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Inhibition of Human Class 3 Aldehyde Dehydrogenase, and Sensitization of Tumor Cells That Express Significant Amounts of This Enzyme to Oxazaphosphorines, by Chlorpropamide Analogues

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ABSTRACT. In some cases, acquired as well as constitutive tumor cell resistance to a group of otherwise clinically useful antineoplastic agents collectively referred to as oxazaphosphorines, e.g. cyclophosphamide and mafosfamide, can be accounted for by relatively elevated cellular levels of an enzyme, viz. cytosolic class 3 aldehyde dehydrogenase (ALDH-3), that catalyzes their detoxification. *Ergo*, inhibitors of ALDH-3 could be of clinical value since their inclusion in the therapeutic protocol would be expected to sensitize such cells to these agents. Identified in the present investigation were two chlorpropamide analogues showing promise in that regard, viz. (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester (NPI-2) and 4-chloro-*N*-methoxy-*N*-[(propylamino)carbonyl]benzenesulfonamide (API-2). Each inhibited NAD-linked benzaldehyde oxidation catalyzed by ALDH-3s purified from human breast adenocarcinoma MCF-7/0/CAT cells (IC_{50} values were 16 and 0.75 μM, respectively) and human normal stomach mucosa (IC_{50} values were 202 and 5 μM, respectively). The differential sensitivities of stomach mucosa ALDH-3 and breast tumor ALDH-3 to each of the two inhibitors can be viewed as further evidence that the latter is a subtle variant of the former. Human class 1 (ALDH-1) and class 2 (ALDH-2) aldehyde dehydrogenases were much less sensitive to NPI-2; IC_{50} values were >300 μM in each case. API-2, however, did not exhibit a similar degree of specificity; IC_{50} values for ALDH-1 and ALDH-2 were 7.5 and 0.08 μM, respectively. Each sensitized MCF-7/0/CAT cells to mafosfamide; the LC_{90} value decreased from >2 mM to 175 and 200 μM, respectively. Thus, the therapeutic potential of combining NPI-2 or API-2 with oxazaphosphorines is established. BIOCHEM PHARMACOL 55;4:465–474, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; cyclophosphamide; aldophosphamide; oxazaphosphorines; chlorpropamide analogues; breast cancer; drug resistance; gossypol.

ALDH-3§ is a demonstrated molecular determinant of cellular sensitivity to the cytotoxic action of certain widely used antineoplastic prodrugs collectively referred to as oxazaphosphorines, e.g. cyclophosphamide, ifosfamide, 4-hydroperoxycyclophosphamide, 4-hydroperoxyifosfamide and mafosfamide (cellular sensitivity to these drugs decreases as cellular levels of ALDH-3 increase) [1–10]. Thus, of therapeutic significance, relatively elevated levels of this enzyme can account for intrinsic, transient acquired, and stable acquired, resistance to the oxazaphosphorines on the part of malignant cells [2–4, 7, 9]. Resistance to the oxazaphosphorines mediated by ALDH-3 is due ostensibly to the enzyme-catalyzed oxidative detoxification of aldophosphamide, the pivotal metabolite of these prodrugs [2, 4, 7, 9, 10].

Inhibition of ALDH-3, therefore, would be expected to sensitize otherwise relatively insensitive tumor cells to the oxazaphosphorines when relatively high cellular levels of

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§ *Abbreviations:* ALDH-1, class 1 aldehyde dehydrogenase; ALDH-2, class 2 aldehyde dehydrogenase; ALDH-3, cytosolic class 3 aldehyde dehydrogenase; API-1, 4-chloro-*N*-ethyl-*N*-[(propylamino)carbonyl]benzenesulfonamide; API-2, 4-chloro-*N*-methoxy-*N*-[(propylamino)carbonyl]benzenesulfonamide; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IC_{50}, concentration of agent required to effect 50% inhibition of enzyme catalysis; LC_{90}, concentration of drug required to effect 90% cell-kill; MCF-7/0/CAT cells, human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to induce ALDH-3; μIU, micro-International Unit of enzyme activity (μmol NAD(P)H formed/min in the case of dehydrogenase activity, and μmol *p*-nitrophenol formed/min in the case of carboxylesterase or phosphatase activity); NPI-1, (benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester; NPI-2, (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester; NPI-3, *N*-acetyl-*N*-(acetyloxy)-4-chlorobenzenesulfonamide; rALDH-1, recombinant ALDH-1; rALDH-2, recombinant ALDH-2; tALDH-3 and nALDH-3, ALDH-3s purified from human tumor and normal cells/tissues, respectively; yALDH, yeast aldehyde dehydrogenase.

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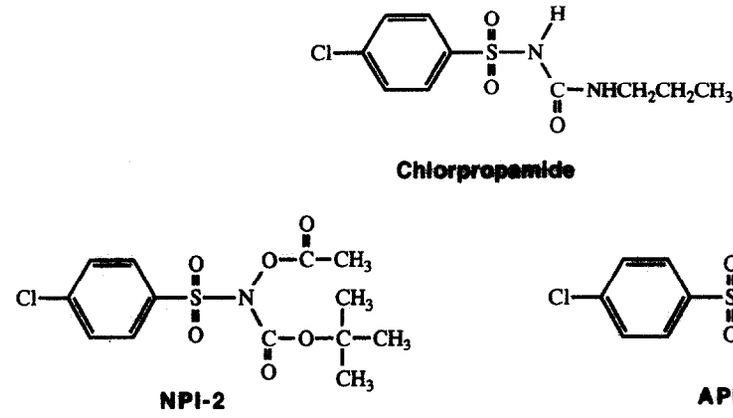

Chlorpropamide: 1-(*p*-chlorobenzenesulfonyl)-3-*n*-propylurea

NPI-2: (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester

API-2: 4-chloro-N-methoxy-N-[(propylamino)carbonyl]benzenesulfonamide

FIG. 1. Structures of chlorpropamide and two analogues thereof.

ALDH-3 are the basis for the relative insensitivity. Thus, inhibitors of ALDH-3 could be of therapeutic value. However, known inhibitors of ALDH-1 and/or ALDH-2, e.g. disulfiram and chloral hydrate, do not, or only minimally, inhibit ALDH-3 [2, 4] and, predictably, do not sensitize tumor cells to the oxazaphosphorines when such cells are insensitive to these agents because of relatively high ALDH-3 levels [reviewed in Ref. 8]. Indeed, to date, only gossypol has been shown to inhibit ALDH-3 [11], although at least one chlorpropamide analogue showed promise in that regard [12], and inhibition of ALDH-3-catalyzed oxidative detoxification of aldophosphamide could be effected by alternative substrates, e.g. benzaldehyde and 4-(diethylamino)benzaldehyde [4, 7, 8].

Like the alcohol deterrents disulfiram and cyanamide, the oral hypoglycemic agent chlorpropamide is thought to be a pro-inhibitor of the aldehyde dehydrogenases, most notably ALDH-2, that catalyze the oxidation of ethanol-derived acetaldehyde [13]. Putative metabolites of cyanamide and chlorpropamide include nitroxyl (HNO) and *n*-propylisocyanate, respectively; they have been postulated to be the metabolites that inhibit hepatic aldehyde dehydrogenase-catalyzed reactions [14–16].

Based on these premises, a number of chlorpropamide analogues, viz. N^1-hydroxy-substituted ester, N^1-methoxy and N^1-alkyl derivatives, intended to be prodrugs with the potential of giving rise to HNO or *n*-propylisocyanate, have been designed and synthesized as potential alcohol deterrents [14–16].

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze the hydrolysis of esters in addition to catalyzing the oxidation of aldehydes [8, 17–19]. Whether catalysis of hydrolytic reactions by these enzymes is of physiological or pharmacological consequence is not known.

The ester analogues were therefore designed with the intent of exploiting the esterolytic activities exhibited by the aldehyde dehydrogenases, viz. to release HNO, a potent inhibitor of aldehyde dehydrogenase-catalyzed oxidations, upon ester hydrolysis catalyzed by these enzymes [16]. Indeed, some of the compounds that were synthesized have been shown to undergo hydrolytic cleavage catalyzed by yALDH and to inhibit yALDH-catalyzed acetaldehyde oxidation.

The N^1-methoxy and N^1-alkyl analogues, on the other hand, were designed to release *n*-propylisocyanate, a potent inhibitor of yeast and rodent hepatic aldehyde dehydrogenases, without the necessity of any enzyme participation [14, 15]. Some of the compounds that were synthesized have been shown to inhibit yeast and rodent hepatic mitochondrial aldehyde dehydrogenase-catalyzed oxidation, to decrease acetaldehyde clearance in rodents given ethanol, and to be devoid of a hypoglycemic effect.

Two of the ester analogues, viz. NPI-1 and NPI-3, and one N^1-ethyl analogue, viz. API-1, were studied more extensively [12]. They were found to inhibit ALDH-3 but they were not very potent in doing so, nor were they very selective, i.e. they inhibited ALDH-1 and/or ALDH-2 equally as well. Nonetheless, these findings encouraged us to ascertain the effects of some additional chlorpropamide analogues (Fig. 1) on the catalytic activities of these enzymes.

The ALDH-3 present in human tumor cells/tissues (tALDH-3), although otherwise seemingly identical to the ALDH-3 present in human normal tissues/fluids (nALDH-3), differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines [reviewed in Ref. 8]. Moreover, tALDH-3 is more sensitive to inhibition by NPI-1, NPI-3,

and API-1 than is nALDH-3 [12]. Hence, both tALDH-3 and nALDH-3 were included in our investigations.

Since aldehyde dehydrogenases catalyze hydrolytic as well as oxidative reactions, the effect of the chlorpropamide analogues on each of these reactions was determined though inhibition of the latter was our principal interest.

The ability of the chlorpropamide analogues to negate the influence of relatively high cellular levels of ALDH-3 on the cellular sensitivity of cultured tumor cells to oxazaphosphorines was also determined.

MATERIALS AND METHODS

Mafosfamide was provided by Dr. J. Pohl, Asta Medica AG. Phosphoramide mustard · cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. *Escherichia coli* [BL21(DE3)pLysS] transfected with pET-19b vector, to which human ALDH-1 cDNA (cloned from human hepatoma Hep G2 cells [20]) was ligated, was provided by Dr. Jan Moreb, University of Florida. A vector, viz. pT7-7, to which human ALDH-2 cDNA (cloned from human liver [21]) was ligated, was provided by Dr. Henry Weiner, Purdue University. Transfection of human ALDH-2 cDNA ligated to the pT7-7 vector into *E. coli* [BL21(DE3)pLysS] was by Drs. P. A. Dockham and L. Sreerama of our laboratory as described by Sambrook *et al.* [22]. Generation and purification of human rALDH-1 and rALDH-2 were as described previously [12]. NPI-2 and API-2 were synthesized as described previously [15, 16]. Chromatographically purified yALDH, human erythrocyte GAPDH, and human placental alkaline phosphatase type XXIV were purchased from the Sigma Chemical Co. All other chemicals and reagents were obtained from the sources listed in previous publications [2, 4, 9, 12, 23].

Human normal stomach mucosa ALDH-3 (nALDH-3) and the ALDH-3 (tALDH-3) present in human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to induce the enzyme (MCF-7/0/CAT cells) were purified as described previously [3, 9].

Primarily to remove dithiothreitol, all of the purified enzymes were transferred from the storage buffer [25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5, supplemented with 1 mM EDTA and 1 mM dithiothreitol] to 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5, with the aid of a PD-10 (Sephadex G-25) column prior to their use.

NAD-linked oxidation of acetaldehyde catalyzed by rALDH-1 and rALDH-2 at 37° and pH 8.1, NAD(P)-linked oxidation of benzaldehyde catalyzed by nALDH-3 and tALDH-3 at 37° and pH 8.1, hydrolysis of *p*-nitrophenyl acetate catalyzed by each of these enzymes at 25° and pH 7.5, NAD-linked oxidation of GAP catalyzed by GAPDH at 37° and pH 7.6, and hydrolysis of *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase at 25° and pH 9.8 were quantified spectrophotometrically as described previously [2, 23–25] except that, whenever previously it had been included, glutathione was omitted from the reaction mixture when aldehyde dehydrogenase activity was quantified. Except where noted, preincubation of the putative inhibitor, viz. NPI-2 or API-2, or vehicle together with the complete reaction mixture except for the substrate was for 5 min. Preincubation temperatures and pH levels were the same as incubation temperatures and pH levels. All reactions were started by the addition of substrate. Stock solutions of NPI-2 and API-2 were prepared in dimethyl sulfoxide and were stored at −20°. The final concentration of dimethyl sulfoxide in the reaction mixture was always 5% (v/v); this concentration of dimethyl sulfoxide did not inhibit any of the enzyme-catalyzed reactions under investigation.

Enzyme · inhibitor complexes were subjected to gel permeation chromatography to determine whether inhibition effected by NPI-2 and API-2 was reversible. Briefly, the complete reaction mixture except for the substrate was first incubated at 37° for 5 min in a volume of 1 mL with vehicle or concentrations of inhibitor that effected >50% inhibition with one exception, viz. inhibition of rALDH-2 by NPI-2. Reaction mixtures were then chilled in an ice-bath for 2 min after which they were placed on a PD-10 (Sephadex G-25) column (2 × 5 cm; 2.5 mL void volume; 10 mL bed volume) that had been equilibrated with 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5. The column was then eluted with 3.5 mL of equilibration buffer, and the resultant eluate was collected and saved for assay of enzyme activity. Gel permeation chromatography was at 4°. Preliminary experiments established that recoveries of free inhibitor and free enzyme protein were <2 and >98%, respectively. Enzyme activities were quantified before and after the gel permeation chromatography as described above.

Human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells were cultured (monolayer), harvested when still in exponential growth, resuspended in growth medium, and checked for viability (usually greater than 95% as judged by trypan blue exclusion) as described previously [2, 9]. Drug exposure and the colony-forming assay used to determine surviving fractions were also as described previously [2]. Briefly, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and then exposed to drug (mafosfamide or phosphoramide mustard) 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. Colonies (≥50 cells) were then visualized with methylene blue dye and counted. Stock solutions of mafosfamide and phosphoramide mustard were prepared by dissolving them in water just before use. In some experiments, cells were preincubated with NPI-2, API-2, or vehicle for 5 min at 37° prior to the addition of mafosfamide or phosphoramide mustard. Stock dimethyl sulfoxide solutions of NPI-2 or API-2 were diluted with drug-exposure medium just before use. The dimethyl sulfoxide concentration in the drug-exposure medium was 0.1% (v/v); this concentration of dimethyl sulfoxide did

not affect the rate of cell proliferation. At the concentrations used, viz. 100 and 50 μM, respectively, NPI-2 and API-2 effected only a small amount of cell-kill (<12%; LC_{90} values were >300 μM in each case), and this was taken into account when calculating the effect of including NPI-1 or API-2 in the drug-exposure medium on LC_{90} values for mafosfamide and phosphoramide mustard.

Computer-assisted unweighted nonlinear regression analysis effected by the STATView statistical program (Brain Power Inc.) was used to generate the curves that best-fit plots of enzyme activities (% of control) as a function of inhibitor concentrations (four to eight) and, subsequently, to estimate the concentration of inhibitor that effected a 50% decrease in catalytic activity (IC_{50}). Double-reciprocal (Lineweaver–Burk) plots of initial catalytic rates as a function of substrate concentrations (at least three and usually five) were used to estimate the K_m and V_{max} values. Except in the case of rALDH-2-catalyzed oxidation of acetaldehyde, K_i values were determined by plotting the slopes of the lines generated by double-reciprocal (Lineweaver–Burk) plots as a function of inhibitor concentrations (three). In the case of rALDH-2-catalyzed oxidation of acetaldehyde, K_i values were determined by plotting the reciprocals of initial catalytic rates as a function of inhibitor concentrations (five) (Dixon plots) because K_m values were relatively small and, thus, K_i values were difficult to ascertain accurately from Lineweaver–Burk plots. In the case of double-reciprocal (Lineweaver–Burk) plots, computer-assisted Wilkinson weighted linear regression analysis [26] effected by the MacWilkins program (Microsoft) was used to generate the best-fit lines. Computer-assisted unweighted linear regression analysis effected by the STATView statistical program was used to generate best-fit lines for all other straight-line functions.

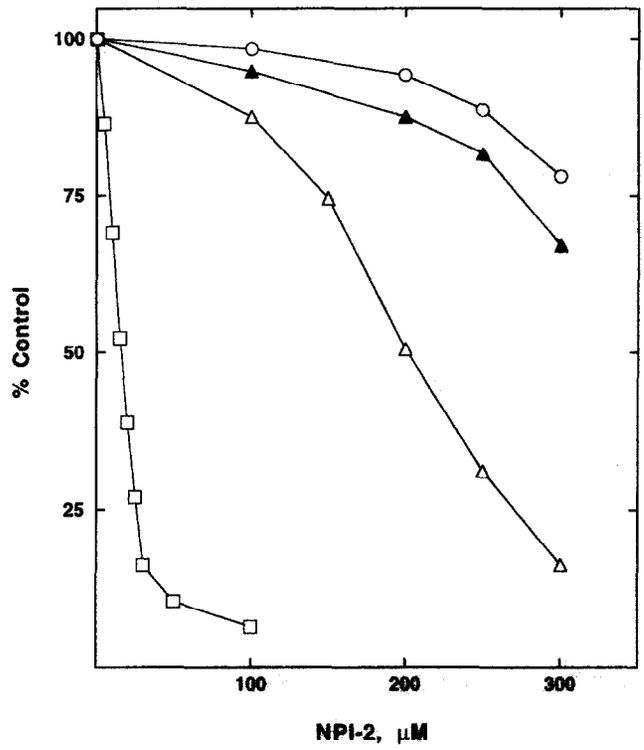

FIG. 2. Inhibition by NPI-2 of the NAD-linked oxidative reactions catalyzed by human aldehyde dehydrogenases: Concentration responses. The sensitivities of rALDH-1 (○), rALDH-2 (▲), nALDH-3 (△), and tALDH-3 (□) to inhibition by NPI-2 were determined as described in Materials and Methods and the footnote to Table 1. Data points are means of duplicate determinations. Control catalytic rates were 0.60, 2.4, 29, and 31 IU/mg for rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively. Best-fit curves, and IC_{50} values estimated therefrom, were generated from the data presented in this figure, as described in "Materials and Methods." The IC_{50} values thus obtained are given in Table 1.

RESULTS

NPI-2 and API-2 were not substrates for the oxidative reactions catalyzed by any of the ALDHs studied.

Oxidative reactions catalyzed by rALDH-1, rALDH-2, the ALDH-3s, yALDH and GAPDH were inhibited by NPI-2 and API-2 (Fig. 2 and Table 1). API-2 was much more potent in that regard. Differential sensitivity to these inhibitors on the part of the human aldehyde dehydrogenases was observed. Thus, as judged by the concentrations of NPI-2 or API-2 required to effect 50% inhibition (IC_{50}), (1) tALDH-3 was, relative to rALDH-1, rALDH-2, nALDH-3, and GAPDH, far more sensitive to inhibition by NPI-2, and (2) tALDH-3 and, to an even greater extent, rALDH-2 were, relative to rALDH-1, nALDH-3, and GAPDH, far more sensitive to API-2. yALDH-catalyzed oxidation was relatively sensitive to inhibition by each agent.

Routinely, preincubation of NPI-2, API-2, or vehicle together with the complete reaction mixture except for substrate, viz. acetaldehyde or benzaldehyde, was for 5 min. Maximum inhibition of aldehyde dehydrogenase-catalyzed oxidation by NPI-2 and API-2 (Fig. 3) was achieved within this time period in all cases except in that of rALDH-2 where a small amount of additional inhibition was observed between min 5 and 6 of preincubation in each case. Striking is the greater preincubation time that was required to achieve maximum inhibition of rALDH-2 by API-2 as compared with that required to achieve maximum inhibition of the other aldehyde dehydrogenases by this agent.

Inhibition could not be reversed by passing ALDH · NPI-2 complexes (enzyme · inhibitor complexes) through a PD-10 (Sephadex G-25) column (Fig. 4). It was somewhat reversed when the rALDH-1 · API-2 and rALDH-2 · API-2 complexes were passed through a PD-10 column. It was largely reversed when either of the ALDH-3 · API-2 complexes were passed through a PD-10 column.

Fifty percent inhibition of a hydrolytic reaction (hydrolysis of *p*-nitrophenyl acetate) catalyzed by the human ALDHs, and of one (hydrolysis of *p*-nitrophenyl phosphate) catalyzed by human placental alkaline phosphatase, was not achieved at the highest concentrations of NPI-2 tested and, except in the case of rALDH-2, was not

TABLE 1. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenase-catalyzed oxidations and hydrolyses: IC_{50} values*

	IC_{50} (μM)					
	NAD-linked oxidation†		NADP-linked oxidation†		Hydrolysis‡	
Enzyme	NPI-2	API-2	NPI-2	API-2	NPI-2	API-2
rALDH-1	>300	7.5			>200	>1000
rALDH-2	>300	0.08			>200	16
nALDH-3	202	5.0	267	0.90	>200	>1000
tALDH-3	16	0.75	39	0.62	>200	>1000
yALDH	15	0.15			50	2.7
GAPDH	111	38				
Alkaline phosphatase					>300	>1000

* Enzymes were incubated with vehicle or 4–8 different concentrations of one of the putative inhibitors for 5 min, substrate was added, and initial catalytic rates were quantified as described in "Materials and Methods." Examples of plots of the primary data thus obtained are shown in Fig. 2. The IC_{50} values were estimated from such data, as described in "Materials and Methods."

† Substrates and cofactors were: acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) or NADP (4 mM) for the ALDH-3s, acetaldehyde (0.8 mM) and NAD (4 mM) for yALDH, and GAP and NAD (1 mM each) for GAPDH. Uninhibited catalytic rates (means of two determinations each made in duplicate) were 0.59, 2.1, 31, 53, 32, 52, 9.2, and 50 IU/mg protein for rALDH-1, rALDH-2, NAD-linked nALDH-3, NADP-linked nALDH-3, NAD-linked tALDH-3, NADP-linked tALDH-3, yALDH, and GAPDH, respectively.

‡ Substrates were *p*-nitrophenyl acetate (500 μM) for the dehydrogenases and *p*-nitrophenyl phosphate (10 mM) for alkaline phosphatase. Uninhibited catalytic rates (means of two determinations each made in duplicate) were 147, 578, 8.7, 9.5, 433, and 15 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3, yALDH, and alkaline phosphatase, respectively.

achieved at the highest concentration of API-2 tested either (Table 1). yALDH-catalyzed hydrolysis was relatively sensitive to inhibition by each agent.

rALDH-2- and yALDH-catalyzed oxidations and hydrolyses appear to be uniquely sensitive to inhibition by N^1-methoxy and N^1-ethyl analogues of chlorpropamide, viz. API-2 (Table 1) and API-1 [12], respectively.

Kinetic constants, viz. K_m, V_{max} and K_i values, defining the catalysis of oxidative reactions by rALDH-1, rALDH-2, nALDH-3, tALDH-3, yALDH, and GAPDH, and inhibi-

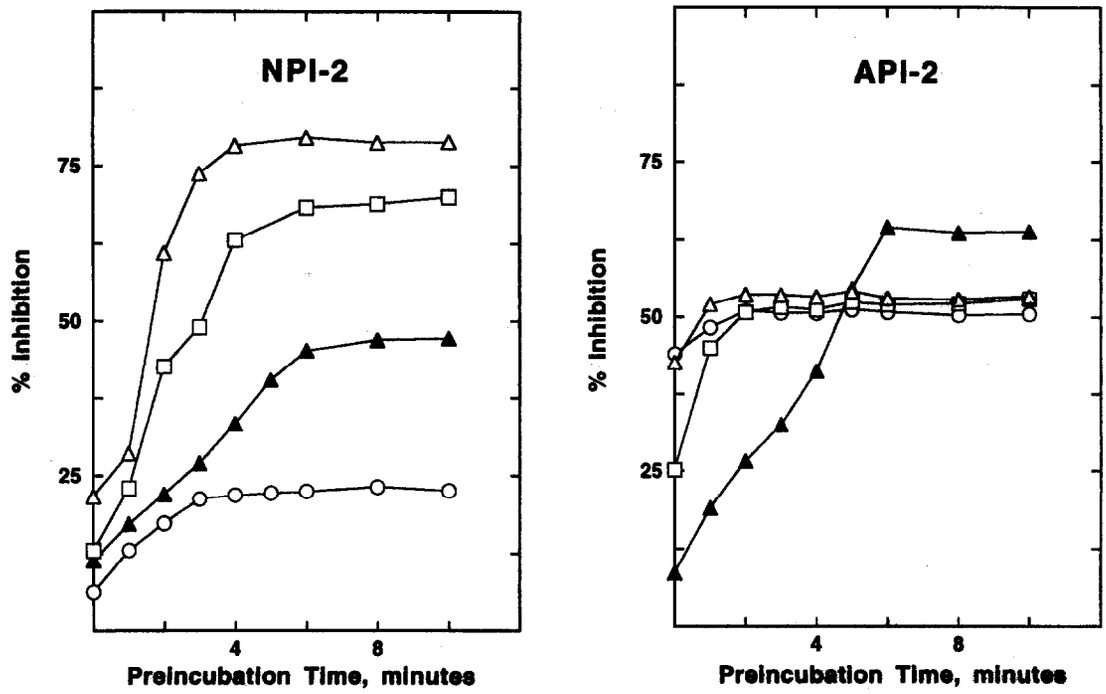

FIG. 3. Inhibition by NPI-2 and API-2 of the NAD-linked oxidative reactions catalyzed by human aldehyde dehydrogenases as a function of preincubation time. Sensitivities of rALDH-1 (○), rALDH-2 (▲), nALDH-3 (△), and tALDH-3 (□) to inhibition by NPI-2 (300, 300, 300, and 30 μM, respectively) and API-2 (7, 0.1, 4, and 0.75 μM, respectively) were quantified. Experimental conditions were as described in "Materials and Methods" and a footnote to Table 1 except that preincubation of NPI-2, API-2, or vehicle together with the complete reaction mixture except for the substrate, viz. acetaldehyde or benzaldehyde, was for the length of time indicated in the figure. Data points are means of duplicate determinations. Mean control catalytic rates at the start, as well as at the end, of the preincubation period were 0.60, 2.5, 33, and 32 IU/mg for rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively.

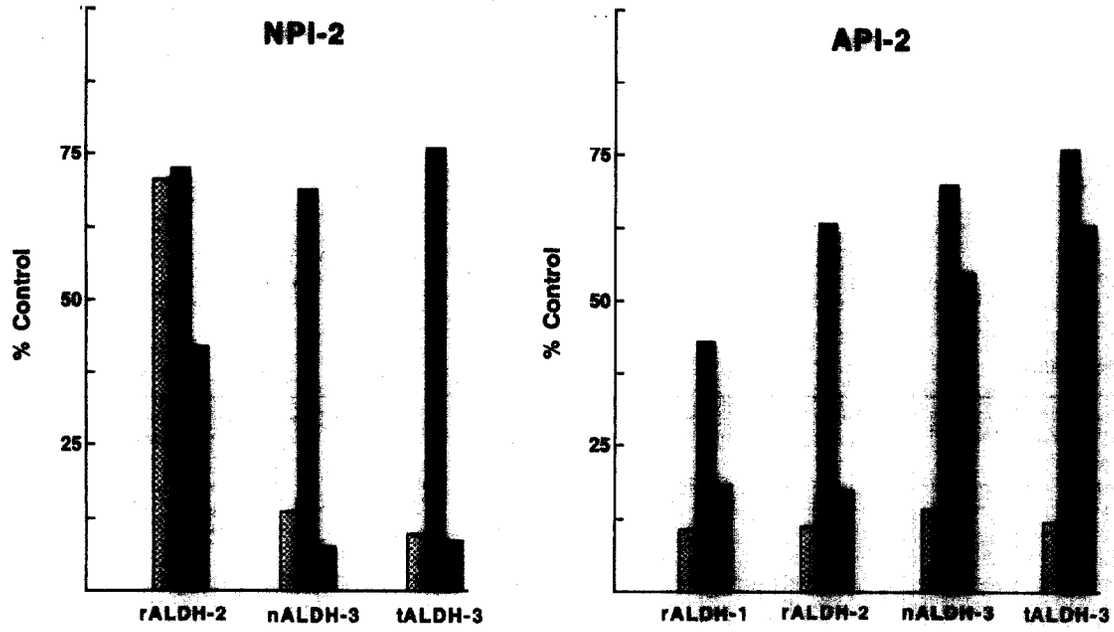

FIG. 4. NPI-2- and API-2-effected inhibition of NAD-linked aldehyde dehydrogenase-catalyzed oxidations: Reversibility studies. Purified enzyme was incubated with vehicle, NPI-2 (300 μM in the cases of rALDH-2 and nALDH-3; 30 μM in the case of tALDH-3), or API-2 (50, 1, 25, and 10 μM in the cases of rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively) for 5 min, and initial catalytic rates were quantified before and after passage through a PD-10 (Sephadex G-25) column as described in "Materials and Methods." Substrates were acetaldehyde (4 and 2 mM; rALDH-1 and rALDH-2, respectively) or benzaldehyde (4 mM; nALDH-3 and tALDH-3). NAD concentrations were 4 (rALDH-1 and rALDH-2) and 1 (nALDH-3 and tALDH-3) mM. Values are means of duplicate determinations made on each of two samples. Mean control (no inhibitor; before passage through the column) rates were 0.60 (rALDH-1), 2.4 (rALDH-2), 36 (nALDH-3), and 32 (tALDH-3) IU/mg. Key: (▩) no inhibitor; after passage through the column; (▨) and (▩) inhibitor; before and after the passage through the column, respectively.

tion thereof, as well as the type of inhibition, viz. competitive or noncompetitive, are given in Table 2. A representative of the plots from which these values were obtained is shown in Fig. 5. The K_m and V_{max} values obtained in the present investigation are in good agreement with those reported previously [2, 4, 9, 11, 12, 23, 25, 27].

As judged by K_i values determined with respect to the substrate, i.e. benzaldehyde, as well as those determined with respect to a cofactor, i.e. NAD or NADP, tALDH-3-catalyzed oxidation was inhibited to a greater extent by NPI-2 than was that catalyzed by nALDH-3 (Table 2). In the case of API-2, the rank order of enzyme sensitivity to the inhibitor was rALDH-2 > tALDH-3 > nALDH-3 > rALDH-1.

Inhibition of nALDH-3- and tALDH-3-catalyzed oxidation by NPI-2 was noncompetitive with respect to the substrate, i.e. benzaldehyde (Table 2 and data not shown). In contrast, inhibition of these enzymes, as well as of rALDH-2, by API-2 was competitive with respect to the substrate, i.e. benzaldehyde or acetaldehyde (Table 2, Fig. 5, and data not shown). Inhibition by NPI-2 and API-2 of human aldehyde dehydrogenase-catalyzed oxidations was always noncompetitive with respect to NAD (Table 2 and data not shown). In contrast, inhibition by NPI-2 and API-2 of ALDH-3-catalyzed oxidation was competitive with respect to NADP (Table 2 and data not shown).

K_i values defining the inhibition of yALDH- and rALDH-2-catalyzed hydrolysis by NPI-2 and API-2 are given in Table 3. Inhibition of yALDH- and rALDH-2-catalyzed hydrolysis by these agents was of the mixed type (data not shown). Similar observations were made in the cases of NPI-1, NPI-3, and API-1 [12].

Addition of NPI-2 (100 μM) or API-2 (50 μM) to the drug-exposure medium prior to exposure to mafosfamide markedly increased the sensitivity of tumor cells that express large amounts of ALDH-3, viz. MCF-7/0/CAT, to the oxazaphosphorine (Fig. 6 and Table 4). As expected, identical treatment of tumor cells that express very small amounts of ALDH-3, viz. MCF-7/0, did not effect a statistically significant ($P > 0.1$; paired t-tests) increase in their sensitivity to mafosfamide. Also as expected because ALDH-3 does not catalyze the detoxification of phosphoramide mustard, the ultimate cytotoxic metabolite of mafosfamide [28], addition of NPI-2 or API-2 to the drug-exposure medium prior to exposure to this agent essentially did not increase the sensitivity of MCF-7/0/CAT cells to it (Table 4).

DISCUSSION

NPI-2 and API-2 each showed some potential as useful inhibitors of tALDH-3, and, therefore, therapeutic potential when combined with an oxazaphosphorine in the treatment of certain cancers, since they were relatively potent and specific inhibitors of this enzyme. They join gossypol in that regard [11]. Attractive is that tALDH-3 is

TABLE 2. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenase-catalyzed oxidations: K_i values*

Enzyme	Substrate and cofactor		K_m† (μM)	V_{max}† (IU/mg)	K_i (μM)			
	Variable (mM)	Fixed (mM)			NPI-2		API-2	
rALDH-1	Acetaldehyde (0.1–1.6)	NAD (4)	434	0.66	ND‡		6.0	(N)§
	NAD (0.05–1)	Acetaldehyde (4)	33	0.63	ND		2.1	(N)
rALDH-2	Acetaldehyde (0.16–2)	NAD (4)	3.6‖	2.4	ND		0.03	(C)
	NAD (0.125–4)	Acetaldehyde (2)	329	2.8	ND		0.08	(N)
nALDH-3	Benzaldehyde (0.1–2)	NAD (1)	417	30	254	(N)	1.7	(C)
	NAD (0.05–1)	Benzaldehyde (4)	47	39	120	(N)	2.1	(N)
	Benzaldehyde (0.2–4)	NADP (4)	446	60	281	(N)	0.02	(C)
	NADP (1–4)	Benzaldehyde (4)	720	67	16	(C)	0.17	(C)
tALDH-3	Benzaldehyde (0.1–2)	NAD (1)	356	31	11	(N)	0.14	(C)
	NAD (0.075–1)	Benzaldehyde (4)	34	31	12	(N)	0.42	(N)
	Benzaldehyde (0.2–4)	NADP (4)	430	59	41	(N)	0.01	(C)
	NADP (0.5–4)	Benzaldehyde (4)	780	64	2.3	(C)	0.04	(C)
yALDH	Acetaldehyde (0.025–0.3)	NAD (4)	32	9.2	10	(N)	0.15	(N)
	NAD (0.5–4)	Acetaldehyde (0.8)	877	8.5	3.6	(C)	0.02	(C)
GAPDH	GAP (0.05–1)	NAD (1)	159	64	ND		31	(N)

* Enzymes were preincubated with vehicle or various concentrations of the putative inhibitor for 5 min, substrate was added, and initial catalytic rates were quantified as described in "Materials and Methods." A representative of the plots of the primary data from which the kinetic constants were obtained is given in Fig. 5.

† N = 2 except in the case of rALDH-1, rALDH-2, and GAPDH where N = 1.

‡ ND = not determined.

§ N: noncompetitive; C: competitive.

‖ Unlikely to be accurate because it is difficult to ascertain K_m values that are less than about 10 μM from the very flat Lineweaver–Burk plots that we generated. Thus, the K_m value was determined to be <0.1 μM when a more appropriate experimental design and method of analysis, viz. integrated Michaelis analysis of a single enzyme-progress curve, was used [23].

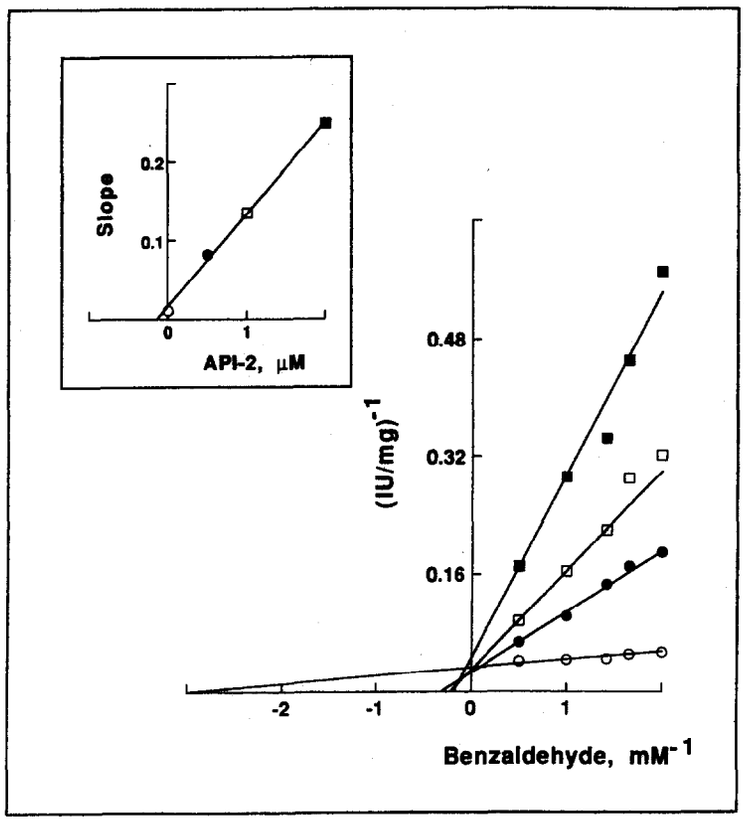

FIG. 5. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by API-2: Lineweaver–Burk plot. tALDH-3 was incubated with 0 (○), 0.5 (●), 1.0 (□), or 2.0 (■) μM API-2 for 5 min, various concentrations of the substrate, benzaldehyde, were added, and initial catalytic rates were quantified as described in "Materials and Methods." The NAD concentration was 1 mM. Data points are means of triplicate determinations. Inset: Slopes generated by the double-reciprocal (Lineweaver–Burk) plots were plotted as a function of API-2 concentrations for the purpose of determining the K_i value. K_m, V_{max}, and K_i values obtained in this experiment were 335 μM, 30 IU/mg, and 0.14 μM, respectively.

TABLE 3. Inhibition by chlorpropamide analogues of yeast and human recombinant class 2 aldehyde dehydrogenase-catalyzed hydrolyses*

Enzyme	*p*-Nitrophenyl acetate (μM)	K_m (μM)	V_{max} (IU/mg)	K_i (μM) NPI-2	K_i (μM) API-2
rALDH-2	50–400	30	584	ND†	11
yALDH	40–800	31	318	45	0.28

* Enzymes were preincubated with vehicle or various concentrations of the putative inhibitor for 5 min, substrate (*p*-nitrophenyl acetate) was added, initial catalytic rates were quantified, and K_i values were determined as described in "Materials and Methods."

† ND = not determined; K_i value was not determined because inhibition was less than 20% at the highest concentration of inhibitor tested (Table 1).

differentially sensitive to inhibition effected by NPI-2 and that this inhibition is apparently irreversible. Differentially sensitive to inhibition by API-2 are tALDH-3 and rALDH-2, with the latter being the most sensitive. Thus, at first glance, although clearly of potential value as an alcohol deterrent given its greater specificity for rALDH-2, API-2 would not appear to have any future as a clinically useful inhibitor of tALDH-3 since it would inhibit ALDH-2 to an even greater extent. In fact, this is not the case because ALDH-2 is an enzyme that humans can apparently do without since 30–50% of Orientals lack a functional ALDH-2 and do not suffer any recognized ill-effects as a consequence thereof except for those following the ingestion of alcohol [29].

Whether these agents will inhibit tALDH-3 *in vivo* at doses that do not cause untoward effects remains to be tested. However, experiments with a limited number of animals showed that API-2, 1 mmol/kg, i.p., did inhibit enzyme-catalyzed oxidation of acetaldehyde in rats, as judged by the markedly elevated plasma levels of acetaldehyde that were observed when the animals were treated with this agent prior to being given ethanol [15]. On the other hand, as judged by the same criteria, NPI-2, 1 mmol/kg, i.p., failed to inhibit enzyme-catalyzed oxidation of acetaldehyde in rats [16]. Offered as the likely explanation was that NPI-2 was hydrolyzed prematurely by plasma esterases, thereby prematurely giving rise to the short-lived cytotoxic metabolite, i.e. before reaching the liver. However, the findings reported herein are consistent with another explanation. The aldehyde dehydrogenases that are thought to catalyze the bulk of acetaldehyde oxidation *in vivo*, viz. ALDH-2 and, to a lesser extent, ALDH-1, are simply not very sensitive to the inhibitory action of NPI-2. In contrast, the ALDH-3s, especially tALDH-3, are. Thus, the possibility that tolerated doses of NPI-2 will inhibit tALDH-3 *in vivo* remains viable.

NPI-2 is an ester analogue of N^1-hydroxy-substituted chlorpropamide that, putatively, is without enzyme inhibitory activity *per se*, but that gives rise to nitroxyl upon ester hydrolysis catalyzed by the aldehyde dehydrogenases, which then irreversibly inhibits them. Inhibition of aldehyde dehydrogenase-catalyzed oxidations by NPI-2 was indeed

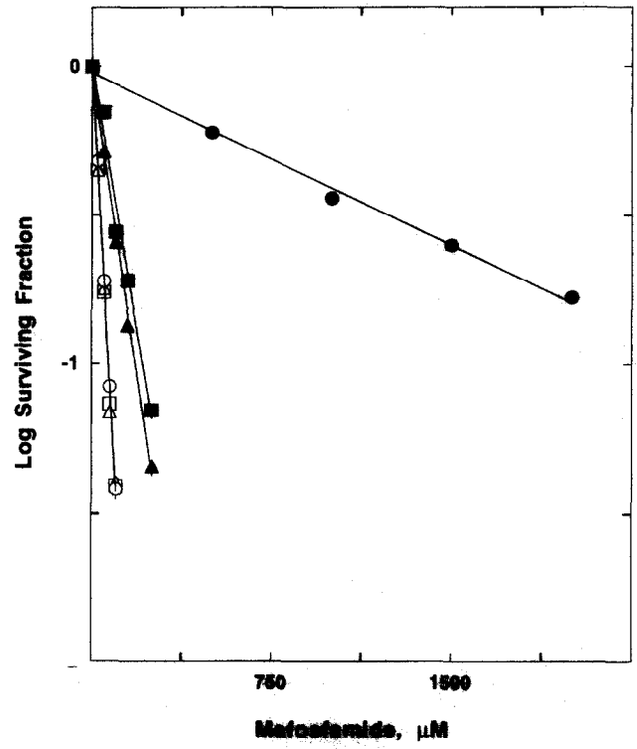

FIG. 6. Sensitivities of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide in the presence and absence of NPI-2 or API-2. Exponentially growing MCF-7/0 cells were cultured in the presence of vehicle (○, △, □; MCF-7/0) or 30 μM catechol (●, ▲, ■; MCF-7/0/CAT) for 5 days after which time they were each harvested and incubated with vehicle (○, ●), 100 μM NPI-2 (△, ▲), or 50 μM API-2 (□, ■) for 5 min at 37°. Mafosfamide was added and incubation was continued for an additional 30 min. The cells were then harvested and grown in drug-free growth medium for 15 days. The colony-forming assay described in "Materials and Methods" was used to determine surviving fractions. Data points are means of triplicate determinations. Cellular levels of aldehyde dehydrogenase activities (cofactor and substrate were 4 mM each of NADP and benzaldehyde, respectively) were 1.9 and 648 mIU/10^7 cells in 105,000 *g* supernatant fractions obtained from Lubrol-treated whole homogenates of MCF-7/0 and MCF-7/0/CAT cells, respectively.

found to be apparently irreversible (Fig. 4). However, as judged by the relative rates at which yALDH, rALDH-1, rALDH-2, nALDH-3, and tALDH-3 catalyze the hydrolysis of *p*-nitrophenyl acetate (see footnote to Table 1), yALDH, rALDH-1, and rALDH-2 would be expected to catalyze the bioactivation of NPI-2 at a much faster rate. These enzymes are also much more sensitive to Piloty's acid than are nALDH-3 and tALDH-3 [12]. Piloty's acid spontaneously gives rise to HNO; the inhibitory action of Piloty's acid is thought to be effected by HNO, rather than by the parent compound [30]. Thus, the expectation was that yALDH-, rALDH-1-, and rALDH-2-catalyzed oxidation would be much more sensitive to inhibition by NPI-2 than would be that catalyzed by nALDH-3 and tALDH-3. As before when two structural analogues of NPI-2 were tested [12], this expectation was not realized. Why is not

TABLE 4. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide and phosphoramide mustard in the presence and absence of NPI-2 or API-2*

Cell line	ALDH-3 (mIU/10^7 cells)	Inhibitor	LC_{90} (μM) Mafosfamide	LC_{90} (μM) Phosphoramide mustard
MCF-7/0	2	None	65	800
		NPI-2	60	ND†
		API-2	60	ND
MCF-7/0/CAT	665	None	>2000	1350
		NPI-2	175	1300
		API-2	200	1400

* Human breast adenocarcinoma MCF-7/0 cells were cultured in the presence of vehicle (MCF-7/0) or 30 μM catechol (MCF-7/0/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. Then the cells (1×10^5 cells/mL) were incubated with NPI-2 (100 μM), API-2 (50 μM), or vehicle for 5 min at 37° after which time various concentrations of mafosfamide, phosphoramide mustard, or vehicle were added, and incubation was continued as before for 30 min at 37°. The colony-forming assay described in "Materials and Methods" was used to determine surviving fractions. The LC_{90} values were obtained from plots of log surviving fractions versus concentrations of drug (Fig. 6). Values are means of LC_{90}s obtained in two experiments. Cellular levels of ALDH-3 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde; 4 mM each of cofactor and substrate) in 105,000 *g* supernatant fractions obtained from Lubrol-treated whole homogenates of tumor cells were determined as described in "Materials and Methods."

† ND = not determined.

known. A detailed speculative discussion in that regard has been presented in a previous publication [12].

API-2 is a N^1-methoxy analogue of chlorpropamide that, putatively, is also without enzyme inhibitory activity *per se*, but that gives rise to *n*-propylisocyanate, a potent, presumably reversible, inhibitor of aldehyde dehydrogenases, without the necessity of any enzyme participation [15]. In harmony with this notion is our finding that inhibition of aldehyde dehydrogenase-catalyzed oxidation was partially reversible. Unexplained is the relatively greater preincubation time that was required to achieve maximum inhibition of rALDH-2 by API-2 as compared with that required to achieve maximum inhibition by API-2 of the other aldehyde dehydrogenases (Fig. 3).

Interestingly, but not totally unexpected, tALDH-3, as compared with nALDH-3, was significantly more sensitive to inhibition by each of the two chlorpropamide analogues. This observation further substantiates the notion that tALDH-3, putatively tumor-specific, is a subtle varient of nALDH-3 [8].

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