Inhibition of Recombinant Human Mitochondrial and Cytosolic Aldehyde Dehydrogenases by Two Candidates for the Active Metabolites of Disulfiram[†]

Jennifer P. Lam,[‡] Dennis C. Mays,[‡] and James J. Lipsky*,[‡,§]

Clinical Pharmacology Unit, Department of Pharmacology, and Department of Internal Medicine, Mayo Medical School, Mayo Clinic/Foundation, Rochester, Minnesota 55905

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ABSTRACT: We expressed recombinant human cytosolic (ALDH1, high K_m) and mitochondrial aldehyde dehydrogenase (ALDH2, low K_m) in *Escherichia coli* and purified the enzymes to homogeneity to examine the nature of inhibition of human ALDH by disulfiram, its confirmed metabolite *S*-methyl *N*,*N*-diethylthiocarbamate (MeDTC) sulfoxide, and its proposed metabolite MeDTC sulfone. Disulfiram, MeDTC sulfoxide, and MeDTC sulfone, respectively, were potent inhibitors with IC_{50} values of 0.15 ± 0.02 μM, 0.27 ± 0.04 μM, and 0.12 ± 0.02 μM for ALDH1, and 1.45 ± 0.40 μM, 1.16 ± 0.56, and 0.40 ± 0.10 μM for ALDH2. Extensive dialysis did not restore the activity of the inactivated enzyme, indicating irreversible inhibition. Both the esterase and dehydrogenase activities of ALDH2 were inhibited to the same extent by MeDTC sulfone and sulfoxide, suggesting that both catalytic sites are closely linked. The time course of inhibition of ALDH appeared to be first-order for both MeDTC sulfone and MeDTC sulfoxide. Kitz and Wilson plots of the half-life of inactivation versus 1/[inhibitor] indicated that the reactions between ALDH and inhibitors were bimolecular. The pseudobimolecular rate constants (k_3/K_I) for the ALDH−inhibitor reactions were 1×10^5, 1×10^4, 3×10^3, and 1×10^3 s^{-1} M^{-1} ALDH1−sulfone, ALDH1−sulfoxide, ALDH2−sulfone, and ALDH2−sulfoxide, respectively. ALDH2 was not significantly protected from inactivation from either MeDTC sulfoxide or MeDTC sulfone by NAD alone, but high concentrations of NAD and acetaldehyde completely prevented inhibition. Since disulfiram is rapidly metabolized *in vivo*, it is believed that disulfiram is too short-lived to inhibit ALDH directly. The results of our study indicate that MeDTC sulfoxide and sulfone are potent inhibitors of human ALDH and are reasonable candidates for the proximal inhibitors of ALDH following disulfiram administration.

Disulfiram, used in aversion therapy for alcoholism, leads to inhibition of hepatic aldehyde dehydrogenase (ALDH)[1] activity. Ingestion of ethanol by an individual on disulfiram therapy causes acetaldehyde to accumulate which produces several undesirable effects including nausea, vomiting, flushing, and tachycardia (*1*, *2*). *In vivo*, disulfiram is very rapidly reduced to *N*,*N*-diethyldithiocarbamate (*3*), which is metabolized to *S*-methyl *N*,*N*-diethyldithiocarbamate (*4*) and *S*-methyl *N*,*N*-diethylthiocarbamate (MeDTC) (*5*). These metabolites are very weak or noninhibitors of ALDH *in vitro*, suggesting further metabolism is required to inhibit the enzyme (*6*, *7*). Additional evidence for inhibition of ALDH by an active metabolite of disulfiram is the delay in inhibition of ALDH *in vivo*; in the rat, maximal inactivation of ALDH occurs 8 h after administration of disulfiram (*8*). In previous investigations, MeDTC sulfoxide and MeDTC sulfone (confirmed and proposed metabolites of disulfiram, respectively; Figure 1) have been shown to be inhibitors of nonhuman ALDH (*9*, *10*, *11*).

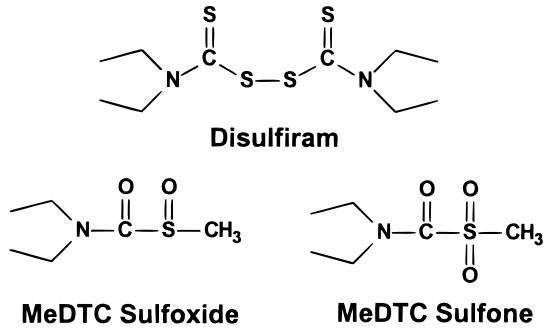

FIGURE 1: ALDH inhibitors used in this study.

There have been no reports on the effects of MeDTC sulfone and sulfoxide on human ALDH, nor of disulfiram on purified recombinant human ALDH. In this study, we examined the effects of these inhibitors on the cytosolic (ALDH1, high K_m) and mitochondrial (ALDH2, low K_m) forms of recombinant human ALDH.

EXPERIMENTAL PROCEDURES

Materials. The recombinant human ALDH2 (mitochondrial) and ALDH1 (cytosolic) cDNAs in pT7-7 (*12*) were generous gifts from Dr. Henry Weiner (Department of

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* Address correspondence to this author at the Clinical Pharmacology Unit, Mayo Foundation, Guggenheim 6, Rochester, MN 55905. Email: lipsky.james@mayo.edu. Fax: (507) 284−4521.

[‡] Clinical Pharmacology Unit, Department of Pharmacology.

[§] Department of Internal Medicine.

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; ALDH1, cytosolic ALDH; ALDH2, mitochondrial ALDH; DDC, *N*,*N*-diethyldithiocarbamate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; IC_{50}, concentration causing 50% inhibition; MeDDC, *S*-methyl *N*,*N*-diethyldithiocarbamate; MeDTC, *S*-methyl *N*,*N*-diethylthiocarbamate; mAU, milliabsorbance unit(s); *p*-HAP, *p*-hydroxyacetophenone; PMSF, phenylmethanesulfonyl fluoride.

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Biochemistry, Purdue University, West Lafayette, IN). *S*-Methyl *N*,*N*-diethylthiocarbamate sulfoxide (MeDTC sulfoxide) and *S*-methyl *N*,*N*-diethylthiocarbamate sulfone (MeDTC sulfone) were prepared as previously described (*10*, *11*). BCA Protein Assay kit and Slide-A-Lyzer cassettes were obtained from Pierce (Rockford, IL).

Expression and Purification of Recombinant Human ALDH. The full-length cDNAs encoding the human ALDH1 and ALDH2 were expressed in *E. coli* and purified using a procedure modified from the method published by Zheng et al. (*12*). Cells carrying the plasmid containing the specific cDNA were grown in 2 L of Terrific Broth supplemented with 100 μg/mL ampicillin. When the culture reached an OD_{600} between 0.4 and 0.6, IPTG was added to a final concentration of 4 mM, and the culture was then allowed to grow overnight at room temperature (27 °C) with shaking. The bacterial cells were harvested by centrifugation at 7000*g* for 15 min. The pellet was resuspended in sonication buffer (20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.025% β-mercaptoethanol, and 10 μM PMSF) and lysed by sonication using a Branson sonifier at output 6 for 10 × 20-s bursts. The lysate was then centrifuged at 18000*g* for 15 min to remove cell debris, and 125 mg of protamine sulfate per liter of culture was added to the supernatant and mixed well. After incubating on ice for 10 min, the protamine sulfate precipitate was removed by centrifuging at 18000*g* for 15 min, and the clarified lysate was dialyzed for 5−6 h against 2 changes of 1 L each of DEAE column equilibration buffer (20 mM sodium phosphate, pH 6.1, and 0.025% β-mercaptoethanol). The sample was centrifuged after dialysis and then loaded onto a DEAE Sepharose Fast-Flow column using an automatic FPLC system. The protein was eluted using a 0−200 mM NaCl gradient. Fractions containing ALDH activity were subsequently loaded onto a *p*-HAP affinity column. The *p*-HAP affinity resin was synthesized according to Ghenbot and Weiner (*13*). The purified enzyme was dialyzed extensively (4 changes of 1 L of buffer each over 2 days) against *p*-HAP column equilibration buffer (20 mM sodium phosphate, pH 7.4, 0.1 mM DTT, 1 mM EDTA, and 50 mM sodium chloride) and stored in 200−500 μL aliquots at −80 °C. The final protein concentrations were from 0.5 to 8.6 mg/mL as determined by BCA Kinetic Protein Assay using bovine serum albumin as the standard. The specific activities of ALDH1 and ALDH2 were 150−350 and 600−1200 nmol of NADH min^{-1} (mg of protein)$^{-1}$, respectively.

ALDH Activity Assays. The microtiter-based assay for ALDH was performed for the dehydrogenase activity as described (*14*) with the following modifications: purified recombinant human ALDH was used instead of detergent-solubilized rat mitochondria, rotenone and pyrazole were omitted, 200-μL triplicate aliquots were added to the microwells, and the acetaldehyde and NAD were added together in 25 μL of buffer G (0.05 M sodium pyrophosphate, pH 8.8). Protein concentrations in the reaction mixture were 30.5−44 μg/mL (0.14−0.2 μM as the tetramer) for ALDH1, and 4.4−13.3 μg/mL (0.02−0.06 μM as the tetramer) for ALDH2.

Inhibitor Studies. Purified ALDH1 or ALDH2 at concentrations that produced control velocities between 10 and 25 mAU/min was preincubated in buffer G (pH 8.8) with inhibitor (0.1−8 μM) or methanol (vehicle) for 15 min. The inhibitor was added in 7 μL of methanol in a total reaction volume of 700 μL. Triplicate aliquots of 200 μL were transferred into wells of a microtiter plate. The reaction was initiated by adding NAD (500 μM final concentration) and acetaldehyde (160 μM for ALDH2 and 1 mM for ALDH1) in 25 μL of buffer G. Production of NADH from the oxidation of acetaldehyde was monitored by measuring the change in absorbance at 340 nm for 3 min. The esterase activity was measured by adding 100 μM *p*-nitrophenyl acetate as substrate and monitoring the rate of *p*-nitrophenol formation at 405 nm for 3 min. The relatively high rate of spontaneous *p*-nitrophenyl acetate hydrolysis (5−6 mAU/min) at pH 8.8 necessitated the use of twice the standard amount of protein in these reactions to obtain velocities of the esterase reaction (30−40 mAU/min) that were sufficiently above background. In experiments with two consecutive measurements of the same plate, a concentration−inhibition curve for each inhibitor was generated first using *p*-nitrophenyl acetate as substrate and measuring esterase activity, and then NAD and acetaldehyde were added and dehydrogenase activity was measured. In experiments in which three consecutive measurements were taken, the order was reversed, and the NAD/acetaldehyde solution was added first and monitored for dehydrogenase activity. The esterase reaction was then measured after *p*-nitrophenyl acetate was added to the same wells. The plate was monitored again for dehydrogenase activity after the supplemental NAD and acetaldehyde were added to the wells. The time that elapsed between the readings was approximately 6 min.

To examine the reversibility of ALDH inhibition, ALDH1 was preincubated with 0.4 μM disulfiram, 2 μM MeDTC sulfoxide, 1 μM MeDTC sulfone, or methanol (vehicle control) for 10 min before dialysis. Similarly, ALDH2 was preincubated with 2 μM disulfiram, 2 μM MeDTC sulfoxide, 1 μM MeDTC sulfone, or methanol. Half of each incubation mixture (1.5 mL) was transferred to a Slide-A-Lyzer dialysis cassette and placed in a beaker with 150 mL of buffer G. The remaining half was allowed to incubate at room temperature. After 15 min of dialysis, approximately 0.75 mL of the dialysate was removed for an intermediate measurement, and the remaining 0.75 mL in the cassette was dialyzed in 150 mL of fresh buffer G for another 15 min. The dehydrogenase activity of each sample was determined before dialysis to verify that the inhibition was greater than 70%. Activity was also measured in the undialyzed reaction mixtures incubated for the same length of time (30 min) as the dialyzed samples.

In the NAD protection assay, ALDH2 was incubated with 500 μM NAD or buffer G (vehicle) for 2 min before inhibitor (2 μM MeDTC sulfone or 4 μM MeDTC sulfoxide) or methanol (vehicle) was added and further incubated for 10 min. At the end of the total incubation time, buffer G (vehicle) or NAD was added to the respective reaction mixes, and triplicate 200 μL aliquots were immediately transferred into a microtiter plate. The activity was read after 25 μL of acetaldehyde (160 μM) was added to the wells. The final reaction volume was 700 μL before transferring aliquots into the microwells. The experiments to evaluate protection by NAD in combination with acetaldehyde were similar to those performed with the NAD alone; the enzyme was preincubated with both 1 mM NAD and 2 mM acetaldehyde for 2 min before contact with inhibitor, and the activity was measured after a 10-min incubation with the inhibitor.

Table 1: Inactivation of Human Recombinant ALDH[a]

inhibitor	IC_{50} (μM) cytosolic (ALDH1) *n*		IC_{50} (μM) mitochondrial (ALDH2) *n*	
disulfiram	4	0.15 ± 0.10	5	1.45 ± 0.40
MeDTC sulfoxide	4	0.27 ± 0.04	4	1.16 ± 0.56
MeDTC sulfone	3	0.12 ± 0.02	4	0.40 ± 0.10

[a] Experimental methods are described in Figure 2. Values are mean ± SD.

The time course of ALDH inactivation was determined by varying the length of time between the addition of inhibitor and the addition of substrate and cofactor to the enzyme. For each time point, 693 μL of diluted enzyme was added to 7 μL of inhibitor, and 200 μL of the mixture was transferred to each of three wells of the microplate. At the appropriate time, 25 μL of acetaldehyde and NAD solution was added to the wells immediately before measuring the activities. For the zero time point, inhibitor was added together with substrate, and the activity was measured immediately.

RESULTS

MeDTC sulfone, MeDTC sulfoxide, and the parent compound disulfiram were all potent inhibitors of recombinant human ALDH1 (cytosolic) and ALDH2 (mitochondrial) with IC_{50} values from 0.12 to 1.45 μM (Table 1). Typical concentration−inhibition curves are shown in Figure 2. ALDH2 was 3−10 times less sensitive than ALDH1 to all the inhibitors.

In addition to catalyzing the oxidation of acetaldehyde to acetic acid, ALDH hydrolyzes a variety of esters via an NAD-independent reaction (*15*, *16*). Therefore, we studied the effect of MeDTC sulfoxide and sulfone upon the ability of each recombinant human enzyme to catalyze the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol. This esterase reaction is shown in Figure 3. The esterase and dehydrogenase activities were measured consecutively in the same microwell plate. In some incubations, the esterase activity was measured first, prior to the addition of NAD (Figure 4A,B). NAD was added together with acetaldehyde immediately before the dehydrogenase reaction was measured. In assays where the dehydrogenase activity was measured prior to the esterase activity (Figure 4C), the control velocities of the uninhibited esterase reaction were about 6 times faster (210−250 mAU/min) than the control velocities measured without NAD and acetaldehyde (37−40 mAU/min). In all cases in Figure 4, plots of the percent of inhibition of the dehydrogenase and esterase activities versus concentration of inhibitor were superimposable, indicating that these reactions were inhibited to the same extent when measured as separate events.

Since the inhibition of ALDH in solubilized rat liver mitochondria by MeDTC sulfoxide and sulfone has been shown to be irreversible (*10*, *11*), we examined the effect of dialysis upon inhibited recombinant human enzymes by these inhibitors as well as disulfiram. Compared to undialyzed inhibited enzyme, the activity of ALDH was not appreciably restored after 30 min of dialysis at room temperature against the equivalent of 20 000 volumes of buffer G at pH 8.8 (Table 2).

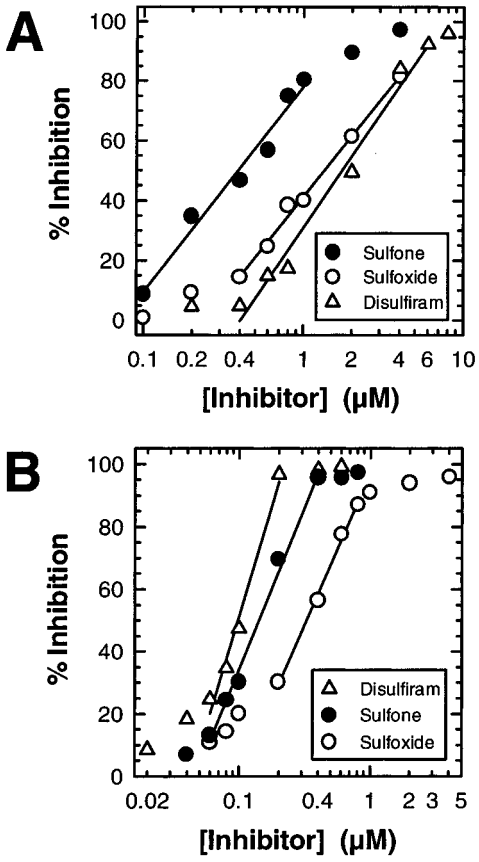

FIGURE 2: Representative concentration−inhibition curves of recombinant human ALDH. Purified ALDH2 (A) or ALDH1 (B) was preincubated with inhibitor (0.1−8 μM) or methanol (vehicle control) for 15 min. The reaction was initiated by adding NAD and acetaldehyde, and production of NADH from the oxidation of acetaldehyde was monitored by measuring the change in absorbance at 340 nm over 3 min.

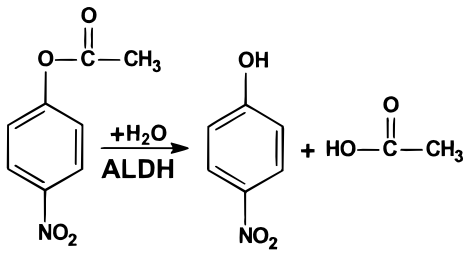

FIGURE 3: Hydrolysis of *p*-nitrophenyl acetate by ALDH.

Plots of ALDH activity versus preincubation time with various concentrations of inhibitor are shown in Figure 5. The end-point velocity observed for each concentration of inhibitor was subtracted from the measured velocity at each of the time points, and the time zero point was set to 100% activity. The resulting semi-log plots of the percent of remaining enzyme activity versus time were linear, as the inactivation of the enzyme displayed apparent first-order kinetics for both MeDTC sulfone and MeDTC sulfoxide. Increasing the concentration of inhibitors resulted in more rapid loss of activity. Both MeDTC sulfone and MeDTC sulfoxide inhibited ALDH1 more rapidly than ALDH2, with the sulfone being the more potent and faster-reacting inhibitor. The data for the time dependence of inactivation were analyzed according to Kitz and Wilson (*17*) based on

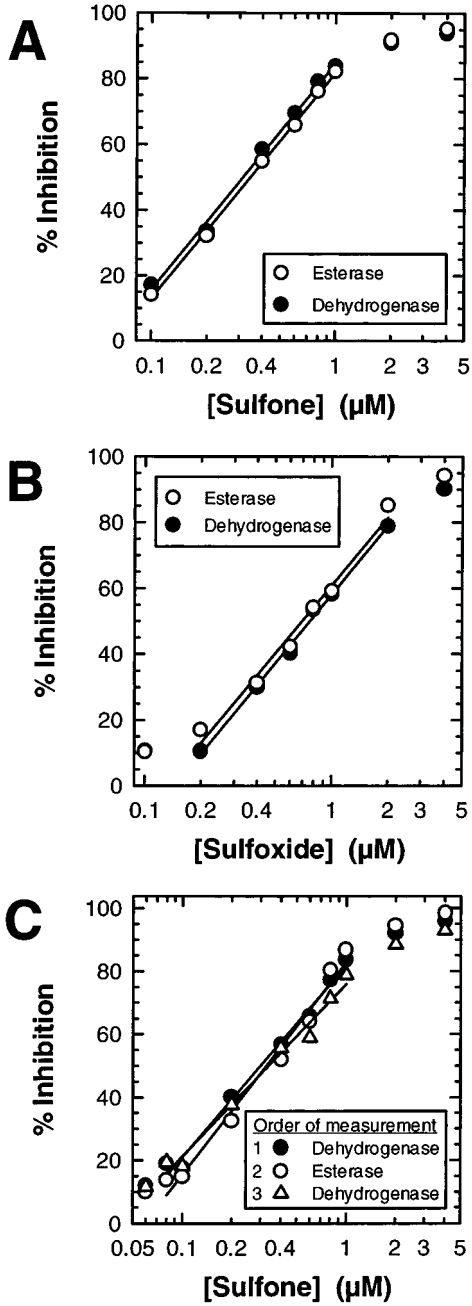

FIGURE 4: Inhibition of dehydrogenase and esterase activities of ALDH2 by MeDTC sulfoxide and sulfone. The incubation conditions for Figure 2 were as described under Experimental Procedures. Esterase activity was measured by adding *p*-nitrophenyl acetate (100 μM final concentration) as substrate, and monitoring for 3 min the increase in absorbance at 405 nm for the production of *p*-nitrophenol. After preincubation of ALDH for 15 min with MeDTC sulfone (A) or MeDTC sulfoxide (B), esterase activity was measured. Immediately afterward acetaldehyde and NAD were added together to the same wells, and the plate was monitored at 340 nm for dehydrogenase activity. In (C), the order was reversed so that the dehydrogenase activity was measured first, followed by the measurement of esterase activity. The plate was read again for dehydrogenase activity after addition of supplemental acetaldehyde.

the assumption that the enzyme is a reagent in the following reaction:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E{-}I,\ K_I = k_2/k_1$$

where the enzyme, E, and the inhibitor, I, form a reversible complex (E•I) which proceeds to an irreversibly inactivated enzyme (E−I). The first-order rate constant, k_{obs}, from the semilog plots of enzyme activity versus time in Figure 5 is related to k_3 and K_I by eq 1 (*18*).

$$k_{obs} = k_3[I]/(K_I + [I]) \quad (1)$$

This equation predicts saturation kinetics; thereby, plots of $1/k_{obs}$ (or $t_{1/2}$) versus 1/[I] should be linear. The *y*-axis intercept is equal to the $t_{1/2}$ of inactivation, and the *x*-intercept is equal to $-1/K_I$. The Kitz−Wilson plots of the kinetics data are shown in Figure 6. These plots are linear but intercept the axes very near the origin, indicating that saturation kinetics were not observed under our experimental conditions. Measurements of the rate of inactivation of ALDH at higher concentrations of inhibitor were difficult due to the rapidity of the reaction. A rapid-mixing method may overcome this obstacle. Since saturation kinetics were not observed, plots of k_{obs} versus [I] were made (Figure 7). The slope of the plot is equal to the pseudobimolecular rate constant k_3/K_I, which has the units of s^{-1} M^{-1} and is a measure of the relative reactivity of the inhibitor−enzyme combinations (*18*). The pseudobimolecular rate constants are summarized in Table 3.

In our previous work with solubilized mitochondria from rat liver, NAD, in a concentration-dependent manner, protected ALDH from inhibition by MeDTC sulfone and sulfoxide (*10, 11*). Therefore, we determined if similar protection would occur with the recombinant human enzymes. Preincubation of the inhibitors with ALDH2 in the presence of 500 μM NAD alone resulted in only partial protection of human ALDH2 against inactivation by the sulfone (78% inhibition unprotected versus 61% inhibition protected) and no protection against the sulfoxide (70% inhibition unprotected versus 69% inhibition protected). These data are an average of three experiments. However, when human ALDH2 was incubated with 2 mM acetaldehyde and 1 mM NAD for 2 min before adding inhibitor and activity was measured after 2 min of incubation with inhibitor, the enzyme was almost completely protected from inhibition by 2 μM MeDTC sulfone (79% inhibition unprotected versus 0.5% inhibition protected) and 4 μM MeDTC sulfoxide (76% inhibition unprotected versus 5% inhibition protected).

Table 2: Effect of Dialysis on Inactivation of ALDH[a]

	ALDH activity (% control)			
	cytosolic (ALDH1)		mitochondrial (ALDH2)	
inhibitor	undialyzed	dialyzed	undialyzed	dialyzed
disulfiram	9	8	1	6
MeDTC sulfoxide	10	17	10	13
MeDTC sulfone	5	11	16	19

[a] ALDH1 or ALDH2 was preincubated for 10 min with disulfiram, MeDTC sulfoxide, MeDTC sulfone, or vehicle, and the incubation mixtures were dialyzed as described under Experimental Procedures. The final activity of the inhibited enzyme was measured after 30 min of dialysis and expressed as percent of control activity of uninhibited enzyme. Values are the average of two determinations.

DISCUSSION

In this study, MeDTC sulfone and MeDTC sulfoxide (candidates for the active metabolites of disulfiram), as well as disulfiram, were potent inhibitors of both human recom-

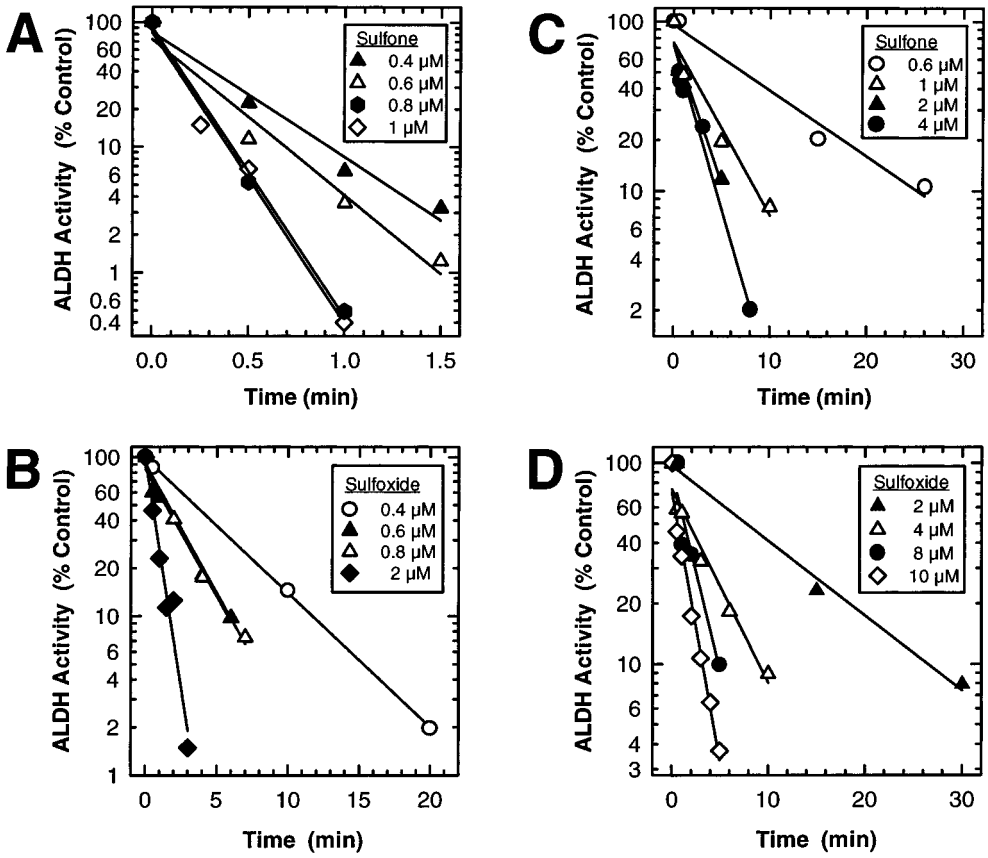

FIGURE 5: Time course of inactivation of recombinant human ALDH by MeDTC sulfoxide and sulfone. ALDH1 (0.14−0.23 μM) (A and B) or ALDH2 (0.03−0.14 μM) (C and D), was incubated with MeDTC sulfone (A and C) or MeDTC sulfoxide (B and D), and activity was measured at different times as described under Experimental Procedures.

binant ALDH1 and ALDH2 (Table 1). These data for the human ALDH2 are comparable with results obtained in our laboratory with ALDH in detergent-solubilized rat liver mitochondria (*10*, *11*). In previous studies by other investigators with ALDH purified from human and nonhuman liver, the mitochondrial isoform was very resistant to inhibition by disulfiram with values for the IC_{50} much greater than 50 μM (*19*, *20*, *21*). This is in contrast to our results with human recombinant ALDH in the present investigation, in which the IC_{50} for disulfiram was less than 2 μM for ALDH2. This discrepancy in findings could be due to difference in technique and the source of human ALDH preparations. However, our results clearly show that ALDH2 is susceptible to inactivation by low concentrations of disulfiram. In comparison to ALDH2, ALDH1 was 10 times more sensitive to disulfiram inhibition, and 3−4 times more sensitive to MeDTC sulfone and sulfoxide.

The current theory is that the mitochondrial isoform is responsible for metabolism of most of the acetaldehyde produced after ethanol ingestion (*21*−*24*). This view is supported by the observation that many individuals who are deficient in mitochondrial ALDH activity exhibit very high peak acetaldehyde levels (up to 125 μM after 0.4 g/kg ethanol) in the blood after ingestion of ethanol (*25*). However, involvement of other ALDH isoenzymes in acetaldehyde metabolism is suggested because about 20% of the individuals who are deficient in mitochondrial ALDH activity still exhibit low peak blood levels of acetaldehyde (<20 μM after 0.4 g/kg ethanol) (*26*). Our findings that disulfiram, MeDTC sulfoxide, and MeDTC sulfone are potent inhibitors of both the cytosolic and mitochondrial isoforms of ALDH

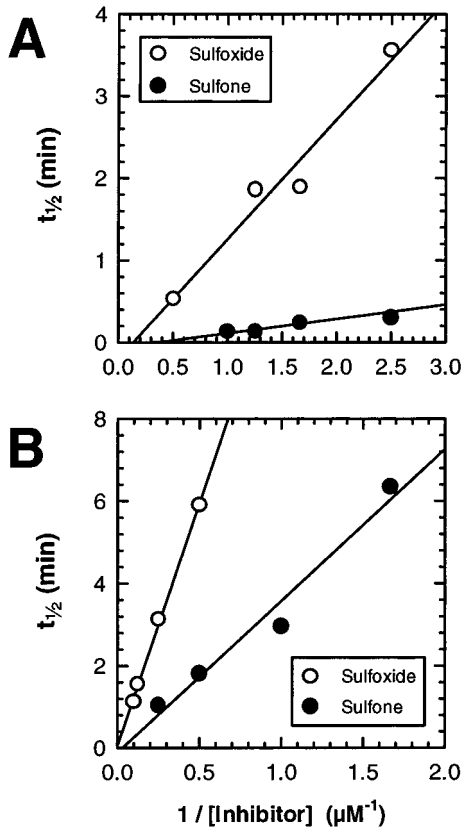

FIGURE 6: Kitz and Wilson plots of the inactivation of ALDH. The half-life of inactivation, $t_{1/2}$, of ALDH2 (A) and of ALDH1 (B) versus 1/[I] for the data in Figure 5 is shown.

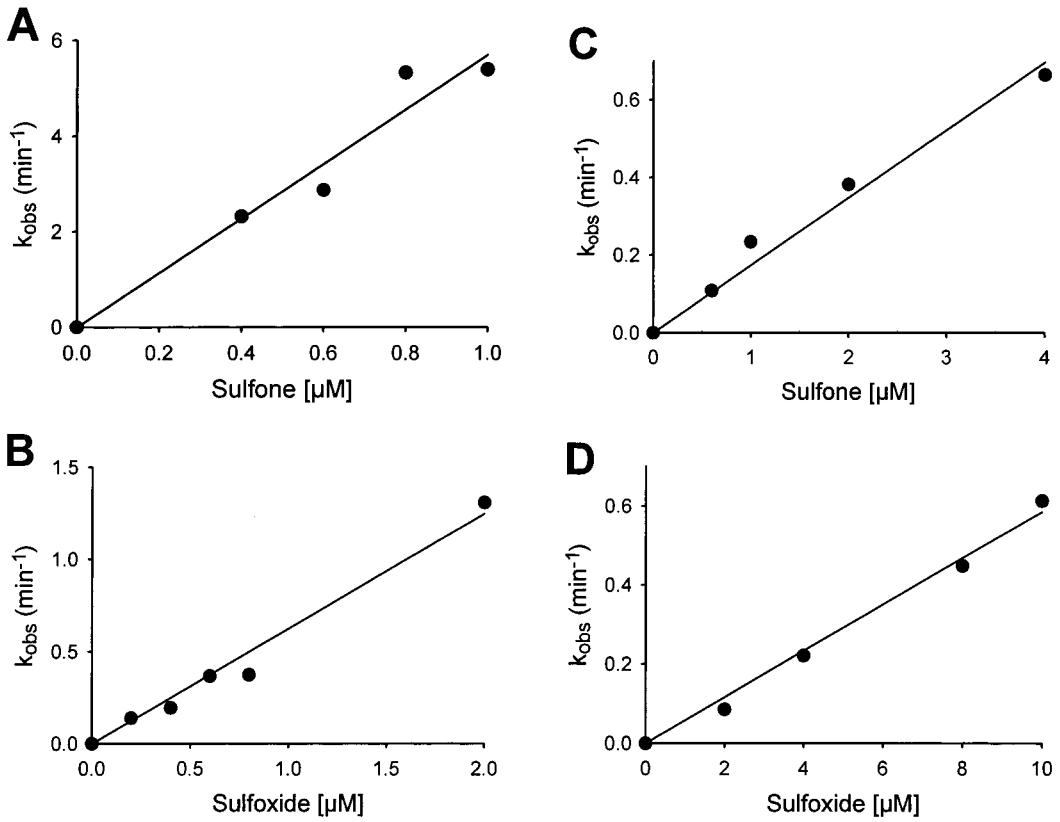

FIGURE 7: k_{obs} versus [I] for inactivation of ALDH. k_{obs} (=0.693/$t_{1/2}$) versus [I] are plotted for the time-dependent inactivation of ALDH1 (A and B) and ALDH2 (C and D) for the corresponding data in Figure 5.

Table 3: Pseudobimolecular Rate Constants for Inactivation of ALDH[a]

inhibitor	k_3/K_I (s^{-1} M^{-1})	
	cytosolic (ALDH1)	mitochondrial (ALDH2)
MeDTC sulfoxide	1.0×10^4	9.8×10^2
MeDTC sulfone	9.5×10^4	2.9×10^3

[a] Values are the slopes of the plots of k_{obs} versus [I] in Figure 7.

leave open the possibility that inhibition of cytosolic ALDH *in vivo* contributes to the action of disulfiram.

MeDTC sulfone was more potent than MeDTC sulfoxide at inhibiting both ALDH isoforms studied. Although MeDTC sulfoxide is a known metabolite of disulfiram in the rat, the formation of the sulfone has not been detected in this species (*27*). Neither MeDTC sulfoxide nor MeDTC sulfone have been detected in humans given disulfiram. As noted above, the general consensus is that the mitochondrial isoform of ALDH, located in the mitochondrial matrix (*28*), is the physiologically important enzyme in the metabolism of acetaldehyde. Therefore, an inhibitor formed in the endoplasmic reticulum or cytosol must enter the mitochondria in order to inhibit ALDH2 directly. Our previous experiments showed that MeDTC sulfoxide and sulfone were approximately equipotent inhibitors of ALDH in detergent-solubilized mitochondria from rat liver. In contrast, MeDTC sulfoxide was 10 times more potent than MeDTC sulfone in intact mitochondria, indicating that the sulfoxide is better able to enter the mitochondria than the sulfone (*11*). Due to the greater chemical reactivity of the sulfone as compared to the sulfoxide, normal cell constituents may react with the sulfone and thereby limit its entry into the mitochondria (*11, 29, 30*). Our experiments do not preclude the possibility that MeDTC sulfone is formed inside the mitochondria near the target enzyme. The results of our current study are consistent with MeDTC sulfoxide and MeDTC sulfone being active metabolites of disulfiram in humans. Additionally, MeDTC sulfoxide has been proposed as a therapeutic agent in the treatment of alcoholism (*31*). Our results support that it is effective in inhibiting human isoforms of ALDH.

ALDH catalyzes both an NAD-dependent dehydrogenase reaction in the metabolism of aldehydes as well as the NAD-independent hydrolysis of esters (*15, 16, 32*). The effect of MeDTC sulfone and sulfoxide upon the two different activities of ALDH may provide some insight into the mechanism of these reactions. The esterase activity is compatible with a proposed mechanism for the dehydrogenation of aldehydes in which an enzyme−thioester covalent intermediate is formed and subsequently hydrolyzed to release acetic acid and the active enzyme (*15, 16, 33*). The concentration−inactivation curves with MeDTC sulfoxide and sulfone were essentially the same for both the esterase and dehydrogenase activities of ALDH (Figure 4). One interpretation of this result is both enzyme activities are inactivated by a similar mechanism, which is consistent with the proposal of one active site for both functions (*34*), as opposed to an alternative proposal of two separate sites for these activities (*35, 36*). It was also noted that the esterase activity of ALDH2 was enhanced about 6-fold in the presence of 500 μM NAD (data not shown). Takahashi and Weiner (*37*) have also observed stimulation of esterase activity by NAD in equine hepatic mitochondrial ALDH.

The inactivation of ALDH by MeDTC sulfone and sulfoxide is irreversible in rat liver mitochondrial ALDH (*10, 11*). The results in this study show the same phenomenon, i.e., irreversible inactivation, with the human isoforms of ALDH (Table 2). This finding is in agreement with the

observation that disulfiram administration irreversibly inactivates ALDH in experimental animals, as recovery of ALDH activity is blocked by cycloheximide (*38*). This finding indicates that recovery of ALDH activity requires synthesis of new protein.

The nature of the inhibition of ALDH by the sulfone and sulfoxide was further delineated by our experiments which examined the time course of enzyme inactivation. Both MeDTC sulfone and sulfoxide inactivated recombinant human ALDH in a time-dependent manner (Figure 5), which is consistent with previous results obtained with rat liver mitochondrial ALDH (*10, 11*). At concentrations of inhibitor in excess of enzyme, apparent first-order kinetics were observed for both inhibitors with each isoform of the enzyme. Kitz and Wilson plots demonstrated a linear relationship between the half-lives of inactivation and the reciprocal of inhibitor concentration (Figure 6). These plots intercepted the *y*-axis near zero, indicating that the reactions of the inhibitors with ALDH were not saturable (i.e., $[I] << K_I$) under our experimental conditions. The nonsaturability of the inactivation reaction is consistent with a simple bimolecular reaction (*39*), whose rate constant (k_3/K_I) can be obtained from the slope of the plot of k_{obs} versus [I] (*18*) (Figure 7). The linearity of these plots confirms our interpretation of the data (*40*). The pseudobimolecular rate constants for the ALDH−inhibitor combinations (Table 3) indicate that the reactivity of ALDH1−sulfone > ALDH1−sulfoxide > ALDH2−sulfone > ALDH2−sulfoxide (a 100-fold difference in relative reactivity).

In our previous studies using detergent-solubilized rat liver mitochondria, ALDH was completely protected from inactivation by MeDTC sulfone and sulfoxide by NAD, but not by acetaldehyde alone (*11*). Similar substrate/cofactor protection from inhibition by MeDTC sulfoxide was observed with purified recombinant rat liver mitochondrial ALDH (Lipsky, unpublished results). In the "protection" experiments conducted with recombinant human mitochondrial enzyme, there was protection only by preincubation of the enzyme with high concentrations of both cofactor and substrate.

In conclusion, we have demonstrated that disulfiram, its known metabolite MeDTC sulfoxide, and its proposed metabolite MeDTC sulfone inhibit human mitochondrial and cytosolic ALDH. MeDTC sulfoxide and sulfone are time-dependent and irreversible inactivators of human ALDH. Since it is believed that disulfiram is too short-lived *in vivo* to inhibit ALDH directly, our results indicate that MeDTC sulfoxide and sulfone are reasonable candidates for the proximal inhibitors of ALDH subsequent to disulfiram administration.

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