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Inhibition of mouse cytosolic aldehyde dehydrogenase by 4-(diethylamino)benzaldehyde

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Increased NAD-dependent aldehyde dehydrogenase (EC 1.2.1.3) activity (ALDH) has been demonstrated to be a mechanism of antitumor drug resistance to the alkylating agent cyclophosphamide (CP) *in vivo* and to the activated analog 4-hydroperoxycyclophosphamide (4-HC) *in vitro* [1, 2]. CP is a pro-drug requiring cytochrome P-450 hydroxylation for activation. Prior to release of the active alkylating metabolite phosphoramide mustard, CP passes through an aldehyde intermediate, aldophosphamide [3]. ALDH catalyzes the oxidation of aldophosphamide to the inactive metabolite carboxyphosphamide [4, 5]. Tumor cell resistance to CP is thus conferred by an increased expression of ALDH activity. This mechanism has been best characterized in the murine L1210 leukemia cell lines which are sensitive (L1210/0) and resistant (L1210/CPA) to CP.

One attempt to reverse the drug-resistant phenotype has been the use of ALDH-specific inhibitors. Disulfiram (DS), diethyldithiocarbamate (DDTC), cyanamide, and 1-aminocyclopropanol have been shown to inhibit ALDH activity in rat [6] and human [7] liver. DS and cyanamide appear to act as irreversible inhibitors *in vivo* and are currently used in alcohol aversion therapy as they elicit elevated blood acetaldehyde levels following ethanol ingestion [8, 9]. These inhibitors have been used experimentally to revert L1210/CPA cells to a CP-sensitive phenotype in soft agar survival and alkaline elution assays [10] and to identify murine and human hematopoietic progenitor cells whose mechanism of resistance to oxazaphosphorines may be due to high ALDH levels [11, 12].

It has been proposed in the case of the human liver E1 (cytosolic) isozyme [13] that DS, as it attacks Cysteine-302 [14], is initially cleaved into two DDTC molecules, one of which forms a mixed disulfide adduct on the enzyme. This bound DDTC is then displaced by another enzymic sulfhydryl, resulting in an inactivated enzyme containing an intramolecular disulfide bond. DS also has been shown to inhibit dopamine β-hydroxylase [15] and may alter other proteins or enzymes by acting as a general sulfhydryl oxidizing agent [16]. The *in vivo* and *in vitro* cellular toxicities due to the nonspecific interactions of DS and DDTC have limited their use as ALDH inhibitors. Though cyanamide does not elicit many of the same toxicities as DS, its use is dependent on the presence of catalase activity for formation of the active inhibitory species [17].

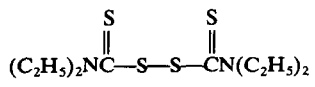
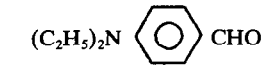
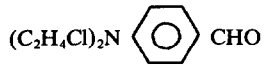
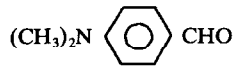
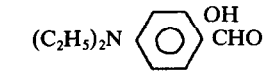
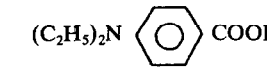
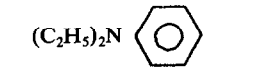
Therefore, an ALDH specific, competitive type (reversible) inhibitor not requiring enzymatic activation would be preferred for *in vivo* inhibition of ALDH. Since DS binds specifically to a single reactive cysteine residue (Cys-302), we decided to investigate the role of the diethylamino groups in directing the binding of ALDH inhibitors to the enzyme active site. In an attempt to mimic disulfiram and substrate binding, several non-thiol-containing inhibitors incorporating dialkylamino and carbonyl substituent groups were screened as potential inhibitors by direct spectrofluorometric enzyme assay. 4-(Diethylamino) benzaldehyde (DEAB) was identified as a potent, partial competitive inhibitor of the cytosolic (but not mitochondrial) isozyme of mouse ALDH. In addition, DEAB was shown to confer sensitivity to L1210/CPA cells using *in vitro* cell survival assays.

Materials and methods

NAD, aprotinin, leupeptin, and disulfiram were purchased from the Sigma Chemical Co. (St. Louis, MO). 4-(Diethylamino)benzaldehyde (DEAB), 4-(dimethylamino)benzaldehyde (DMAB), 4-(diethylamino)benzoic acid (DEABA), 4-(diethylamino)salicylaldehyde (DEAS), and *N*,*N*-diethylaniline (DEA) were obtained from the Aldrich Chemical Co. (Milwaukee, WI), and propionaldehyde and benzaldehyde were from the Kodak Chemical Co. (Rochester, NY). 4-Bis(2-chloroethyl)aminobenzaldehyde (BAM) was synthesized as previously described [18].

Female BD2F1 mice, weighing 20–25 g, were used for i.p. passage of L1210/CPA cells. Ascites tumor cells (5×10^8 cells/mouse) were collected on day 8 post-injection and cooled in phosphate-buffered saline (PBS) at 4°. Cells were washed three times in cold PBS and resuspended in freeze/thaw buffer (0.1 M sodium phosphate, pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 200 units/ml aprotinin, and 50 μg/ml leupeptin) with 200 μl/5×10^7 cells. The cells were subjected to three cycles of freezing/thawing in a solid CO_2/methanol bath and centrifuged for 10 min at 5000 *g*. The lipid layer was discarded, and the supernatant fraction was removed and centrifuged for 45 min at 48,000 *g*. The supernatant fraction was collected (protein concentration approximately 10 mg/ml) and used for ALDH assays. The WEHI-3 cell line, a mouse myelomonocytic leukemia line (gift from James Ihle, Frederick Cancer Research Center, Frederick, MD) was grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The 1C1C7 cell line, a mouse hepatoma line (gift of Mary DeLong, Department of Pharmacology, Johns Hopkins School of Medicine, Baltimore, MD), was maintained in

Table 1. Structure–activity relationship for the inhibition of the cytosolic ALDH isozyme

		% Inhibition
	Disulfiram (DS)	95 ± 3.1
	4-(Diethylamino)benzaldehyde (DEAB)	90 ± 3.5
	4-(Bischloroethylamino)benzaldehyde (BAM)	77 ± 2.4
	4-(Dimethylamino)benzaldehyde (DMAB)	33 ± 1.6
	4-(Diethylamino)salicylaldehyde (DEAS)	16 ± 1.8
	4-(Diethylamino)benzoic acid (DEABA)	0
	*N,N*-Diethylaniline (DEA)	0

Inhibition studies were performed on cytosolic ALDH from L1210/CPA cells. Assays contained 5 μM inhibitor, 100 μM NAD, and 1 mM propionaldehyde. None of the compounds tested were substrates for ALDH. ALDH activity in the absence of inhibitor was 28.9 nmol NADH/min/mg protein. Each percent inhibition value is the mean ± SEM from at least four measurements from two enzyme preparations.

culture in Minimum Essential Medium (α-MEM). Mouse liver extract was prepared by homogenization in 4 vol. of 0.25 M sucrose. Cytosolic preparations of cell and tissue extracts were done as above for L1210 cells. Mitochondrial fractions were prepared by centrifugation of cell or tissue homogenates for 10 min at 500 *g* to pellet nuclei and cell debris. The supernatant fraction was removed and centrifuged for 10 min at 7500 *g* to pellet mitochondria. The mitochondrial pellet was resuspended in 0.25% Triton X-100, incubated on ice for 30 min, and centrifuged for 30 min at 48,000 *g*. This supernatant fraction was used for the mitochondrial ALDH assay.

ALDH activity was measured in a spectrofluorometric assay at 25° by following the appearance of NADH (excitation 350, emission 450). Standard assay conditions were as follows in 1 ml final reaction volume: assay buffer (0.1 M potassium phosphate, pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol); 100 μM NAD^+; 1 mM propionaldehyde or benzaldehyde (prepared in distilled H_2O). Background activity (less than 10% of substrate activity) was established with NAD and enzyme, and the reaction was initiated by the addition of aldehyde substrate. Initial rates of reaction (1–3 min) were measured. Inhibition studies were performed by incubating enzyme, NAD, and inhibitor for 10 min at 25° prior to addition of substrate. DS and DEAB stock solutions were prepared in 100% dimethyl sulfoxide (DMSO) and diluted in water. Final reaction mixtures contained less than 0.1% DMSO. DS inhibition assays were done in assay buffer without 2-mercaptoethanol.

In vitro clonogenic cell survival studies were performed using the method of Chu and Fischer [19]. Cells were treated with 50 μM DEAB (prepared in 10% DMSO) for 5 min prior to exposure to 4-HC for 30 min at 37°, washed free of inhibitor and drug, and plated in semisolid agar.

* J. E. Russo and J. Hilton, manuscript submitted for publication.

Results and discussion

Structure–activity relationships of potential ALDH inhibitors are shown in Table 1. These studies identified DEAB as a potent inhibitor of cytosolic ALDH activity from L1210/CPA cells, comparable to DS. This inhibition profile was also obtained when the compounds were tested with cytoplasmic ALDH purified to electrophoretic homogeneity from L1210/CPA cells by affinity chromatography.* From the structure–activity studies of this class of compounds, it appears that two functional groups, a dialkylamino group and an aldehyde group, are necessary for inhibition of cytosolic ALDH. This was evident by the activity of benzaldehyde (data not presented) as an enzyme substrate and the lack of inhibition by DEA. Additionally, replacement of the aldehyde moiety in DEAB with a carboxyl group in DEABA eliminated the inhibitory effect of this compound. Also, the alkyl chain length appeared to be important since cytosolic ALDH inhibition increased from 33 to 90% upon substitution of a diethyl for a dimethyl group in DEAB versus DMAB.

Disulfiram has been reported to be selective for the cytosolic isozyme of ALDH isolated from sheep [20] and horse [21] liver, with the mitochondrial isozyme being relatively insensitive to DS action. To further characterize the inhibitory action of DEAB, isozyme specificity was determined by screening DEAB against cytoplasmic and mitochondrial fractions from two other mouse cell lines, WEHI-3 and 1C1C7 hepatoma, and mouse liver. These results (Table 2) show that DEAB and DS possess identical isozyme inhibition specificity. Both DEAB and DS were potent (>90% inhibition at 5 μM DEAB or DS) inhibitors of the cytoplasmic isozyme obtained from L1210/CPA, 1C1C7, or mouse liver but demonstrated little activity against the mitochondrial isozyme of these tissues. However, both DS and DEAB were inactive against cytosolic ALDH from WEHI-3 cells. Moreover, DEAB was a better substrate for the WEHI-3 isozyme than pro-

Table 2. Effect of DEAB on cytosolic and mitochondrial ALDH activity

Source	% Inhibition			
	Cytosolic		Mitochondrial	
	DEAB	DS	DEAB	DS
L1210/CPA	90 ± 2.2	98 ± 1.7		
1C1C7	90 ± 3.5	96 ± 3.2	28 ± 0.7	20 ± 2.6
WEHI-3	−10 ± 2.0	0	25 ± 0.8	22 ± 2.4
Mouse liver	92 ± 1.3	93 ± 3.3	25 ± 1.7	30 ± 1.3

Inhibition assays contained 5 μM DEAB or DS, 100 μM NAD, and 1 mM propionaldehyde. Control ALDH activities in the absence of inhibitor were as follows (specific activity = nmol NADH/min/mg protein): *Cytosolic ALDH:* L1210/CPA, 26.5; 1C1C7, 4.2; mouse liver, 13.2; and WEHI-3, 11.6. *Mitochondrial ALDH:* L1210/CPA, <0.1; 1C1C7, 7.3; mouse liver, 8.2; and WEHI-3, 3.6. Each percent inhibition value is the mean ± SEM from at least four measurements from two enzyme preparations.

pionaldehyde or benzaldehyde. This is of interest since our antisera raised against the cytosolic L1210/CPA ALDH isozyme gave a strong signal in Western blot analysis with ALDH from L1210/CPA, 1C1C7, and mouse liver cytosolic extracts but showed no cross-reactivity against the WEHI-3 cytosolic isozyme. Additionally, this antisera did not cross-react against any mouse mitochondrial isozyme tested.*

The kinetics of DEAB inhibition were studied with the L1210/CPA enzyme and the Michaelis inhibition constant was estimated from initial rates of reaction using the Hunter–Downs linear transformation [22] (Fig. 1). This analysis yielded a $K_i = 0.04$ μM with DEAB acting as a partial competitive inhibitor of ALDH (the reaction $EI + S \rightarrow EIS$ is hindered whereas breakdown of the *ES* or *EIS* complex to product is unaffected [23]). This suggests that DEAB may be bound at or near the enzyme active site via its diethylamino group, with the aldehyde portion of DEAB blocking the substrate binding site on the enzyme. This is consistent with the structure–activity results in which DEA was not an inhibitor (possibly since it does not contain an aldehyde group to block substrate binding), and DMAB was a less potent inhibitor (the longer alkyl chain of DEAB may allow for tighter binding). The inability of DEABA and DEAS to inhibit ALDH may be attributed to a charge effect by which the carboxyl group of DEABA and the phenolic group of DEAS are ionized and unable to bind at the active site. This charge effect is supported by the report of Hempel *et al.* [14] of a hydrophobic cleft surrounding the active site of the cytoplasmic ALDH isozyme as deduced from amino acid sequence analysis.

The above studies were performed using initial rates (1–3 min). However, when the inhibited reaction was followed over time, ALDH activity slowly recovered (>30 min) to its uninhibited rate. One possibility for this phenomenon is that DEAB is a tightly bound, slowly catalyzed substrate reaction and that, once it is oxidized to DEABA, the presence of the charged group displaces the compound from the active site. The ability for DEAB to be oxidized by ALDH is supported by the findings of the property of the variant WEHI isozyme in which DEAB can be turned over very rapidly. Characterization of the WEHI enzyme should be helpful in further studies of the reaction kinetics of DEAB. Additional information on the steric requirements for inhibition may be provided by the use of the following compounds: 4-(dipropylamino)benzaldehyde, to test for optimal alkyl chain length; 5-(diethylamino)-pentanal, to test for the necessity of the aromatic ring of DEAB in providing steric hindrance at the aldehyde binding site; and tetramethyl thiuram disulfide, to test for the role of the diethylamino group in DS inhibition.

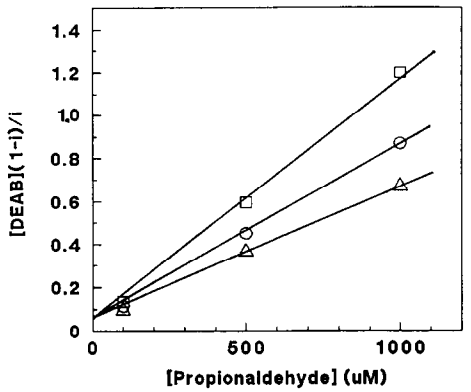


Fig. 1. DEAB inhibition of cytosolic aldehyde dehydrogenase from L1210/CPA cells. A Hunter–Downs linear transformation was used to derive the K_i of DEAB (K_i = 0.04 μM) and to analyze the method of inhibition. i (fractional inhibition) = $[1 - V_i/V_0)]$ where V_i = inhibited velocity and V_0 = uninhibited velocity. The K_i value was obtained from the y-intercept intersection, and partial competitive inhibition was indicated by a decreasing slope as inhibitor concentration was increased. Points are the mean of at least three measurements from one enzyme preparation. Key: (△) 1.0 μM DEAB, (○) 0.5 μM DEAB, and (□) 0.2 μM DEAB.

To test the ability of DEAB to inhibit ALDH intracellularly, a clonogenic cell survival assay was used to follow the sensitivity of L1210/0 and L1210/CPA cells to the alkylating agent 4-HC in the presence and absence of DEAB. These results (Fig. 2) demonstrate that DEAB also inhibited ALDH intracellularly, as seen by the sensitivity conferred on L1210/CPA cells by 4-HC when treated with 50 μM DEAB. At this dose of inhibitor, no potentiation in sensitivity to 4-HC was seen in L1210/0 cells. These results correlate with the measurement of a 200-fold greater ALDH activity (propionaldehyde as substrate) in L1210/CPA cells (specific activity = 26.8 ± 2.9 nmol NADH/min/mg protein) compared to L1210/0 (sp. act. = 0.15 ± 0.04), thus making the L1210/CPA cells "susceptible" to DEAB action. Additionally, unlike DS [10], there was no evidence of cytotoxicity to DEAB up to 50 μM.

In summary, we have identified DEAB as a potent inhibitor of mouse cytosolic ALDH (in purified form, in cell or tissue homogenates, or in whole cells) and have partially characterized the structural requirements for this novel class of non-thiol ALDH inhibitors. Preliminary results

* J. E. Russo and J. Hilton, manuscript submitted for publication.

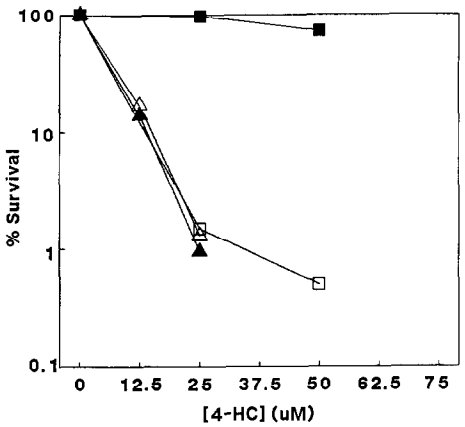


Fig. 2. Effect of DEAB on L1210/0 and L1210/CPA survival. Cells were treated with 50 μM DEAB or vehicle for 5 min prior to exposure to 4-hydroperoxycyclophosphamide (4-HC) for 30 min at 37°. The cells were then washed free of inhibitor and drug and plated in semisolid agar. Survival values are the mean of at least two survival assays. Key: (□) L1210/CPA [+DEAB], (■) L1210/CPA [−DEAB], (△) L1210/0 (+DEAB), and (▲) L1210/0 [−DEAB].

(data not presented) indicate that DEAB was also an effective ALDH inhibitor *in vivo* in mice as well as of the human cytosolic ALDH isozyme in liver, erythrocytes, and the HEPG2 hepatoma cell line.

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Pharmacology Laboratory
Johns Hopkins Oncology Center
Johns Hopkins School of Medicine
Baltimore, MD 21205, U.S.A.

JAMES E. RUSSO*
DENISE HAUQUITZ
JOHN HILTON

* Address all correspondence to: James E. Russo, Pharmacology Laboratory, Rm. 1-120, Johns Hopkins Oncology Center, 600 North Wolfe St., Baltimore, MD 21205.

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Inhibition of the oxidation of the urinary bladder carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine by pyrazole and 4-substituted pyrazoles

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N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BHBN*) is widely used as an experimental urinary bladder carcinogen [1]. The principal urinary metabolite of BHBN in the rat is BCPN, formed by oxidation of the alcoholic group of BHBN to the carboxyl group [1, 2]. BCPN itself is a urinary bladder carcinogen, and it has been suggested that BCPN is the proximate carcinogenic metabolite of BHBN [1]. To study further the biochemical mechanism of the organospecificity of BHBN in cancer induction, it was necessary to find some means of inhibiting the metabolism of BHBN to BCPN. The enzymological aspects of the conversion of BHBN to BCPN have not been studied. The principal enzymes involved in oxidation of alcohols are the NAD^+-dependent alcohol dehydrogenases and aldehyde dehydrogenases, which are relatively specific for NAD^+ as coenzyme but exhibit broad substrate specificity with respect to alcohols and aldehydes [3, 4]. One would expect BHBN to be oxidized by an alcohol dehydrogenase to form the aldehyde intermediate BFPN (Fig. 1). BFPN has not been detected as a urinary metabolite of BHBN, or as a metabolite in isolated hepatocytes, because it is very rapidly

* Abbreviations: BHBN, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine; BFPN, *N*-butyl-*N*-(3-formylpropyl)nitrosamine; BCPN, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.