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S-METHYL *N*,*N*-DIETHYLTHIOCARBAMATE SULFONE, A POTENTIAL METABOLITE OF DISULFIRAM AND POTENT INHIBITOR OF LOW K_m MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

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Abstract—Disulfiram inhibits hepatic aldehyde dehydrogenase (ALDH) causing an accumulation of acetaldehyde after ethanol ingestion. It is thought that disulfiram is too short-lived *in vivo* to directly inhibit ALDH, but instead is biotransformed to reactive metabolites that inhibit the enzyme. *S*-Methyl *N*,*N*-diethylthiocarbamate (MeDTC) sulfoxide has been identified in the blood of animals given disulfiram and is a potent inhibitor of ALDH (Hart and Faiman, *Biochem Pharmacol* **46**: 2285–2290, 1993). MeDTC sulfone is a logical metabolite of MeDTC sulfoxide. Therefore, we investigated the effects of MeDTC sulfone on the activity of rat hepatic low K_m mitochondrial ALDH, the major enzyme in the metabolism of acetaldehyde. MeDTC sulfone inhibited the low K_m mitochondrial ALDH *in vitro* with an IC_{50} of 0.42 ± 0.04 μM (mean ± SD, N = 5) compared with disulfiram, which had an IC_{50} of 7.5 ± 1.2 μM under the same conditions. The inhibition of ALDH by MeDTC sulfone was time dependent. The decline in ALDH activity followed pseudo first-order kinetics with an apparent half-life of 2.1 min at 0.6 μM MeDTC sulfone. Inhibition of ALDH by MeDTC sulfone was apparently irreversible; dilution of the inhibited enzyme did not restore lost activity. The substrate (acetaldehyde, 80 μM) and cofactor (NAD, 0.5 mM) together completely protected ALDH from inhibition by MeDTC sulfone; substrate alone partially protected the enzyme. Addition of either thiol-containing compound glutathione (GSH) or dithiothreitol (DTT) to MeDTC sulfone before incubation with the enzyme increased the IC_{50} of MeDTC sulfone by 7- to 14-fold. Neither GSH nor DTT could restore lost ALDH activity after exposure of the enzyme to MeDTC sulfone. Results of these studies indicate that MeDTC sulfone, a potential metabolite of disulfiram, is a potent, irreversible inhibitor of low K_m mitochondrial ALDH.

Key words: disulfiram; disulfiram metabolism; disulfiram-ethanol reaction; aldehyde dehydrogenase; *S*-methyl diethylthiocarbamate sulfone; irreversible enzyme inhibition

Disulfiram, bis(diethylthiocarbamoyl) disulfide, used in aversion therapy for alcoholism, inhibits hepatic ALDH‖ causing an accumulation of acetaldehyde after ethanol ingestion [1, 2]. The resulting increased tissue levels of acetaldehyde cause a spectrum of undesirable side-effects, including flushing, nausea, vomiting, and tachycardia, which is referred to as the disulfiram-ethanol reaction. Disulfiram presumably inhibits aldehyde dehydrogenase *in vitro* by formation of mixed disulfide bonds with essential sulfhydryl groups in the protein [3]. However, this mechanism is unlikely to be important *in vivo* because disulfiram is reduced very rapidly by glutathione reductase in erythrocytes and by sulfhydryl groups in proteins to DDC [4] (Fig. 1). DDC is a very weak inhibitor of ALDH *in vitro* [5], suggesting that further metabolism is required to inhibit the enzyme. The current thinking is that DDC is methylated to form MeDDC, which is metabolically activated by oxidative desulfuration [6] and sulfoxidation forming MeDTC and MeDTC sulfoxide [7–9]. Alternatively, oxidation of the thioether sulfur of MeDDC may occur [10]. Further metabolic oxidation of MeDTC sulfoxide to MeDTC sulfone is logical (Fig. 1). There have been numerous reports of *S*-oxidation of sulfoxides, including dimethyl sulfoxide [11, 12], sulfinpyrazone [13], and sulindac [14, 15]. Furthermore, the thiocarbamate herbicide metabolite EPTC sulfoxide, which is chemically similar to MeDTC sulfoxide, is converted to its corresponding sulfone in mouse liver microsomes [16].

While this work was in progress, Nagendra *et al.* [17] described in a short report inhibition of ALDH

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‖ Abbreviations: ALDH, aldehyde dehydrogenase; DDC, *N*,*N*-diethyldithiocarbamate; DCI, desorption chemical ionization; DTT, dithiothreitol; EPTC, *S*-ethyl dipropylthiocarbamate; GSH, glutathione; ITMS, ion trap mass spectrometry; MeDDC, *S*-methyl *N*,*N*-diethyldithiocarbamate; and MeDTC, *S*-methyl *N*,*N*-diethylthiocarbamate.

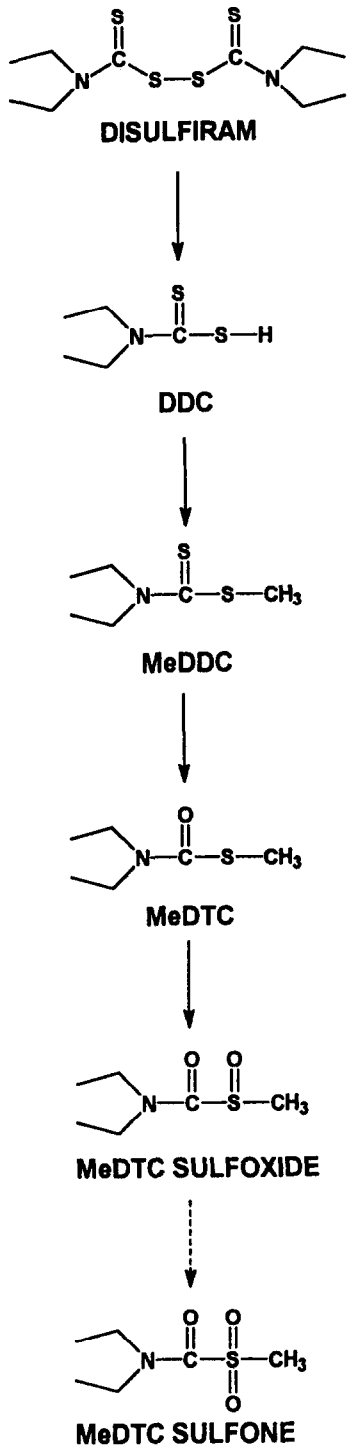


Fig. 1. Proposed metabolic pathway for the formation of MeDTC sulfone. The solid and dashed arrows indicate confirmed and proposed reactions, respectively.

by MeDTC sulfone *in vivo* and *in vitro*. These authors were unable to detect the metabolism of MeDTC sulfoxide to MeDTC sulfone and concluded that it was unlikely that MeDTC sulfone was formed [17]. However, the high reactivity of MeDTC sulfone may preclude its detection in biological matrices. Similar difficulties have been encountered in the detection of EPTC sulfone because of its high reactivity with protein [18]. Glutathione conjugates of disulfiram metabolites have been detected in the bile of rats treated with disulfiram [19]. These glutathione adducts were identified as *S*-methyl *N*,*N*-diethylthiocarbamoylates, which the authors proposed were derived from MeDTC sulfoxide and/or MeDTC sulfone, further supporting the idea that MeDTC sulfone is a possible metabolite of MeDTC sulfoxide [19]. We report here, in detail, the rapid, irreversible inhibition of rat hepatic mitochondrial low K_m ALDH by MeDTC sulfone.

MATERIALS AND METHODS

Materials. Disulfiram (tetraethylthiuram disulfide; $C_{10}H_{20}N_2S_4$; mol. wt 296.54), recrystallized twice in ethanol before use (m.p. 71–72°), rotenone, and pyrazole were purchased from Sigma (St. Louis, MO). NAD (grade 1 free acid, 100%) was from Boehringer Mannheim (Mannheim, Germany). Acetaldehyde, DTT, GSH, sodium deoxycholate, and sodium pyrophosphate were obtained from Aldrich (Milwaukee, WI). Acetonitrile, methanol (Burdick & Jackson UV grade) and ethanol (redistilled before use) were purchased from Baxter (McGaw Park, IL).

S-Methyl N,N-diethylthiocarbamate sulfone. MeDTC was synthesized as described by Faiman and coworkers [5]. Initially, MeDTC sulfone was synthesized by oxidation of MeDTC with $NaIO_4$ and purified by preparative reverse phase HPLC. Because the yield of MeDTC sulfone by this method was <5% (the major product was MeDTC sulfoxide), subsequent material was synthesized by combining MeDTC with 2.2 equivalents of *m*-chloroperoxybenzoic acid at 0° in chloroform. The product was purified by silica gel chromatography (230–400 mesh) using 20% ethyl acetate in hexane. MeDTC sulfone was a clear liquid at room temperature (yield 65%) that was ≥99% pure by HPLC. Elemental analysis: found C 40.21%, H 7.31%, and N 7.81%; calculated for $C_6H_{13}O_3SN$: C 40.14%, H 7.15% and N 7.73%. MeDTC sulfone was characterized by ^{1}H- and ^{13}C-NMR spectroscopy: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 3.75 (q, 2H, J = 7.1 Hz), 3.42 (q, 2H, J = 7.2 Hz), 3.13 (s, 3H), 1.30 (t, 3H, J = 6.9 Hz), 1.23 (t, 3H, J = 7.2 Hz; ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 160.6, 42.6, 41.5, 39.5, 13.7, and 12.1. The mass spectrum of MeDTC sulfone was obtained with a quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA). The sample was ionized by DCI from a desorption probe built in-house. Gas pressure in the ion trap chamber was 5×10^{-5} torr using methane as the reagent gas and helium as the buffer gas. Instrument control, data acquisition, and data analysis were performed using Ion Catcher Mass Spectrometry software (Dr. N. Yates, University of Florida, Gainesville, FL). The mass spectrum of MeDTC sulfone is shown in Fig. 2. The ^{1}H-NMR and mass spectroscopy data for MeDTC sulfone are similar to those reported previously [17].

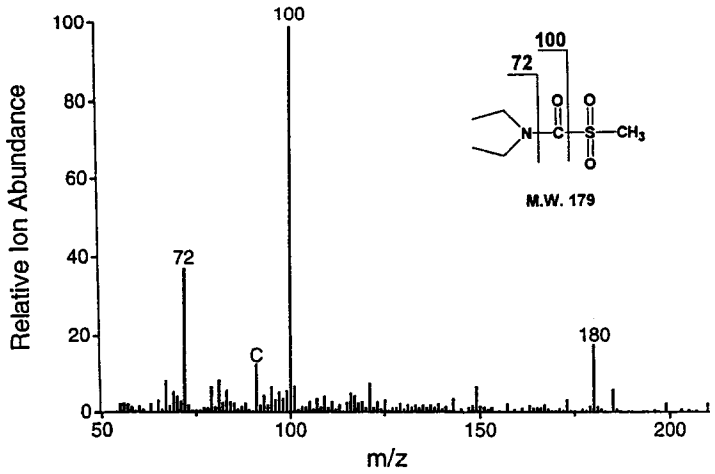


Fig. 2. Mass spectrum of MeDTC sulfone by DCI-ITMS. The molecular ion (MH^+) at m/z 180 and the fragment ions at m/z 100 and 72 are diagnostic for MeDTC sulfone. "C" indicates a contaminant ion in the ionization source of the mass spectrometer.

Low K_m *aldehyde dehydrogenase assay.* Untreated, male Sprague–Dawley rats weighing 250–300 g (Harlen Sprague Dawley, Indianapolis, IN), after equilibration for 1 week in a controlled environment with free access to lab chow and water, were fasted overnight, anesthetized with CO_2, and decapitated. The livers were removed and mitochondria were isolated according to the method of Pedersen *et al.* [20]. The protein concentrations were determined by the BCA protein assay [21] adapted for a microplate reader using standards of bovine serum albumin (Pierce, Rockford, IL). The isolated mitochondria (10–20 mg protein/mL) were stored at −80° in 0.5- to 1-mL portions. The mitochondria were thawed immediately before use; the unused portion was discarded because substantial loss of ALDH activity was noted in mitochondria that were thawed and refrozen. The activity of mitochondrial low K_m aldehyde dehydrogenase (aldehyde: NAD(P) oxidoreductase, EC 1.2.1.3) was assayed at 22° by following the formation of NADH spectrophotometrically at 340 nm [22] using a procedure modified for a microplate reader (Molecular Devices, Menlo Park, CA). Except where noted all assays contained the following: 50 mM sodium pyrophosphate buffer (pH 8.8), pyrazole (100 μM final concentration, added in 10 μL of methanol), rotenone (2 μM final concentration, added in 1.4 μL of methanol); rat liver mitochondria (0.5 to 2.5 mg protein/mL); sodium deoxycholate (0.25 mg/mg mitochondrial protein); and disulfiram (1, 2, 4, 8, 10, or 20 μM) or MeDTC sulfone (0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, or 4 μM) in methanol. An equal volume of methanol (7 μL) was added to control assays without inhibitor. The preincubation volume was 700 μL. Triplicate aliquots (175 μL) of the preincubation mixture were transferred to wells in the microplate, NAD (0.5 mM final concentration in 25 μL of buffer) was added, and the reaction started by adding acetaldehyde (80 μM final concentration in 25 μL of buffer). The order of addition of inhibitor, substrate, and cofactor was varied where noted. Data acquisition was initiated within 1 min of addition of the final component to the incubation mixture, except where noted. Values for the IC_{50} were obtained by linear regression analysis of the percent inhibition of ALDH activity versus the log concentration of inhibitor along the linear portion of the curve (3–5 data points). For graphical presentation only, log concentration–response data were fit by nonlinear regression.

HPLC analysis of MeDTC sulfone. MeDTC sulfone concentration and purity were measured with a Pharmacia-LKB HPLC system (Uppsala, Sweden) consisting of a model 2157 autoinjector, two model 2150 pumps with a static mixer, a 2152 LC controller, and a model 2140 photodiode array detector. Samples (5 μL in ethanol or acetonitrile) were injected directly into the HPLC equipped with a Microsorb 4.6 × 100 mm C18 column (Cat. No. 80–200, pore size 100 Å, particle size 3 μm, Rainin, Worburn, MA). Analytes were separated with a mobile phase of H_2O/acetonitrile (0.5 mL/min) using a linear gradient from 10 to 90% acetonitrile over 18 min. Absorbance at 210 nm was monitored continuously with a Hitachi model D-2500 integrator. UV spectra (190–370 nm) of the analytes were obtained and analyzed using the Pharmacia-LKB Wavescan program. MeDTC sulfoxide, MeDTC sulfone, MeDTC, and MeDDC eluted at 4.0, 12.3, 14.9, and 18.1 min, respectively.

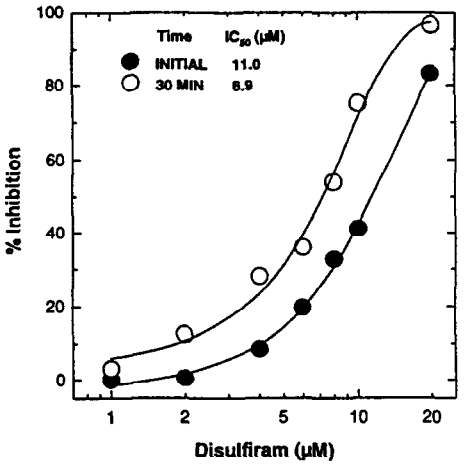
RESULTS

MeDTC sulfone was found to be a potent inhibitor of low K_m mitochondrial ALDH with an IC_{50} of 0.42 ± 0.04 μM. Under the same assay conditions, disulfiram had an IC_{50} of 7.5 ± 1.2 μM (Table 1). As shown in Fig. 3, there was an increase in the potency of MeDTC sulfone and disulfiram in their inhibition of ALDH when incubated with the enzyme for 30 min.

Table 1. Inhibition of aldehyde dehydrogenase by MeDTC sulfone and disulfiram

Inhibitor	IC_{50} (μM)
Disulfiram	7.5 ± 1.2*
MeDTC sulfone	0.42 ± 0.04

Either disulfiram (1–20 μM), MeDTC sulfone (0.1–4 μM), or an equivalent volume of vehicle (methanol) was added to rat liver mitochondria (0.5 mg protein/mL), solubilized with sodium deoxycholate (0.25 mg/mg protein). After 5 min, NAD (0.5 mM) and acetaldehyde (80 μM) were added to start the reaction. Low K_m ALDH activity was measured by following the formation of NADH spectrophotometrically at 340 nm, as described in Materials and Methods. The reaction velocity in control incubations without inhibitor was 13.5 ± 1.2 nmol NADH formed/min/mg protein. The IC_{50} values were calculated from the plot of the percent inhibition of ALDH activity versus log concentration of inhibitor along the linear portion of the curve. Values are mean ± SD of five assays with triplicate incubations at each concentration of inhibitor.



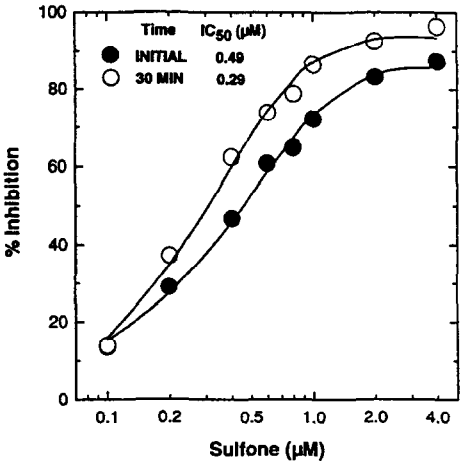


Fig. 3. Inhibition profiles of low K_m mitochondrial ALDH by disulfiram and MeDTC sulfone. Assay conditions are as described in Table 1 and in Materials and Methods. Either MeDTC sulfone (0.1–4 μM), disulfiram (1–20 μM), or an equivalent volume of vehicle (7 μL of methanol) was added to solubilized rat liver mitochondria (0.5 mg protein/mL). Triplicate aliquots of each mitochondrial mixture were transferred to a microtiter plate. Five minutes later, NAD (0.5 mM) and acetaldehyde (80 μM) were added to start the reaction (initial time). Low K_m ALDH activity at 22° was measured by following the formation of NADH spectrophotometrically at 340 nm for 3 min as described in Materials and Methods. After preincubation with inhibitor or vehicle for 30 min at room temperature, another set of triplicate aliquots of the mitochondrial mixture was transferred to a microtiter plate, and ALDH activity was measured immediately as described above (30 min). Each point is the mean ± SD of three determinations (SD are smaller than the symbols). Inhibition by MeDTC sulfone and disulfiram was studied separately but with the same mitochondrial preparation. The reaction velocities at 5 and 30 min, respectively, in control incubations without inhibitor were 11.9 ± 0.1 and 7.3 ± 0.1 nmol NADH/min/mg protein in the experiment with disulfiram and 10.8 ± 0.2 and 6.7 ± 0.4 nmol NADH/min/mg protein in the experiment with MeDTC sulfone.

The inhibition by MeDTC sulfone was apparently irreversible in that dilution did not decrease the degree of inhibition of ALDH (Fig. 4). After MeDTC sulfone was added to the enzyme, the preparation was rapidly diluted 10-fold, and the activity was measured after an additional 15 and 30 min. As shown in Fig. 4, the enzyme remained inhibited for up to 30 min despite the 10-fold dilution in MeDTC sulfone. Slow dissociation of a very tightly bound reversible enzyme–inactivator complex is an alternate explanation for these data. However, it is noteworthy that if such an enzyme–inactivator complex existed, it was stable in our experiments to addition of cofactor and a large excess of acetaldehyde (500 μM). To perform the reversibility experiments presented in Fig. 4, it was necessary to use a 5-fold higher concentration of protein (2.5 mg/mL) than was used in the experiments in Fig. 3 and Table 1 (0.5 mg/mL). Comparing these experiments, the IC_{50} of MeDTC sulfone was proportional to the protein concentration in the incubation: 2.1 μM at 2.5 mg/mL versus 0.42 to 0.49 μM at 0.5 mg/mL.

The apparent irreversible nature of the enzyme inhibition allowed for a more detailed examination of the time–dependence of inhibition of ALDH. MeDTC sulfone was added to the incubation mixture containing ALDH, and at timed intervals (5–300 sec) the ALDH activity was measured. As shown in Fig. 5, the inactivation of ALDH was time dependent. The decline in ALDH activity appeared to follow pseudo first-order kinetics with an apparent half-life of inactivation of 2.1 min at 0.6 μM MeDTC sulfone.

The mechanism of the inhibition by MeDTC sulfone was further examined by investigating the effects of acetaldehyde, the endogenous substrate of ALDH, on enzyme inactivation. Figure 6 shows the time course of the reaction measured spectrophotometrically as the formation of NADH. Line *A* shows the uninhibited reaction without MeDTC sulfone. The V_{max} for this reaction was approximately 15 nmol NADH/min/mg protein. Line *B* is the time

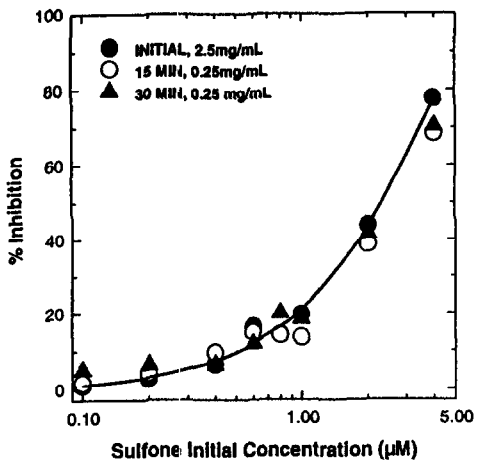


Fig. 4. Effect of dilution on inhibition of ALDH by MeDTC sulfone. MeDTC sulfone (0.1–4 μM initial concentration) or an equivalent volume of methanol was added to solubilized mitochondria (2.5 mg protein/mL) as described in Materials and Methods. Two 100-μL portions of each of these mixtures were immediately diluted 10-fold with 50 mM pyrophosphate buffer (pH 8.8); another portion was assayed after 8 min (initial time) for ALDH activity as described in Materials and Methods, except that 500 μM acetaldehyde was added to start the reaction. The higher substrate concentration was required at this higher protein concentration to maintain linearity of the reaction during the 3-min assay. There was <5% contribution to the reaction from the high K_m ALDH at 500 μM acetaldehyde (Lipsky JJ, unpublished observation). After 15 and 30 min, triplicate samples of the 10-fold diluted aliquots of mitochondria (0.5 mg protein/mL) containing MeDTC sulfone were added to a microtiter plate and assayed for ALDH as described above. The average velocities in control incubations without inhibitor were 10.3 ± 0.1, 6.6 ± 0.1 and 5.5 ± 0.1 nmol NADH/min/mg protein at 8, 15 and 30 min, respectively.

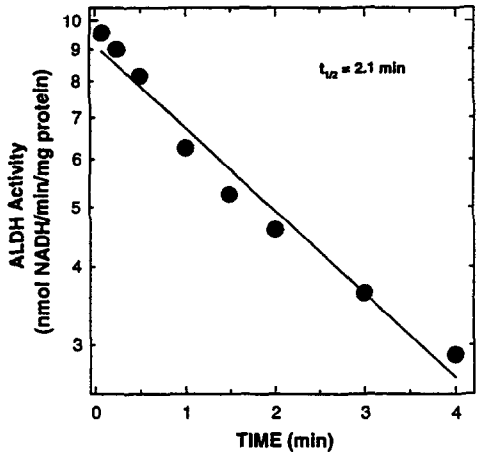


Fig. 5. Early time course of inhibition of ALDH by MeDTC sulfone. MeDTC sulfone (0.6 μM final concentration) or vehicle was added directly to wells in the microtiter plate containing solubilized mitochondria (0.5 mg/mL final concentration) and all other components of the incubation except NAD and acetaldehyde. At timed intervals from 5 to 600 sec, a mixture of acetaldehyde (80 μM) and NAD (0.5 mM) was added to triplicate incubation mixtures, and ALDH activity was measured immediately as described in Materials and Methods. ALDH activities in control incubations without inhibitor were 14.1 ± 0.1, 12.3 ± 0.3, and 9.7 ± 0.3 nmol NADH/min/mg protein (N = 3) at 5, 240 and 600 sec, respectively. ALDH activity in the incubations containing MeDTC sulfone at each time point (average of 3 determinations; SD are smaller than the symbols) was the observed activity minus the end-value activity at 600 sec (3.6 ± 0.1 nmol NADH/min/mg protein). Data are from one of two experiments that gave similar results.

course of the reaction in the presence of 0.6 μM sulfone, which was added 2 min prior to NAD and acetaldehyde. The V_{max} for this reaction was 6.6 nmol NADH/min/mg protein, which corresponds to an inhibition of 55%. Line *C* shows the reaction when MeDTC sulfone was mixed with the acetaldehyde, and this mixture was used to start the reaction in incubations that already contained NAD. No inhibition of the reaction could be detected under these conditions. Finally, line *D* shows the time course of the reaction after it was initiated by the addition of NAD. Under this condition, the sulfone and acetaldehyde were added together to the enzyme but prior to the addition of NAD. The V_{max} was 11.7 nmol NAD/min/mg protein, which corresponds to an inhibition of 21%. Therefore, there was substrate protection that was enhanced when NAD was added prior to the acetaldehyde. Similar results were obtained with 4 μM MeDTC sulfone (Table 2). In a related experiment not shown, NAD and 80 μM acetaldehyde were added to ALDH. After the reaction was allowed to run for 2 min, 4 μM MeDTC sulfone or vehicle was added to the incubation, and the reaction velocity was measured over the next 1.5 min. The reaction velocity was 14.2 nmol NADH/min/mg protein before the addition of MeDTC sulfone compared with 14.0 nmol NADH/min/mg protein after the addition of MeDTC sulfone. These results further demonstrate protection of the enzyme by substrate and cofactor.

To determine if the thiol-containing compounds DTT or GSH could protect the ALDH from inhibition, MeDTC sulfone was added to 50 μM DTT or 50 μM GSH, and 1 min later this mixture was added to the enzyme. Results in Fig. 7 show that 50 μM DTT was able to partially protect ALDH with a shift in the IC_{50} of MeDTC sulfone from 0.52 μM in the absence of DTT to 3.7 μM in its presence. GSH (50 μM) was a better protector of ALDH and increased the IC_{50} 14-fold to 7.1 μM. However, the addition of DTT or GSH to ALDH previously exposed to MeDTC sulfone did not restore lost enzyme activity (Fig. 8).

DISCUSSION

The results of this investigation demonstrated that MeDTC sulfone is a potent inhibitor of low K_m mitochondrial ALDH. The inhibition was rapid and compared with that of disulfiram, more potent. Although MeDTC sulfone has not yet been isolated

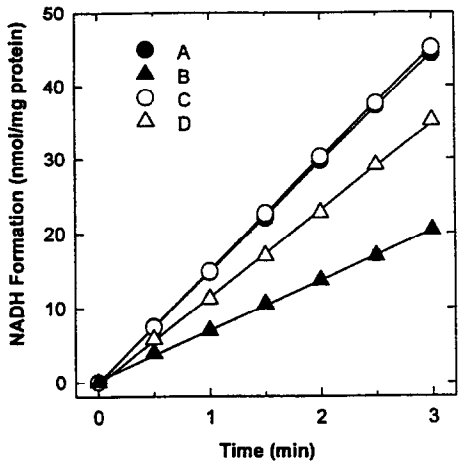


Fig. 6. Effect of substrate and cofactor on inhibition of ALDH by MeDTC sulfone. ALDH activity was measured in rat liver solubilized mitochondria (0.5 mg protein/mL) with a microplate reader, as described in Materials and Methods, using 80 μM acetaldehyde and 0.5 mM NAD. Line *A*: Control reaction with vehicle (no inhibitor). The velocity for this reaction was 14.8 nmol NADH/min/mg protein. Line *B*: Reaction in the presence of 0.6 μM MeDTC sulfone, which was added to the mitochondria prior to NAD and acetaldehyde. Line *C*: Reaction in which MeDTC sulfone (0.6 μM final concentration) was added to a solution of acetaldehyde. The ALDH reaction was started by adding this mixture to mitochondria that already contained NAD. Line *D*: Reaction initiated by the addition of NAD to mitochondria. Under this condition, the MeDTC sulfone and acetaldehyde were added together to the mitochondria but prior to the addition of NAD. Optical density was simultaneously recorded for 3 min at 0.1-min intervals in 6 replicate incubations for each of the 4 groups. Each point is the mean ± SD (most error bars are smaller than the symbols). Only the data at 0.5-min intervals are presented for clarity. The lines were generated by linear regression analysis.

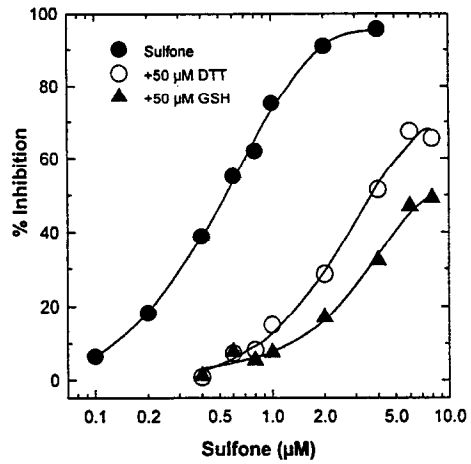


Fig. 7. Effects of GSH and DTT on the inhibition of mitochondrial ALDH by MeDTC sulfone. DTT (50 μM), GSH (50 μM), or vehicle (10 μL of 50 mM pyrophosphate buffer, pH 8.8) was added to solutions containing MeDTC sulfone (0.1–4 μM final concentration). After approximately 1 min, solubilized rat liver mitochondria (0.58 mg protein/mL) were added to each solution, and triplicate aliquots were immediately transferred to wells of the micotiter plate. NAD (0.5 mM) and acetaldehyde (80 μM) were added immediately, and ALDH activity was measured as described in Materials and Methods. The reaction velocities in control incubations without MeDTC sulfone were 11.4 ± 0.2 (vehicle), 11.2 ± 0.0 (50 μM DTT), and 11.2 ± 0.1 (50 μM GSH) nmol/min/mg protein. Data are from one of two experiments that gave similar results.

Table 2. Substrate and cofactor effects on inhibition of ALDH by MeDTC sulfone

Incubation condition	ALDH activity (nmol/min/mg protein)	
	0.6 μM Sulfone	4.0 μM Sulfone
Control (no sulfone)	14.8 ± 0.1 (100)	15.0 ± 0.2 (100)
Sulfone first	6.6 ± 0.2 (45)	0.8 ± 0.1 (5)
NAD first	15.2 ± 0.1 (103)	14.2 ± 0.1 (95)
NAD last	11.7 ± 0.1 (79)	8.4 ± 0.1 (56)

Low K_m mitochondrial ALDH activity was measured as described in Materials and Methods. Other experimental details are described in Fig. 6. Values are the means ± SD of 6 determinations with percent control activity in parentheses.

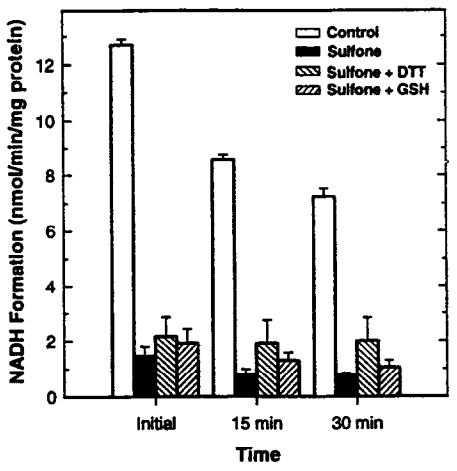


Fig. 8. Lack of reversal by DTT or GSH of inhibition of ALDH by MeDTC sulfone. This experiment was similar to the one described in Fig. 7 except that vehicle, 100 μM DTT, or 100 μM GSH was added to solubilized mitochondria (0.5 mg protein/mL) that had been exposed previously to MeDTC sulfone (2 μM) for 1 min. After 5 min (initial time), 15 min, and 30 min, triplicate aliquots of each mixture were transferred to microtiter plates. NAD (0.5 mM) and acetaldehyde (80 μM) were added immediately, and the ALDH activity was measured as described in Materials and Methods. Each point is the combined mean ± SD (N = 6) of two experiments.

in vivo or *in vitro* [17], the current understanding of the metabolic pathway of disulfiram makes the formation of MeDTC sulfone a potential metabolite. Oxidation by cytochromes P450 or flavin monooxygenase of sulfoxides to sulfones has been reported for other compounds [16, 23]. It is clear that *in vivo* disulfiram is reduced rapidly to DDC, which undergoes methylation to MeDDC. This latter metabolite has been shown to undergo at least two oxidative steps: oxidative desulfuration to MeDTC, followed by sulfoxidation to MeDTC sulfoxide (Fig. 1). Both MeDTC and MeDTC sulfoxide have been hypothesized to be the active metabolite of disulfiram. However, MeDTC is not very active *in vitro* ([5] and Lipsky, unpublished results). Faiman and coworkers were able to detect MeDTC sulfoxide [7] but not MeDTC sulfone [17] in the plasma of rats treated with disulfiram. Although the potency of MeDTC sulfoxide as an inhibitor of ALDH makes it an attractive compound for an active metabolite [24], there is no clear evidence that it is the ultimate, or only, active metabolite of disulfiram. Whether or not further oxidation of MeDTC sulfoxide occurs *in vivo* remains to be determined.

It will be of interest to ascertain if the MeDTC sulfone can be found in plasma of animals treated with disulfiram by using alternative techniques to those described by Nagendra *et al.* [17]. For example, EPTC sulfone could be detected in incubations of EPTC sulfoxide with mouse liver microsomes, only after blocking the sites on protein that were presumably carbamoylated by EPTC sulfone [16, 25]. It appears that detection of MeDTC sulfone may be similarly difficult as it apparently readily reacts with ALDH and possibly other cellular constituents. The work of Jin and coworkers [19] indicates that reactive metabolites of disulfiram (possibly MeDTC sulfoxide and/or sulfone) form conjugates with glutathione. But it is not possible from their data to distinguish whether the glutathione adduct is derived from the sulfone or sulfoxide [19]. Identification of the chemical species covalently bound to ALDH *in vivo* may help determine whether the MeDTC sulfone is an important reactive metabolite of disulfiram.

The inhibition of ALDH by the MeDTC sulfone apparently was irreversible (Fig. 4). This is consistent with the observation that disulfiram is an irreversible inhibitor of ALDH in humans and experimental animals [26]. Administration of cycloheximide, which inhibits protein synthesis, delays recovery of ALDH activity following disulfiram administration in experimental animals [26].

The protection of ALDH by its physiological substrate acetaldehyde from inhibition by MeDTC sulfone may point toward a mechanistic explanation of the inhibition (Fig. 6, Table 2). One interpretation of this finding is that MeDTC sulfone reacts with an essential sulfhydryl group in the active site of the enzyme, similar to the reactions described by Pietruszko and coworkers [27, 28]. This sulfhydryl group may be protected from MeDTC sulfone when acetaldehyde is bound to the enzyme. The mechanistic studies with various forms of ALDH [29–32] demonstrate ordered binding of NAD and substrate. NAD binds first and enhances binding of the substrate by $\geq$500-fold [29]. Enhanced binding of acetaldehyde in the presence of NAD is consistent with our finding of essentially complete protection of ALDH from inhibition by MeDTC sulfone in the presence of both acetaldehyde and NAD compared with the partial protection with acetaldehyde alone (Fig. 6, Table 2).

The protection of ALDH by the thiol-containing compounds DTT and GSH was not stoichiometric. This may indicate that adduct formation between either of these agents and the sulfone is slow or that the presumed MeDTC sulfone-thiol adduct itself may have activity against ALDH. It is also possible that the protective effect may be due to an interaction of the sulfhydryl reagents and ALDH. Since GSH is an endogenous substance in the liver, the GSH status of the liver may be an important factor in the relative efficacy of disulfiram in a particular individual. The fact that inhibition of ALDH by MeDTC sulfone could not be restored by GSH or DTT is further evidence of the irreversible nature of the inhibition. Previous studies have shown that inhibition of hepatic ALDH by *in vivo* treatment of rats with disulfiram could not be reversed by DTT treatment *ex vivo* [26].

ALDH contains several cysteine residues, one of which, cysteine-302, is highly conserved and thought to reside at the catalytic site of the enzyme [27, 28]. Chemical modification of cysteine-302 on human ALDH 2 (low K_m mitochondrial form) by a ketone analog of an insect pheromone irreversibly inhibits the enzyme [33]. It is tempting to speculate that MeDTC sulfone may be reacting with this or other important cysteine residues. Further studies of ALDH modified by MeDTC sulfone will be necessary to elucidate the exact nature of this inhibition.

In conclusion, our results show that MeDTC sulfone is a candidate for an active metabolite of disulfiram. Further studies may indicate whether or not this is correct. It is also possible that MeDTC sulfone may be a useful pharmacologic tool for investigating the mechanism of inhibition of ALDH by metabolites of disulfiram.

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