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DISULFIRAM METABOLISM AS A REQUIREMENT FOR THE INHIBITION OF RAT LIVER MITOCHONDRIAL LOW K_m ALDEHYDE DEHYDROGENASE

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Abstract—In humans and animals, disulfiram produces a disulfiram–ethanol reaction after an ethanol challenge, the basis of which is the inhibition of liver aldehyde dehydrogenase (ALDH). Disulfiram and the metabolites diethyldithiocarbamate (DDTC), diethyldithiocarbamate-methyl ester (DDTC-Me), and *S*-methyl-*N*,*N*-diethylthiolcarbamate (DETC-Me) were studied in order to determine the role of bioactivation in disulfiram's action as an inhibitor of rat liver mitochondrial low K_m ALDH (RLM low K_m ALDH). In *in vitro* studies, disulfiram and DDTC (0.01 to 2.0 mM) both inhibited RLM low K_m ALDH in a concentration-dependent manner. The addition of rat liver microsomes to the mitochondrial incubation did not further increase disulfiram-induced RLM low K_m ALDH inhibition. However, DDTC-induced RLM low K_m ALDH inhibition was increased further, but only at DDTC concentrations <0.05 mM. DDTC-Me and DETC-Me (2.0 mM) similarly exhibited an increased RLM low K_m ALDH inhibition after the addition of liver microsomes. In *in vivo* studies, disulfiram (75 mg/kg), DDTC (114 mg/kg), DDTC-Me (41.2 mg/kg) or DETC-Me (18.6 mg/kg) administered i.p. to female rats inhibited RLM low K_m ALDH. Inhibition of drug metabolism by pretreatment of rats with the cytochrome P450 inhibitor *N*-octylimidazole (NOI) (20 mg/kg, i.p.) prior to either disulfiram, DDTC, DDTC-Me or DETC-Me administration blocked the inhibition of RLM low K_m ALDH. The *in vitro* and *in vivo* data support the conclusion that bioactivation of disulfiram to a reactive chemical species is required for RLM low K_m ALDH inhibition and a disulfiram–ethanol reaction.

Disulfiram has been used as a pharmacological deterrent for the treatment of alcoholism since it was first reported to produce adverse effects after ethanol ingestion [1]. The basis for its action is the inhibition of liver aldehyde dehydrogenase (ALDH‡), and the subsequent increase in acetaldehyde in tissue fluids after ethanol ingestion. Clinically, this is characterized by nausea, tachycardia, hypotension, and other adverse symptoms, and is referred to as the disulfiram–ethanol reaction [2].

The inhibition of liver ALDH by disulfiram has been studied extensively for many years, and several mechanisms by which liver ALDH is inhibited by disulfiram have been proposed. These include the suggestion that disulfiram is a competitive inhibitor of NAD^+ [3], and that disulfiram forms mixed disulfides with protein thiols [4]. A better understanding of the mechanism of ALDH inhibition was provided by the studies of Vallari and Pietruszko [5] with human liver cytoplasmic ALDH. Those studies suggested that disulfiram "oxidized essential sulfhydryl groups" initially forming a diethyldithiocarbamate (DDTC)-adduct followed by the formation of internal disulfide bonds. These findings were extended by Kitson [6] who suggested that the initial reaction was rapid and was responsible for the loss of ALDH activity.

Disulfiram is reduced *in vivo* by both plasma glutathione reductase [7] and albumin [8] to diethyldithiocarbamate (DDTC), which is relatively inactive as an ALDH inhibitor *in vitro* but is found to be active *in vivo*. The ALDH inhibitory profile produced by DDTC is similar to that observed with disulfiram [9]. The ubiquitous nature of endogenous catalase, methemoglobin, cytochrome *c*, and xanthine oxidase has provided an explanation for the mechanism by which DDTC is oxidized to disulfiram *in vivo*. Recently, oxyhemoglobin has been shown to oxidize DDTC to disulfiram and hydrogen peroxide [10], further supporting the oxidation of DDTC to disulfiram *in vivo* and the inhibition of ALDH. These potential oxidative mechanisms have accounted for the suggestion that an equilibrium exists between the reduction of disulfiram to DDTC, the reoxidation of DDTC to disulfiram, the inactivation of ALDH by disulfiram, further metabolism and excretion of DDTC, and the *de novo* synthesis of ALDH [11].

The seminal finding that the disulfiram metabolite diethyldithiocarbamate-methyl ester (DDTC-Me) inhibits RLM low K_m ALDH *in vivo* [12] provided the first evidence that the metabolism of disulfiram and not reoxidation of DDTC to disulfiram may be important in the inhibition of ALDH. This suggested

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‡ Abbreviations: ALDH, aldehyde dehydrogenase; RLM low K_m ALDH, rat liver mitochondrial low K_m aldehyde dehydrogenase; DDTC, diethyldithiocarbamate; DDTC-Me, diethyldithiocarbamate-methyl ester; DETC-Me, *S*-methyl-*N*,*N*-diethylthiolcarbamate; and NOI, *N*-octylimidazole.

that disulfiram *per se* may not be the active chemical species responsible for the *in vivo* inhibition of ALDH as previously believed. In subsequent comparative studies, disulfiram, and the metabolites DDTC and DDTC-Me all inhibited RLM low K_m ALDH, with DDTC-Me being the most potent. In addition, all produced a disulfiram–ethanol reaction after an ethanol challenge [13]. These findings thus supported the hypothesis that bioactivation of disulfiram was necessary for the *in vivo* inhibition of ALDH. Since DDTC-Me is relatively inactive *in vitro* as a low K_m ALDH inhibitor, but is active *in vivo* [12], this suggested that further metabolism of DDTC-Me was necessary for the *in vivo* inhibition of ALDH. This led to the discovery of *S*-methyl-*N*,*N*-diethylthiolcarbamate (DETC-Me), its isolation and identification in rats after disulfiram administration, and the description of its ALDH inhibitory properties [14]. The potent RLM low K_m ALDH inhibitory characteristics of DETC-Me have since been characterized in detail [15]. DETC-Me formation *in vivo* after disulfiram administration to rats and humans also has been confirmed recently by Johansson *et al.* [16]. The studies to be described now provide further evidence that disulfiram metabolism is required for the inhibition of RLM low K_m ALDH *in vivo*, and examine the apparent role of cytochrome P450 in the formation of an active chemical species produced from disulfiram and which apparently is responsible for ALDH inhibition.

METHODS

Animals. Sprague–Dawley Charles River-derived female rats (200–300 g) were used throughout the studies. The rats were obtained from a colony maintained in the Animal Care Unit of The University of Kansas. Rats were maintained on a 12-hr light–dark cycle with access to laboratory chow and water *ad lib*. Animals were fasted overnight prior to any drug administration, with water allowed *ad lib*.

In vivo *drug administration.* *N*-Octylimidazole (NOI) was suspended in saline and dissolved by the addition of a few drops of 0.5 N HCl. Rats were treated with NOI (20 mg/kg; 3 mL/kg, i.p.) 30 min before the i.p. administration of either disulfiram (75 mg/kg), DDTC (114 mg/kg), DDTC-Me (41.2 mg/kg), or DETC-Me (18.6 mg/kg). Two hours later, the rats were killed by decapitation; each liver was excised, the mitochondria were isolated, and the RLM low K_m ALDH was determined. In addition, one group of DDTC-treated rats was killed 8 hr after DDTC administration. Controls received saline/HCl solution instead of NOI.

Mitochondrial isolation. A portion of the liver was excised and immediately placed on ice. The liver tissue was minced and homogenized in 10 vol. (w/v) of a sucrose buffer (0.25 M sucrose, 5 mM Tris–HCl, 0.5 mM Na_2EDTA, pH 7.2), with four passes over the pestle. To remove nuclei and cellular debris, the homogenate was centrifuged for 10 min at 700 *g*, the supernatant was removed and placed into another centrifuge tube, and the pellet was discarded. To isolate the mitochondria, the supernatant was centrifuged for 10 min at 4300 *g*, the supernatant discarded, and the pellet washed once by resuspending the pellet with sucrose buffer and recentrifuged. After the final wash of the mitochondrial pellet, the pellet was resuspended in 0.1 M phosphate buffer (pH 7.2). Protein concentration was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

Microsomal preparation. Rats were killed, and their livers removed, weighed and placed in cold 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM Na_2EDTA and 1.12% KCl. All steps in the isolation were carried out at 0–4°. The liver was minced and homogenized (glass homogenizer tube and teflon pestle, with four passes over the pestle) in 4 vol. of 0.1 M phosphate buffer. The homogenate was centrifuged for 15 min at 3000 *g*. The supernatant obtained was centrifuged for 20 min at 12,000 *g*, and the resulting supernatant then centrifuged for 60 min at 105,000 *g*. The microsomal pellet was washed once with 0.1 M phosphate buffer containing KCl, and then resuspended in 0.1 M phosphate buffer without KCl. The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [17] using bovine serum albumin as the standard. The microsomes were frozen and stored at −70° until used.

In vivo *low* K_m *aldehyde dehydrogenase activity determination.* Approximately 0.5 g of liver removed from either the drug-treated or control rats was homogenized in 0.25 M sucrose and differential centrifugation was carried out to isolate the mitochondria [18]. The mitochondria then were solubilized with 1 mg sodium deoxycholate/mg mitochondrial protein, and mitochondrial low K_m and total (high K_m and low K_m) ALDH activities were determined by the method of Tottmar *et al.* [18]. Concentrations of 50 μM and 5 mM acetaldehyde were used as the substrate for the low K_m and the total ALDH, respectively. High K_m activity was calculated by subtracting the low K_m activity from the total activity. ALDH activity reflects inhibition end points.

In vitro *low* K_m *aldehyde dehydrogenase activity determination.* Mitochondria were isolated from the liver of untreated rats, as described, and resuspended in 0.1 M phosphate buffer (pH 7.4). Incubations were carried out with 2 mg mitochondrial protein containing various concentrations of disulfiram, DDTC, DDTC-Me or DETC-Me dissolved in ethanol in a total volume of 1.5 mL. Incubations were carried out for 60 min at 37°, and the mitochondria were isolated by centrifugation, solubilized, and assayed for low K_m ALDH by the method of Tottmar *et al.* [18]. Control incubations were prepared in an identical manner except that disulfiram, DDTC, and DDTC-Me were excluded. ALDH activity reflects inhibition end points.

In vitro *P450 substrate studies.* Mitochondria were isolated from the liver of untreated rats as described, and suspended in 0.1 M phosphate buffer (pH 7.4). Incubation mixtures contained 2 mg mitochondrial protein, and various concentrations of disulfiram, DDTC, DDTC-Me or DETC-Me dissolved in

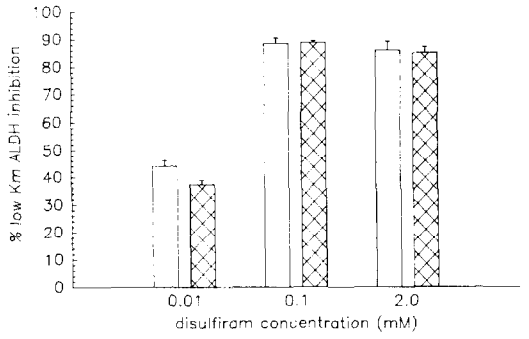

Fig. 1. Effect of a microsomal activating system on RLM low K_m ALDH inhibition by disulfiram. Rat liver mitochondrial preparations were incubated in either the presence ⊠ or absence □ of a liver microsomal activating system for 60 min at 37°, and RLM low K_m ALDH was determined (see Methods). The data are the means ± SEM from four replicate incubations. Control RLM low K_m ALDH was 9.41 ± 0.45 nmol NADH formed/min/mg protein.

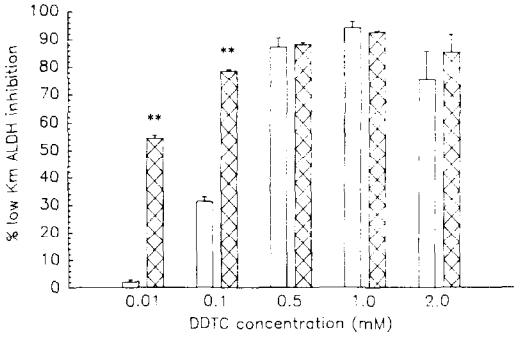

Fig. 2. Effect of a microsomal activating system on RLM low K_m ALDH inhibition by diethyldithiocarbamate (DDTC). Rat liver mitochondrial preparations were incubated in either the presence ⊠ or absence □ of a liver microsomal activating system for 60 min at 37°, and RLM low K_m ALDH was determined (see Methods). The data are the means ± SEM from four replicate incubations. Control RLM low K_m ALDH was 11.6 ± 0.44 nmol NADH formed/min/mg protein. Key: (**) $P < 0.01$ when compared to group without microsomes added.

ethanol. The mitochondria were incubated in either the presence or absence of a microsomal activating system containing an NADPH-generating system (1 unit glucose-6-phosphate dehydrogenase, 10 μmol glucose-6-phosphate, 1 μmol NADP) and 2.0 mg of microsomal protein prepared as previously described. The total assay volume was 1.5 mL. In control incubations, 100 μL of absolute ethanol was substituted for 100 μL of disulfiram or the respective metabolites. Replicate tubes were incubated at 37° for 60 min. After incubation, the mitochondria were again isolated by centrifugation for 10 min and the 2 mg of mitochondrial protein was resuspended in 625 μL of sucrose buffer. Sodium deoxycholate (2 mg) was added to solubilize the mitochondrial membranes, and the RLM low K_m ALDH was determined by the method of Tottmar *et al.* [18] using 0.05 mM acetaldehyde as the substrate. Reference cuvettes contained all reaction components except acetaldehyde. No background activity was detected.

Data analysis. Differences between two group means were determined by using Student's *t*-test. For testing differences between more than two groups, a one-way analysis of variance was performed followed by *a posteriori* multiple comparisons of group means carried out by the Student–Newman–Keuls analysis.

RESULTS

In vitro studies describing the effects of disulfiram, DDTC, DDTC-Me and DETC-Me on RLM low K_m ALDH with and without the microsomal activating system added are given in Figs. 1–3. Disulfiram, at a concentration of 0.01, 0.1 or 2 mM, inhibited RLM low K_m ALDH 44, 89 and 86%, respectively. The addition of liver microsomes to the *in vitro* mitochondrial incubation did not increase RLM low K_m ALDH inhibition (Fig. 1). DDTC, at concentrations of 0.01 and 0.1 mM inhibited RLM

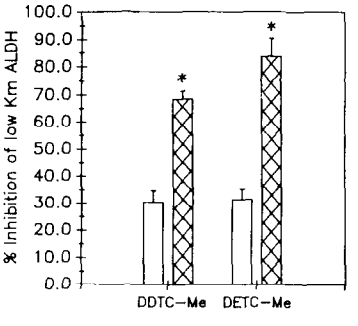

Fig. 3. Effect of a microsomal activating system on RLM low K_m ALDH inhibition by diethyldithiocarbamate-methyl ester (DDTC-Me) and *S*-methyl-*N*,*N*-diethyl-thiolcarbamate (DETC-Me). Rat liver mitochondrial preparations were incubated with 2.0 mM DDTC-Me or DETC-Me in either the presence ⊠ or absence □ of a liver microsomal activating system for 60 min at 37°, and RLM low K_m ALDH was determined (see Methods). The data are the means ± SEM from four replicate incubations. Control RLM low K_m ALDH was 11.1 ± 0.18 nmol NADH formed/min/mg protein. Key: (*) $P < 0.05$ when compared to group without microsomes added.

low K_m ALDH 2 and 31%, with this inhibition increasing to 54 and 78%, respectively, after the addition of rat liver microsomes to the incubation (Fig. 2). Maximal inhibition of RLM low K_m ALDH appears to have been reached at concentrations of DDTC greater than 0.5 mM, and therefore the addition of liver microsomes did not further increase ALDH inhibition. Also, the incubation of rat liver mitochondria with 2.0 mM DDTC-Me and DETC-Me both produced a 30% inhibition of the low K_m ALDH. Inclusion of the cytochrome P450 containing microsomal system in the incubation increased RLM low K_m ALDH inhibition to 70 and 85%, respectively (Fig. 3). These data suggest that further metabolism

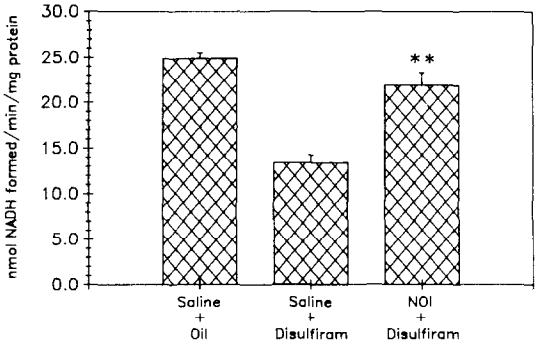

Fig. 4. Effect of *N*-octylimidazole (NOI) on *in vivo* RLM low K_m ALDH inhibition by disulfiram. NOI (20 mg/kg, i.p.) was administered 30 min prior to disulfiram (75 mg/kg, i.p.). The rats were killed 2 hr later and RLM low K_m ALDH activity was determined (see Methods). The data are the means ± SEM from four rats. Key: (**) $P < 0.01$ when compared to saline plus disulfiram group.

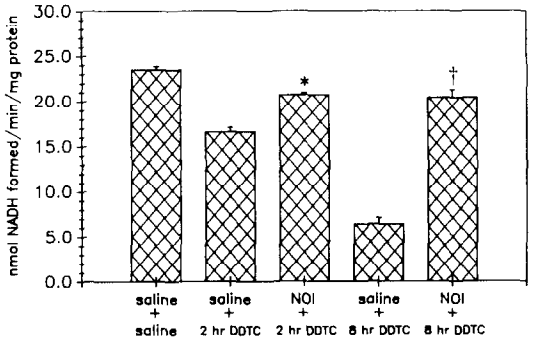

Fig. 5. Effect of *N*-octylimidazole (NOI) on *in vivo* RLM low K_m ALDH inhibition by diethyldithiocarbamate (DDTC). NOI (20 mg/kg, i.p.) was administered 30 min prior to DDTC (114 mg/kg, i.p.). The rats were killed either 2 or 8 hr later and RLM low K_m ALDH was determined. The data are the means ± SEM from four rats. Key: (*) $P < 0.05$ when compared to saline plus 2 hr DDTC; and (†) $P < 0.05$ when compared to saline plus 8 hr DDTC.

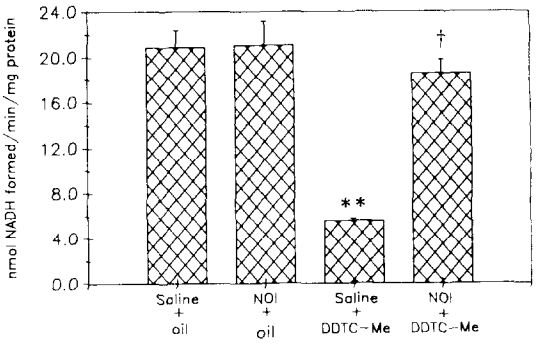

Fig. 6. Effect of *N*-octylimidazole (NOI) on *in vivo* RLM low K_m ALDH inhibition by diethyldithiocarbamate-methyl ester (DDTC-Me). NOI (20 mg/kg, i.p.) was administered 30 min prior to DDTC-Me (41.2 mg/kg, i.p.). The rats were killed 2 hr later and RLM low K_m ALDH activity was determined. The data are the means ± SEM from four rats. Key: (**) $P < 0.01$ when compared to saline plus oil-treated rats; and (†) $P < 0.01$ when compared to saline plus DDTC-Me-treated group.

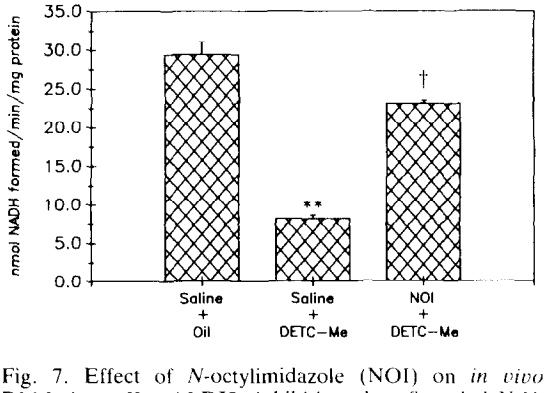

Fig. 7. Effect of *N*-octylimidazole (NOI) on *in vivo* RLM low K_m ALDH inhibition by *S*-methyl-*N*,*N*-diethylthiolcarbamate (DETC-Me). NOI (20 mg/kg, i.p.) was administered 30 min prior to DETC-Me (18.6 mg/kg, i.p.). The rats were killed 2 hr later and RLM low K_m ALDH was determined. The data are the means ± SEM from four rats. Key: (**) $P < 0.01$ when compared to saline plus oil-treated rats; (†) $P < 0.01$ when compared to saline plus DETC-Me-treated rats.

of DDTC, DDTC-Me and DETC-Me is required for the *in vitro* inhibition of RLM low K_m ALDH.

To investigate the importance of cytochrome P450 monooxygenase in the *in vivo* metabolism of disulfiram to the active chemical species responsible for ALDH inhibition, the cytochrome P450 inhibitor NOI was employed [19]. Disulfiram alone inhibited RLM low K_m ALDH 46%. When rats were pretreated with NOI (20 mg/kg, i.p.) 30 min before disulfiram administration and then killed 2 hr later, ALDH was inhibited only 12% (Fig. 4), which was not statistically different from the control group. DDTC also inhibited RLM low K_m ALDH, with ALDH inhibited 30% when rats were killed 2 hr, and 68% when killed 8 hr after DDTC administration. However, NOI pretreatment antagonized DDTC-induced inhibition of RLM low K_m ALDH in that ALDH was inhibited approximately 12% regardless of whether rats were killed 2 or 8 hr after DDTC (Fig. 5). Furthermore, NOI alone had no effect on RLM low K_m ALDH (Fig. 6). NOI also antagonized DDTC-Me- and DETC-Me-induced RLM low K_m ALDH inhibition. For example, DDTC-Me inhibited the low K_m ALDH approximately 72%, but only 10% in rats treated with NOI (Fig. 6), while DETC-Me without prior NOI and after NOI administration inhibited rat liver mitochondrial ALDH 73 and 22%, respectively (Fig. 7). Similar to the studies with DDTC, RLM low K_m ALDH in the NOI plus DETC-Me-treated group was still less than in the control (saline) group, suggesting that the antagonism was not complete.

DISCUSSION

The finding that both disulfiram and DDTC exhibit similar liver ALDH time–response profiles *in vivo* [9], coupled with the relative ineffectiveness of DDTC as an *in vitro* ALDH inhibitor [3, 20], have been the basis for the suggestion that oxidation of DDTC to disulfiram is necessary for the *in vivo* inhibition of liver ALDH by DDTC. It now appears that RLM low K_m ALDH is inhibited *in vivo* not by disulfiram, but rather by a metabolite of disulfiram. Support for this suggestion is provided by the data summarized in Figs. 1–7.

Disulfiram is metabolized to DDTC, DDTC-Me and DETC-Me, all of which inhibit RLM low K_m ALDH *in vivo*, all exhibit similar inhibitory profiles, and all produce a disulfiram–ethanol reaction in rats [12, 13, 15]. NOI antagonism of disulfiram-induced RLM low K_m ALDH inhibition (Fig. 4) suggests that a P450 mediated pathway is important for the formation of a chemical entity responsible for the ALDH inhibition. If disulfiram was the active chemical species *in vivo*, then prior treatment of rats with the cytochrome P450 inhibitor NOI should not have attenuated the disulfiram-induced ALDH inhibition. Disulfiram is reduced rapidly to DDTC by glutathione reductase in erythrocytes [7] and then further metabolized. Formation of DDTC, however, is not a P450-dependent pathway, and therefore antagonism of RLM low K_m ALDH inhibition by NOI in disulfiram-treated rats must involve a metabolic pathway after the reduction of disulfiram to DDTC. Because NOI antagonized the inhibition of RLM low K_m ALDH by DDTC *in vivo* (Fig. 5), this also suggests that a chemical species other than DDTC is responsible for the ALDH inhibition. DDTC has been reported to be oxidized to disulfiram by a cytochrome P450-dependent mechanism *in vitro* [21]. It could be argued that inhibition of cytochrome P450 by NOI blocked the formation of disulfiram from DDTC. However, DDTC is methylated rapidly [22] forming DDTC-Me, which is then further metabolized in the liver to DETC-Me [15]. Both DDTC-Me [12] and DETC-Me [15] are potent *in vivo* RLM low K_m ALDH inhibitors, but not *in vitro*, unless liver microsomes are included in the incubation (Fig. 3). Furthermore, DDTC-Me does not reversibly form DDTC or disulfiram [22, 23], and DETC-Me also did not form DDTC *in vivo* (unpublished results). Yet, inhibition of RLM low K_m ALDH by both DDTC-Me and DETC-Me was blocked by NOI (Figs. 6 and 7). These data therefore suggest that further metabolism of not only DDTC, but also DDTC-Me and DETC-Me must take place *in vivo* in order for RLM low K_m ALDH inhibition to occur.

Inhibition of RLM low K_m ALDH by DDTC was found to be greater when rats were killed 8 hr rather than 2 hr after DDTC administration. The reason for this difference is not clear, but may be due to the poor lipid solubility of the thiol which would require a longer period of time to cross cell membranes in the liver and subsequent methylation [12]. Of interest is that NOI administered prior to DDTC antagonized RLM low K_m ALDH inhibition regardless of whether rats were killed 2 or 8 hr after DDTC dosing, further supporting the importance of subsequent DDTC metabolism in the inhibition of RLM low K_m ALDH.

The apparent importance of cytochrome P450 in disulfiram's mechanism of action is further illustrated from the *in vitro* studies with disulfiram, DDTC, DDTC-Me and DETC-Me (Fig. 1–3). Disulfiram inhibited RLM low K_m ALDH *in vitro* in a concentration-dependent manner (Fig. 1), consistent with studies by others [20, 24]. Inclusion of rat liver microsomes in the mitochondrial incubation did not further increase ALDH inhibition regardless of the concentration of disulfiram in the mitochondrial incubation (Fig. 1). This is to be expected since disulfiram is already in the oxidized state. Inhibition of ALDH *in vitro* by DDTC also was found to be concentration dependent. For example, no inhibition of RLM low K_m ALDH inhibition was found at 0.01 mM, whereas at 0.1 mM, RLM low K_m ALDH was inhibited 30%. At DDTC concentrations of 0.5 mM or greater, RLM low K_m ALDH was inhibited almost 90% (Fig. 2). This concentration dependency for DDTC also has been observed by other investigators [20]. The reason for the concentration dependency exhibited by DDTC is not clear. Contamination of the incubation mixture by disulfiram formed as a result of air oxidation of DDTC may provide one explanation. The possibility that high concentrations of DDTC can inhibit ALDH by a non-specific mechanism also cannot be ruled out. Similarily, a non-specific mechanism could explain the 30% inhibition of RLM low K_m ALDH produced by 2.0 mM DDTC-Me or DETC-Me (Fig. 3), since lower concentrations of both of these methyl esters produced insignificant RLM low K_m ALDH inhibition (unpublished results). The addition of liver microsomes to the mitochondrial incubation containing 0.01 and 0.1 mM DDTC increased ALDH inhibition to 54 and 78%, respectively, whereas at concentrations of 0.5 mM and greater, no additional increase in RLM low K_m ALDH inhibition was observed (Fig. 2). Since RLM low K_m ALDH appears to be inhibited maximally at DDTC concentrations of 0.5 mM and greater, the lack of any additional increase in ALDH inhibitory activity after inclusion of liver microsomes is not unrealistic. The reason for the increase in RLM low K_m ALDH inhibition by low concentrations of DDTC when microsomes were added to the *in vitro* incubation (Fig. 2) is probably due to oxidation of DDTC to disulfiram as proposed by Masuda [21]. Formation of DDTC-Me is unlikely because the liver microsomes added to the *in vitro* mitochondrial incubation containing DDTC lacked *S*-adenosylmethionine, which is required for DDTC methylation to DDTC-Me [22], thus precluding methylation and subsequent metabolism. DDTC-Me and DETC-Me were both relatively inactive as RLM low K_m ALDH inhibitors *in vitro*, unless the P450 system was included, at which time ALDH inhibition increased to 70 and 80%, respectively (Fig. 3). DDTC-Me does not reversibly form either DDTC or disulfiram [23], nor was there any evidence of DDTC-Me formation after incubation of DETC-Me *in vitro* (unpublished results). Thus, inhibition of RLM low K_m ALDH observed after the addition of the microsomal

enzyme system to the liver mitochondria must be due to the metabolism of DDTC-Me and DETC-Me. These *in vitro* data are therefore consistent with the *in vivo* studies.

In conclusion, disulfiram was an effective RLM low K_m ALDH inhibitor *in vitro*. DDTC, DDTC-Me and DETC-Me were ineffective, unless the concentrations used were large, or liver microsomes were included in the incubation. *In vivo* inhibition of RLM low K_m ALDH by DETC- Me is > DDTC-Me > DDTC > disulfiram [15]. It is proposed that *in vivo*, disulfiram *per se* plays only a minor role in RLM low K_m ALDH inhibition, and that a metabolite of disulfiram, yet to be identified, is the chemical species responsible for inhibition of the RLM low K_m ALDH. Although both Vallari and Pietruszko [5] and Kitson [6] have provided an elegant explanation for the mechanism of liver ALDH inhibition by disulfiram *in vitro*, this may not be the mechanism by which liver ALDH is inhibited by disulfiram *in vivo*. The important role of disulfiram metabolism in liver ALDH inhibition may also provide an explanation as to why some individuals receiving disulfiram can drink with impunity and not experience a disulfiram–ethanol reaction [25]. This would occur if the active chemical species responsible for liver ALDH inhibition is not sufficiently formed because of a defect in disulfiram metabolism.

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