generated and used, as described in Materials and Methods, in attempting to visualize (A) purified MCF-7/OAP Type-2 ALDH-3 (Lane 1), the colon C enzyme (Lane 2) and stomach mucosa Type-1 ALDH-3 (Lane 3) after these enzymes had been submitted to isoelectric focusing (non-denaturing conditions), or (B) purified stomach mucosa Type-1 ALDH-3 (Lane 1), the colon C enzyme (Lane 2) and MCF-7/OAP Type-2 ALDH-3 (Lane 3; 40 kDa subunit that is not recognized by anti-stomach mucosa ALDH-3 IgY [1]) after these enzymes had been submitted to SDS–PAGE (denaturing conditions) and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on the gels were 5 μg of each purified enzyme.

Table 3. Kinetic properties of the class-3 aldehyde dehydrogenase purified from human colon carcinoma cell line C*

Substrate (mM)	Cofactor	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)
Benzaldehyde (0.05–4)	NAD	424	32,800	77
	NADP	427	57,246	134
4-(Diethylamino)benzaldehyde (0.02–0.1)	NAD	50	24,630	493
	NADP	41	50,690	1236
4-Pyridinecarboxaldehyde (0.05–4)	NAD	167	30,770	184
	NADP	169	50,000	295
Octanal (0.05–1)	NAD	133	24,000	180
	NADP	153	26,667	174
Acetaldehyde (25–200)	NAD	80,972	20,338	0.25
	NADP	86,331	23,338	0.27
Aldophosphamide (0.16–0.96)	NAD	570	532	0.93
	NADP		0	

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were made in 25 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.05% dithiothreitol, and were added to the reaction mixture in a volume of 100 μL. NAD and NADP concentrations were 1.0 and 4.0 mM, respectively. Each value is the mean of two determinations.

Table 4. Cofactor preference of the class-3 aldehyde dehydrogenase purified from human colon carcinoma cell line C*

Cofactor	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)
NAD (0.01–1)	48	33,000	688
NADP (0.1–4)	940	66,660	71

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were made in 25 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.05% dithiothreitol, and were added to the reaction mixture in a volume of 100 μL. Benzaldehyde (4 mM) was the substrate. Each value is the mean of two determinations.

concentrations of NADP did not activate or inhibit the colon C class-3 aldehyde dehydrogenase (data not presented).

Like other class-3 aldehyde dehydrogenases [1, 3], colon C class-3 aldehyde dehydrogenase exhibited esterolytic activity (8500 mIU/mg protein), and catalysis of *p*-nitrophenyl acetate hydrolysis by colon C class-3 aldehyde dehydrogenase was enhanced by 20 μM NAD and partially inhibited by 100 μM NAD (data not presented).

Colon C class-3 aldehyde dehydrogenase was, like the human stomach mucosa and methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3s [1, 3], heat labile; catalytic activity was completely lost in less than 10 min when the enzyme was incubated at 56° (data not presented).

Disulfiram (50 μM) and chloral hydrate (100 μM) partially inhibited (<30%), and *p*-chloromercuribenzoate (25 μM) completely inhibited, NAD(P)-dependent colon C class-3 aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde, whereas Mg^{2+} (250 and 500 μM) did not inhibit or stimulate the reaction (data not presented). The sensitivity of the colon C enzyme to these agents was virtually identical to that exhibited by human stomach mucosa and methylcholanthrene-induced MCF-7/0 Type-1 ALDH-3s [1, 3].

Collectively, the foregoing observations are consistent with the notion that the colon C class-3 aldehyde dehydrogenase is a Type-1 ALDH-3 identical to that found in methylcholanthrene-treated MCF-7/0 cells but apparently slightly different, since it catalyzes the oxidation of aldophosphamide, from that found in human stomach mucosa.

As judged by LC_{90} values, HCT 116b and RCA cells were, relative to colon C cells, about 10-fold more sensitive to mafosfamide; the three cell lines were equisensitive to phosphoramide mustard (Fig. 5 and Table 5). Inclusion of benzaldehyde or 4-(diethylamino)benzaldehyde, each a relatively good substrate for the colon C class-3 aldehyde dehydrogenase (Table 3), in the drug-exposure medium increased the sensitivity of colon C cells to mafosfamide 10-fold; inclusion of acetaldehyde, a relatively poor substrate for this enzyme (Table 3), did not (Table 5 and Fig. 6). Inclusion of benzaldehyde, 4-(diethylamino)benzaldehyde or acetaldehyde in the incubation medium did not alter the sensitivity of colon C cells to phosphoramide mustard, nor did it alter the sensitivity of HCT 116b and RCA cells to mafosfamide or phosphoramide mustard (Table 5 and Fig. 6).

Collectively, these observations are as expected

Table 5. Sensitivity of human colon carcinoma cell lines, viz. colon C, HCT 116b, and RCA, to mafosfamide and phosphoramide mustard in the presence and absence of various aldehydes*

Cell line	Potential modulator	LC_{90} (μM)	
		Mafosfamide	Phosphoramide mustard
Colon C	None	368 ± 12	1576 ± 146
	Benzaldehyde	36 ± 3	1443 ± 131
	4-(Diethylamino)benzaldehyde	39 ± 2	1620 ± 98
	Acetaldehyde	361 ± 10	1526 ± 121
HCT 116b	None	34 ± 2	1583 ± 145
	Benzaldehyde	33 ± 3	1600 ± 57
	4-(Diethylamino)benzaldehyde	34 ± 3	1640 ± 131
	Acetaldehyde	34 ± 3	1606 ± 102
RCA	None	36 ± 2	1610 ± 102
	Benzaldehyde	38 ± 2	1623 ± 72
	4-(Diethylamino)benzaldehyde	36 ± 4	1626 ± 118
	Acetaldehyde	39 ± 2	1596 ± 95

* Cultured colon C, HCT 116b and RCA cells were incubated with benzaldehyde (5 mM), 4-(diethylamino)benzaldehyde (200 μM), acetaldehyde (5 mM) or vehicle for 5 min at 37° after which time mafosfamide, phosphoramide mustard or vehicle was added and incubation was continued as before for 30 min. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. The LC_{90} (concentration of drug required to effect a 90% cell-kill) values were obtained from plots of log surviving fractions versus concentrations (5–8) of mafosfamide or phosphoramide mustard, as illustrated in Figs. 5 and 6. Values are means ± SEM of three separate determinations.

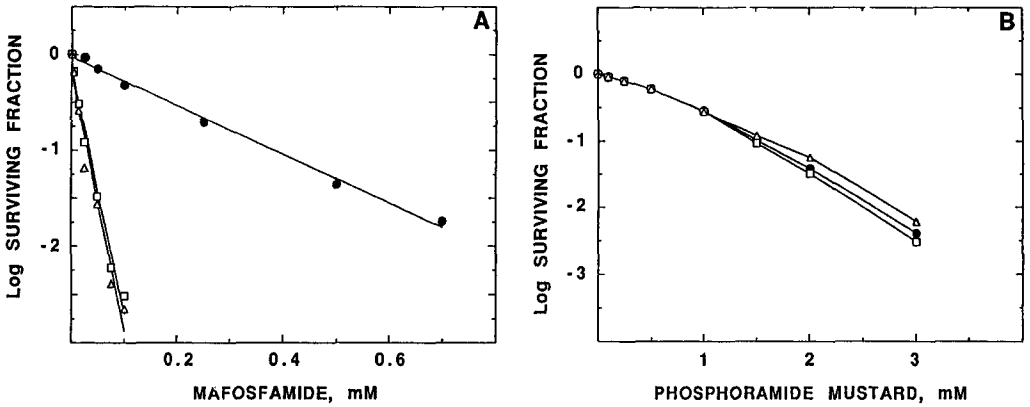

Fig. 5. Sensitivity of human colon carcinoma cell lines, viz. colon C, HCT 116b and RCA, that express markedly different amounts of class-3 aldehyde dehydrogenase to mafosfamide and phosphoramide mustard. Exponentially growing colon C (●), HCT 116b (□) and RCA (Δ) cells were harvested and exposed to mafosfamide (**A**) or phosphoramide mustard (**B**) for 30 min at 37° after which time they were reharvested and grown in a drug-free medium. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures. Aldehyde dehydrogenase activities (4 mM NADP; 4 mM benzaldehyde) in Lubrol®-treated whole homogenates of these cells were 631 (colon C), 2.1 (HCT 116b), and 3.3 (RCA) mIU/10^7 cells.

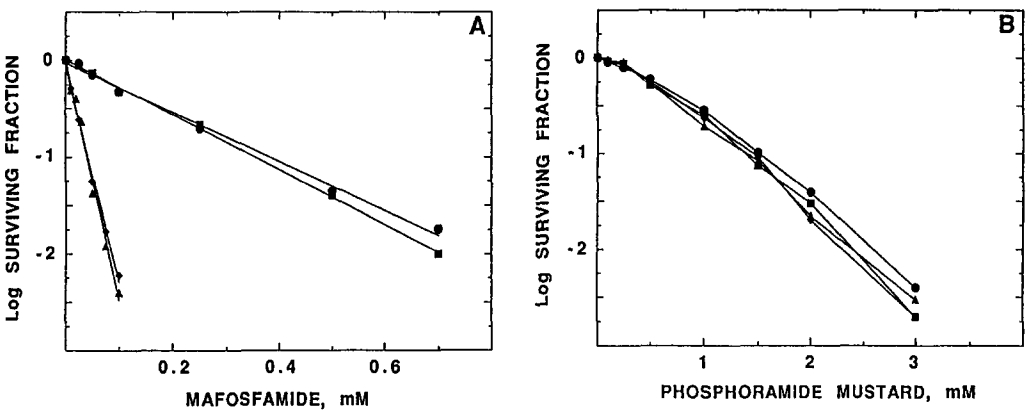

Fig. 6. Sensitivity of colon C cells to mafosfamide and phosphoramide mustard in the presence and absence of benzaldehyde, 4-(diethylamino)benzaldehyde and acetaldehyde. Exponentially growing colon C cells were harvested and preincubated with 5 mM benzaldehyde (◆), 200 μM 4-(diethylamino)benzaldehyde (▲), 5 mM acetaldehyde (■), or vehicle (●) for 5 min at 37°. Mafosfamide (**A**) or phosphoramide mustard (**B**) was then added, and the incubation was continued for an additional 30 min after which time the cells were reharvested, resuspended in drug-free growth medium, and cultured. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures. Aldehyde dehydrogenase activity (4 mM NADP; 4 mM benzaldehyde) in a Lubrol®-treated whole homogenate of the colon C cells was 628 mIU/10^7 cells.

given the relative amounts of class-3 aldehyde dehydrogenase expressed by the three cell lines and that sensitivity to the oxazaphosphorines decreases when the cellular content of an enzyme that catalyzes the detoxification of these agents, e.g. a class-3 aldehyde dehydrogenase, increases.

Of ancillary interest were the following observations.

Along with relatively increased amounts of a class-3 aldehyde dehydrogenase, methylcholanthrene-treated MCF-7/0 cells (as well as MCF-7/OAP cells) express relatively increased amounts of glutathione *S*-transferase and DT-diaphorase [3], as might be expected given that methylcholanthrene-induced expression of each of these enzymes is by a common mechanism, viz. an activated Ah receptor-mediated process [reviewed in Ref. 11]. However, similar relationships between ALDH-3 and glutathione *S*-

Table 6. Glutathione *S*-transferase and DT-diaphorase activities, and glutathione levels, in Lubrol®-treated whole homogenates of human colon carcinoma cell lines, viz. colon C, HCT 116b, and RCA*

Cell line	Enzyme activity (mIU/10^7 cells)		Glutathione (nmol/10^7 cells)
	Glutathione *S*-transferase	DT-diaphorase	
Colon C	866 ± 50	453 ± 15	39 ± 2
HCT 116b	83 ± 3	43 ± 4	19 ± 2
RCA	809 ± 20	421 ± 27	20 ± 2

* Preparation of Lubrol®-treated whole homogenates and quantification of glutathione *S*-transferase activity, DT-diaphorase activity and glutathione levels in such homogenates prepared from 1.5×10^5 to 1.0×10^7 exponentially growing colon C, HCT 116b and RCA cells were as described in Materials and Methods. Values are the means ± SEM of duplicate determinations on each of three samples.

transferase or DT-diaphorase levels were not observed in the present experiments (Tables 1 and 6).

In some models, sensitivity to the oxazaphosphorines appears to be inversely related to cellular glutathione levels [reviewed in Ref. 12]. Glutathione levels were relatively higher in the relatively insensitive colon C cells but only by about 2-fold (Table 6).

Aldehyde dehydrogenase (NAD(P)/benzaldehyde), glutathione *S*-transferase and DT-diaphorase activities were not induced and cell growth was not inhibited when colon C, HCT 116b or RCA cells were grown in the presence of 3, 10, or 20 μM methylcholanthrene for 5 days (data not presented).

Aldehyde dehydrogenase activities in a 105,000 *g* supernatant fraction prepared from human normal colon tissue were 23 (1 mM NAD/4 mM benzaldehyde), 41 (4 mM NADP/4 mM benzaldehyde) and 49 (1 mM NAD/4 mM acetaldehyde) mIU/g tissue. Submission of this preparation to isoelectric focusing followed by staining for aldehyde dehydrogenase activity (NAD/benzaldehyde), and SDS–PAGE followed by electrotransfer of proteins onto a membrane and immunoblot analysis with anti-stomach mucosa Type-1 ALDH-3 IgY, each indicated the presence of very small amounts of Type-1 ALDH-3 in normal colon tissue; relatively greater amounts of ALDH-1 were also present (not shown).

DISCUSSION

It has been demonstrated in recent years that cellular sensitivity to the oxazaphosphorines is inversely proportional to cellular cytosolic class-3 aldehyde dehydrogenase levels, and that overexpression of this enzyme by malignant cells, and thus acquired resistance to the oxazaphosphorines, can be transiently, as well as stably, induced [1–3, 13].

Some time ago, Marselos and Michalopoulos [4] reported that catalytic activity not unlike that effected by cytosolic class-3 aldehyde dehydrogenases varied widely in cytosolic fractions prepared from primary, and especially metastatic, human colon adenocarcinomas, suggesting that expression of relatively large amounts of a cytosolic class-3 aldehyde dehydrogenase may account, at least in some cases, for intrinsic insensitivity to the oxazaphosphorines as well. As shown herein, that proved to be the case.

The operative enzyme in the cultured colon carcinoma model that we examined, viz. colon C, was found to be a Type-1 ALDH-3 identical to that present in methylcholanthrene-treated human breast adenocarcinoma MCF-7/0 cells [3], and very nearly identical to the prototypical Type-1 ALDH-3 found in human stomach mucosa [1], the difference being that the colon C and methylcholanthrene-induced enzymes catalyze the oxidation of aldophosphamide to carboxyphosphamide, albeit not very well, whereas the prototypical stomach mucosa enzyme essentially does not [1, 3, 10]. Whether relatively elevated levels of the prototypical enzyme would be accompanied by relatively decreased sensitivity to the oxazaphosphorines is not known but, depending on the mechanism by which resistance to the oxazaphosphorines is mediated by elevated levels of ALDH-3, the expectation is that it might not [3].

Expression of ALDH-3 appears to be minimal in human normal colon tissue though our experiments do not allow us to pass judgement as to whether it is uniformly minimal; it is stably up-regulated in some, though not all, malignant cells derived therefrom. Of interest is why up-regulation occurs in only some of the malignancies derived from this tissue. Perhaps the malignant colon C cell line originated with the transformation of a normal colon cell that constitutively expressed large amounts of ALDH-3, whereas the HCT 116b and RCA cell lines originated with normal colon cells that did not.

Also of interest is the cellular change prescribing stable up-regulation when it occurs, e.g. in colon C cells. Expression of Type-1 ALDH-3 can be induced in some, but not all, malignant cells by ligands for the Ah receptor [2, 3]. Such agents "coordinately" induce other enzymes, e.g. glutathione *S*-transferase and DT-diaphorase, as well [2, 3, 11, 14–16]. Whereas the Type-1 ALDH-3 level was much higher in colon C cells as compared with that in RCA cells, glutathione *S*-transferase and DT-diaphorase levels were about the same in the two cell lines, suggesting that up-regulation of ALDH-3 expression is not the result of up-regulation of an activated Ah receptor-mediated process.

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