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Inhibition of Recombinant Human Mitochondrial Aldehyde Dehydrogenase by Two Intermediate Metabolites of Disulfiram

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ABSTRACT. Disulfiram is used in aversion therapy for alcoholism. *S*-Methyl-*N*,*N*-diethylthiocarbamate (MeDTC) sulfoxide, a potent inhibitor of the target enzyme mitochondrial aldehyde dehydrogenase (ALDH2), is thought to be the principal active metabolite of disulfiram *in vivo*. We examined the effects on recombinant human ALDH2 of two intermediate metabolites of disulfiram, *S*-methyl-*N*,*N*-diethyldithiocarbamate (MeDDC) sulfoxide and MeDDC sulfine. MeDDC sulfoxide was a potent inhibitor of ALDH2 with an IC_{50} of 2.2 ± 0.5 μM (mean ± SD, $N = 4$) after preincubation with enzyme for 30 min. MeDDC sulfine was a relatively weak inhibitor of ALDH2 under the same conditions with an IC_{50} value of 62 ± 14 μM. The inhibition of ALDH2 by both compounds was irreversible and did not require the cofactor NAD. The latter finding demonstrates that inactivation of ALDH2 is independent of the dehydrogenase activity of the enzyme. GSH blocked almost completely the inhibition by 20 μM of MeDDC sulfoxide and greatly diminished the inhibition by 200 μM of MeDDC sulfine. Inactivation by MeDDC sulfoxide was time dependent. MeDTC sulfoxide was a more potent inhibitor of recombinant human ALDH2 (IC_{50} = 1.4 ± 0.3 μM after preincubation for 15 min) than either of the intermediate metabolites, and its inhibition was unaffected by GSH. Our results suggest that these newer intermediate metabolites of disulfiram, especially the more potent MeDTC sulfoxide, have the potential to inhibit the target enzyme ALDH2 in patients receiving disulfiram. However, until the significance of the interactions of the inhibitors with GSH is more fully understood, the contribution of MeDDC sulfine and MeDDC sulfoxide to the pharmacological effects of disulfiram *in vivo* is uncertain. BIOCHEM PHARMACOL **55**;7: 1099–1103, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. disulfiram; *S*-methyl *N*,*N*-diethyldithiocarbamate sulfine; *S*-methyl *N*,*N*-diethyldithiocarbamate sulfoxide; human recombinant aldehyde dehydrogenase; alcoholism; enzyme inactivation

Disulfiram, the only drug approved for use in aversion therapy for alcoholism, inhibits the metabolism of acetaldehyde, the product of ethanol metabolism, by inhibiting hepatic ALDH2§. Consequently, ingestion of ethanol by an individual on disulfiram therapy causes an accumulation of acetaldehyde, which is associated with a spectrum of undesirable effects called the "disulfiram-ethanol reaction" [1, 2]. *In vivo*, disulfiram is reduced rapidly to DDC [3], which is metabolized further, as shown in Scheme 1. MeDDC, formed primarily by thiol methyl transferase in humans [4], is oxidized by cytochromes P450 and flavin monooxygenase [5]. DDC, MeDDC, and MeDTC are very weak or non-inhibitors of ALDH *in vivo*, suggesting that further metabolism is required to inhibit the enzyme [6–8]. MeDTC sulfoxide and MeDTC sulfone, confirmed and proposed metabolites of disulfiram, respectively, have been shown to be potent inhibitors of recombinantly expressed human mitochondrial and cytosolic ALDH [9] and ALDH activity in rat liver mitochondria [10–12]. Two intermediate metabolites of disulfiram, MeDDC sulfoxide and MeDDC sulfine, have been discovered recently [5]. MeDDC sulfoxide, but not MeDDC sulfine, inhibits ALDH activity in rat liver mitochondria *in vitro* [13, 14]. This is a report of the effects of these intermediate metabolites of disulfiram on recombinant human ALDH2, the target enzyme of disulfiram therapy.

MATERIALS AND METHODS

Materials

The recombinant human ALDH2 (mitochondrial) cDNA in pT7-7 [15] was a gift from Dr. Henry Weiner (Department of Biochemistry, Purdue University). The enzyme was expressed in *Escherichia coli* and purified as previously

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§ *Abbreviations:* ALDH, aldehyde dehydrogenase; ALDH2, mitochondrial isoform of ALDH; DDC, *N*,*N*-diethyldithiocarbamate; MeDDC, *S*-methyl *N*,*N*-diethyldithiocarbamate; and MeDTC, *S*-methyl *N*,*N*-diethylthiocarbamate.

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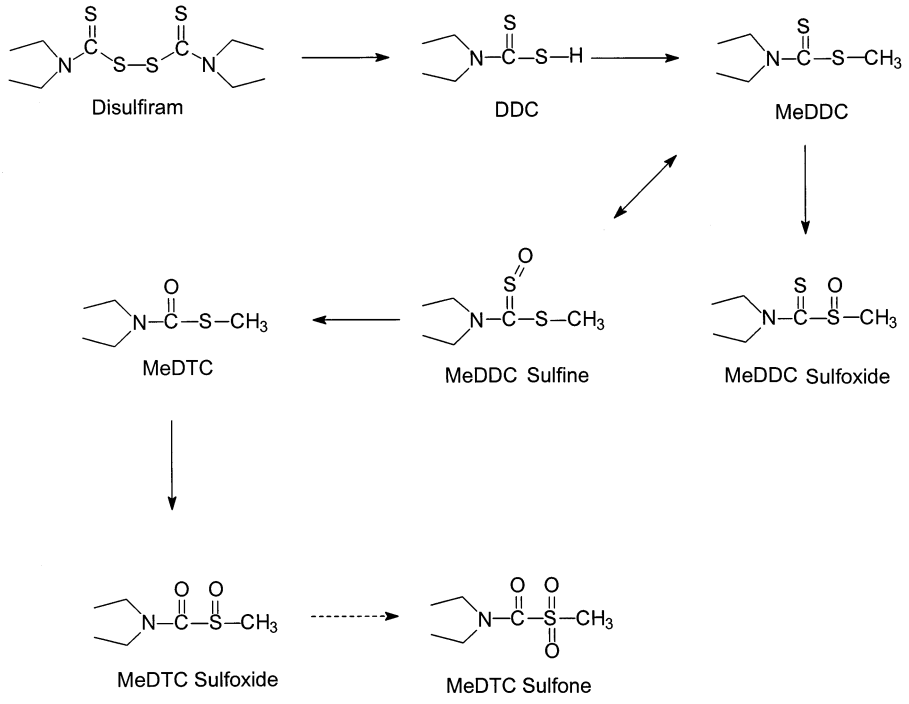

SCHEME 1. Pathways of disulfiram metabolism. The confirmed and proposed pathways are shown by the solid and dashed arrows, respectively.

described [9]. MeDDC [16], MeDTC [6], and MeDTC sulfoxide [12] were prepared as previously described and were determined to be > 99% pure by HPLC-UV. A BCA Protein Assay kit and Slide-A-Lyzer cassettes were obtained from Pierce.

Synthesis of MeDDC sulfine

A solution of the MeDDC (212 mg, 1.3 mmol) in 70 mL of anhydrous CH_2Cl_2, was stirred magnetically and cooled to −10° under argon. A solution of *meta*-chloroperoxybenzoic acid (mCPBA, 285 mg, 1.25 mmol) was then added dropwise, and the resulting mixture was stirred for 30 min. The excess reagent was destroyed by adding a saturated solution of sodium sulfite at −10° and stirring at room temperature. Extraction twice with CH_2Cl_2 followed by washing with a saturated aqueous solution of NacCl and drying with anhydrous $MgSO_4$ gave a crude oil. Purification by silica gel chromatography using 4% MeOH/CH_2Cl_2 furnished the sulfine as a light yellow oil. ^{1}H NMR: d ($CDCl_3$, 300 MHz) 1.28 (t, 6 H, J = 7.1 Hz, $-CH_2-\underline{CH_3}$), 2.23 (s, 3 H, $-\underline{SCH_3}$), 3.91 (q, 4 H, J = 7.1 Hz, $-\underline{CH_2}-CH_3$); ^{13}C NMR: d ($CDCl_3$, 75 MHz) 13.8, 21.2, 46.7; MS: m/z 179 (M^+), 162, 116 (100%), 88; IR (neat) 1493, 1433, 1280, 1030, 958 cm^{-1}. This material was determined to be > 99% pure by HPLC-UV; two minor impurities were identified by retention times and UV spectra as MeDTC (0.2%) and MeDDC (0.3%). Purified neat MeDDC sulfine decomposed rapidly at −20°, primarily to the starting material MeDDC. Decomposition was not detected by ^{1} H NMR in dilute solutions (40 mM of MeDDC sulfine) of deuterium-labeled MeOH, CH_2Cl_2, or benzene stored in the dark at −20° for up to 2 months. Despite the lack of significant decomposition by ^{1}H NMR, in our initial ALDH inhibition studies, the potency of dilute working solutions of MeDDC sulfine in methanol increased with repeated use (see Results and Discussion). These solutions were stored in dark amber glass vials at −20°, but were allowed to come to room temperature before each use. To minimize decomposition, the dilute working solutions of MeDDC sulfine in subsequent experiments were discarded after each use.

Synthesis of MeDDC Sulfoxide

MeDDC sulfoxide was synthesized according to the method of Madan and Faiman [5]. In our hands, this procedure gave both MeDDC sulfoxide and MeDDC sulfine, which then were purified by flash silica gel chromatography using 5% (v/v) MeOH in CH_2Cl_2 as eluent, giving the sulfoxide in 30% yield. ^{1}H NMR: d ($CDCl_3$, 300 MHz) 1.34 (m, 6 H, $-CH_2-\underline{CH_3}$), 2.85 (s, 3 H, $-S\underline{CH_3}$), 3.64–3.90 (m, 3 H, $-\underline{CH_2}-CH_3$), 4.01–4.52 (m, 1 H, $-\underline{CH_2}-CH_3$); ^{13}C NMR: d ($CDCl_3$, 75 MHz) 10.90, 14.48, 42.62, 44.85, 50.45, 202.4); MS: m/z 163 (M^+ −16), 116 (100%), 88. MeDDC sulfoxide was found to be > 99% pure by HPLC.

ALDH Activity Assays

The microtiter-based assay for ALDH was performed for the dehydrogenase activity as described [17] with modifications [9]. Briefly, purified recombinant human ALDH was added to buffer G (0.05 M of sodium pyrophosphate, pH 8.8) in a final volume of 700 μL. Typically triplicate aliquots (200 μL) were added to the wells of a 96-well microliter plate. Acetaldehyde (160-μM final concentration) and NAD

(500 μM) were added together in 25 μL of buffer G to start the dehydrogenase reaction. The protein concentration in the final incubation mixture, unless indicated otherwise, was, 4.4 to 13.3 μg/mL corresponding to 0.02 to 0.06 μM of ALDH2 as the tetramer. NADH produced from the oxidation of acetaldehyde was monitored by measuring the change in absorbance at 340 nm for 3 min.

Inhibitor Studies

Purified ALDH2 at concentrations that produced control velocities of 5–10 nmol NADH/min, was preincubated in buffer G with inhibitor (0.1 to 200 μM) or methanol (vehicle) for 5–30 min. The inhibitor was added in 7 μL of methanol, bringing the total preincubation volume to 700 μL. Triplicate aliquots of 200 μL were transferred into wells of a microliter plate. The ALDH reaction was initiated by adding NAD and acetaldehyde in 25 μL of buffer G.

To examine the reversibility of inhibition, ALDH2 was preincubated at room temperature with 20 μM of MeDDC sulfoxide, 200 μM of MeDDC sulfine, or methanol (vehicle control) for 15 min before dialysis. A 1-mL portion of each preincubation mixture was transferred to a Slide-A-Lyzer dialysis cassette and placed in a beaker with 150 mL of buffer G. Another portion of the preincubation mixture was not dialyzed and maintained at room temperature for 30 min. After 15 min, the dialysis buffer was replaced with another 150 mL of fresh buffer G, and the sample was dialyzed for another 15 min. ALDH activity of each sample was determined before dialysis to verify that the inhibition was greater than 70%. ALDH activity was also measured in the undialyzed samples preincubated for 30 min.

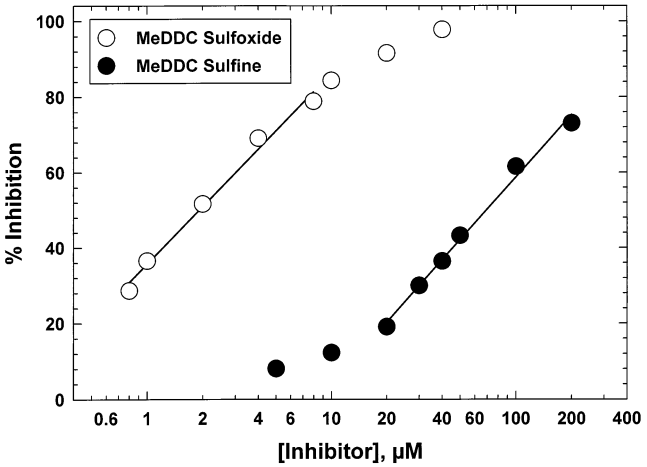

FIG. 1. Representative concentration–inhibition curves of recombinant human ALDH2. Purified ALDH2 was preincubated with inhibitor (0.8 to 200 μM) or methanol (vehicle control) for 30 min. The reaction was initiated by adding NAD and acetaldehyde, and production of NADH from the oxidation of acetaldehyde was monitored by measuring the change in absorbance at 340 nm for 3 min. Data are representative of three experiments.

TABLE 1. Inhibition of recombinant human ALDH2

Inhibitor	IC_{50} (μM)		
	5 min*	15 min	30 min
MeDDC sulfoxide	7.2 ± 1.5	4.1 ± 0.9	2.2 ± 0.5
MeDDC sufline	ND	ND	62 ± 14
MeDTC sulfoxide	ND	1.4 ± 0.3	ND

MeDDC sulfoxide, MeDDC sulfine, MeDTC sulfoxide, or vehicle (methanol) was preincubated at 22–24° with recombinant human ALDH2 for 5, 15, or 30 min. ALDH2 activity was measured as described in Materials and Methods. Values are the means ± SD of 3 to 4 assays with triplicate wells at each concentration of inhibitor. ND = not determined.

*Preincubation time.

RESULTS AND DISCUSSION

MeDDC sulfoxide was a potent inhibitor of recombinant human ALDH2 with an IC_{50} value of 2.2 ± 0.5 μM after preincubation with the enzyme for 30 min (Table 1). MeDDC sulfine was a relatively weak inhibitor with an IC_{50} value of 62 ± 14 μM under the same conditions. In previous studies of ALDH activity in rat liver mitochondria, the IC_{50} values were 10 μM for MeDDC sulfoxide [13], and ≫1000 μM for MeDDC sulfine [14]. In our study, the inhibition of ALDH by both compounds did not require the cofactor NAD, suggesting that dehydrogenase activity is unnecessary for inhibition. Typical concentration–inhibition curves are shown in Fig. 1. The IC_{50} values for inhibition of ALDH2 by MeDDC sulfoxide after preincubation for 5 and 15 min were 7.2 ± 1.5 and 4.1 ± 0.9 μM, respectively, indicating that inhibition was time dependent (Table 1).

As noted previously, the potency of the dilute working solutions of MeDDC sulfine increased after preparation. For example, after 10 days of intermittent use of a solution of 4 mM of MeDDC sulfine, the respective concentrations of MeDDC sulfine, MeDTC, and MeDDC were 3.7, 0.04, and 0.1 mM. During the same 10-day period, the activity of the working solutions, which were diluted 100-fold in the preincubation with ALDH2 (see Materials and Methods), also increased dramatically (apparent IC_{50} = 2 μM). The increased activity could not be explained by the increased concentrations of the very weak inhibitors MeDDC or MeDTC, which have IC_{50} values > 1000 μM (Mays D, unpublished data). The spontaneous deoxygenation of MeDDC sulfine to form MeDDC has been reported previously by Segall and Casida [18]. However, our data indicates that MeDDC sulfine also spontaneously forms a very potent, and yet unidentified, inhibitor of ALDH.

The activity of human ALDH2 inhibited by preincubation for 15 min with 20 μM of MeDDC sulfoxide or 200 μM of MeDDC sulfine, respectively, was unaffected by extensive dialysis (4 ± 6 and 3 ± 3% of control activity for dialyzed enzyme vs 5 ± 6 and 4 ± 5% for undialyzed enzyme; mean ± SD, $N = 3$), indicating that inhibition by these metabolites was irreversible. Similarly, MeDTC sulfoxide, another active metabolize of disulfiram, inhibits

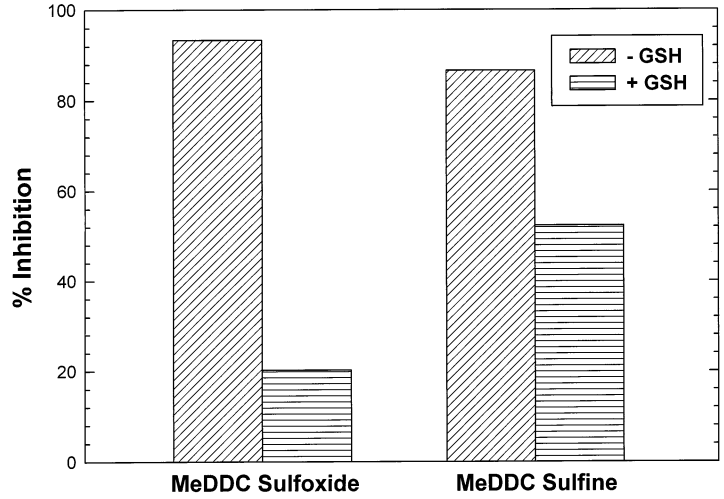

FIG. 2. Effect of GSH on the inactivation of recombinant human ALDH2 by MeDDC sulfoxide and MeDDC sulfine. GSH (0.7 mM) or vehicle (buffer G) was preincubated at room temperature with ALDH2 (0.1 μM) in buffer G for 5 min before adding 20 μM of MeDDC sulfoxide, 200 μM of MeDDC sulfine, or methanol (vehicle control). The reaction mixture was further incubated for 30 min before measuring the ALDH2 activity, as described in Materials and Methods. The results are averages of 2–3 experiments.

ALDH2 activity irreversibly in incubations of solubilized rat liver mitochondria [12] and recombinant human ALDH [9].

Addition of 0.7 mM of GSH to ALDH2 prior to adding the inhibitors protected almost completely against inhibition by 20 μM of MeDDC sulfoxide and partially against inhibition by 200 μM of MeDDC sulfine (Fig. 2). In contrast, inhibition of human ALDH2 by 4 μM of MeDTC sulfoxide was unaffected by GSH (data not shown). In our previous study, GSH also failed to protect ALDH activity from inhibition by MeDTC sulfoxide in rat liver mitochondria [12]. To help elucidate the mechanism of protection by GSH, 20 μM of MeDDC sulfoxide or 200 μM of MeDDC sulfine was incubated separately at room temperature with 0.7 mM of GSH in buffer G without ALDH, and timed samples were analyzed by HPLC. After 15 min, there was no detectable MeDDC sulfoxide (< 0.05 μM), and the concentration of MeDDC sulfine decreased to 120 μM. After 45 min, the concentration of MeDDC sulfine declined further to 53 μM; MeDDC (84 μM) and MeDTC (19 μM) were the major identifiable products, accounting for 70% of the loss of the sulfine. There was no loss of MeDDC sulfine or MeDDC sulfoxide in control incubations in buffer G without GSH over the same period of time. Thus, the protection of ALDH in our experiments appears to be due to a direct chemical reaction of the inhibitors with GSH. Decomposition of MeDDC sulfine to MeDDC by GSH has been reported previously [19].

The reactivity of MeDDC sulfoxide and MeDDC sulfine with GSH *in vitro* raises the question of possible interactions with endogenous GSH, the levels of which are 3–10 mM in normal hepatocytes [20]. The actual concentration of GSH to which the inhibitors would be exposed is uncertain. For example, it has been reported that disulfiram causes oxidative stress and depletion of GSH in some tissues [21, 22]. Therefore, administration of disulfiram could decrease the potential for its metabolites to be scavenged by GSH. Furthermore, electrophilic attack of nucleophilic sites in tissue macromolecules by reactive intermediates formed in hepatocytes still occurs, despite appreciable intracellular levels of GSH. In the classic studies of Mitchell *et al.* [23], it has been clearly shown that an active metabolite of acetaminophen binds to hepatic protein even when intracellular levels of GSH remain above 1 mM. The protection by GSH *in vivo* is dependent on many factors, including the proximity of the site of formation of the reactive intermediate to the target macromolecule and the relative reactivity of the active metabolite toward its cellular targets and non-target molecules (e.g. GSH).

Kinetic studies suggest that ALDH2 is responsible for the metabolism of most of the acetaldehyde produced after ethanol ingestion [24, 25] and that ALDH2 is a logical target of disulfiram therapy. Our investigation has shown that *in vitro* MeDDC sulfoxide is a potent inhibitor of ALDH2 (comparable in potency to MeDTC sulfoxide), and that MeDDC sulfine is a very weak inhibitor of ALDH2. It is noteworthy, however, that GSH effectively blocked the inhibition of ALDH2 by MeDDC sulfoxide and MeDDC sulfine, but had no effect on the inhibition by MeDTC sulfoxide. Until the significance *in vivo* of the GSH findings is known, the contribution of MeDDC sulfine and MeDDC sulfoxide to the inhibition of ALDH2 in individuals receiving disulfiram is uncertain.

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