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Pretranslational and Posttranslational Regulation of Rat Hepatic CYPs 3A2 and 2E1 by Disulfiram

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ABSTRACT. The aldehyde dehydrogenase inhibitor disulfiram (DS) has been used to deter drinking in alcoholics, but it also precipitates pharmacokinetic interactions with coadministered drugs. From previous experiments conducted *in vitro*, it has been proposed that the ethanol-inducible cytochrome P450 2E1 (CYP2E1) is the major target for inhibition by DS, but the inference from reported drug interactions is that the drug inhibits multiple CYPs. The aim of the present study was to evaluate the inhibition of major constitutive CYPs in rat liver by DS. Thus, the effects of DS on activities mediated by CYPs 2A1/2, 2C11, 2E1, and 3A, which constitute ~80% of total CYPs in male rat liver, were evaluated. It was found that CYP2E1-mediated aniline 4-hydroxylase activity was weakly inhibited by DS *in vitro*, but that preincubation of the drug with NADPH-supplemented microsomes to generate metabolites of DS enhanced the extent of inhibition somewhat. In contrast, constitutive testosterone hydroxylases were inhibited effectively at low concentrations of DS (20 μM decreased the activities of all hydroxylation pathways to 40–60% of control), and a preincubation step between DS and NADPH-fortified microsomes enhanced the inhibition of CYP2C11 and 3A2 activities. *In vivo* studies were undertaken in which a single dose of DS (100 mg/kg, i.p.) was administered to rats; 24 hr later, CYP2E1-mediated aniline 4-hydroxylase activity was decreased to about 50% of the activity in untreated control rats. CYP2E1 apoprotein and mRNA were also decreased to 38% of the respective control, and CYP3A apoprotein and CYP3A2 mRNA responded similarly. In contrast, CYP2C11 apoprotein was decreased to 66% of control after DS administration, and CYP2A1 expression was unchanged. These findings establish that multiple CYPs are targets for inhibition by DS and provide a basis for clinically significant drug interactions involving CYPs other than 2E1. In addition, the *in vivo* modulation of CYP function by DS administration is not restricted to enzyme inactivation and may also include down-regulatory effects mediated at a pretranslational level. BIOCHEM PHARMACOL **54**;12: 1323–1329, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. disulfiram; cytochrome P450; mechanism-based inactivation; gene regulation; pharmacokinetic interactions

DS§ (tetraethylthiuram disulfide) has been used in the treatment of alcoholism because it inhibits hepatic aldehyde dehydrogenase activity and thereby promotes the accumulation of acetaldehyde, a toxic metabolite of ethanol [1]. Subsequent studies have reported that other enzymes, including dopamine β-hydroxylase and cytochrome P450 2E1 (CYP2E1), are also inhibited by DS [2, 3]. This effect on CYP2E1 may be significant for nitrosamine activation [4]. Indeed, certain organosulfur compounds have received attention for their potential to inhibit nitrosamine carcinogenesis [5].

CYPs catalyze the oxidation of thiono-sulfur-containing compounds, such as carbon disulfide and parathion [6, 7]. However, CYPs are also inactivated by reactive metabolites formed from these agents, and irreversible heme/protein modification is central to the observed extent of CYP inhibition [8, 9]. It has been suggested that CYP2E1 is the major target for inhibition by DS and other sulfur-containing compounds, but there is evidence that other CYPs may also be susceptible. Thus, DS precipitates pharmacokinetic interactions with antipyrine and centrally acting agents like phenytoin, morphine, amphetamine, meperidine, chlordiazepoxide, hexobarbital, and thiopental [10–12]. Symptoms of toxicity are often exaggerated therapeutic effects, but, with the antiprotozoal drug metronidazole, more serious adverse effects include confusion and acute psychosis [13]. In contrast, tolbutamide elimination is unaffected during DS therapy [10], which suggests that 2C subfamily CYPs may be refractory to inhibition. These observations are consistent with inhibition of multiple CYPs and cannot be explained solely in terms of CYP2E1 inhibition.

The present study assessed the capacity of DS to modulate major constitutive CYPs in rat liver *in vitro* and *in vivo*. Thus, the capacity of DS to inhibit and inactivate CYPs

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§ *Abbreviations:* DS, disulfiram; CYP or P450, cytochrome P450; and NADPH-CYP-reductase, NADPH-cytochrome P450-reductase.

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2A1/2, 2C11, 2E1, and 3A (which from immunochemical determinations constitute about 80% of the total CYP in male rat liver [14, 15]) was evaluated in microsomal incubations *in vitro*. Comparative studies were conducted *in vivo* to evaluate the effects of DS on CYP expression. As anticipated from the findings of earlier studies, CYP2E1 was inhibited by DS *in vitro*, but the drug exerted a more potent effect *in vivo*. CYP2E1 mRNA was markedly down-regulated after a single dose of DS. It also emerged that CYP3A2, the major testosterone 6β-hydroxylase in untreated male rat liver, was down-regulated at a pretranslational level by DS.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Testosterone (sp. act. 56–59 mCi/mmol) and [γ-^{32}P]ATP (sp. act. 5000 Ci/mmol) were obtained from Amersham Australia (North Ryde, NSW, Australia). Steroid standards were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), Steraloids (Wilton, NH, U.S.A.), and the MRC Steroid Reference Collection (Queen Mary's College, London, U.K.). DS and biochemicals used in enzyme assays were purchased from Sigma. HPLC grade solvents and analytical reagent grade chemicals were obtained from Rhone-Poulenc and Ajax (Sydney, Australia), respectively. CYP-specific oligonucleotides were obtained from Bresatec (Adelaide, South Australia), and the 18S RNA oligonucleotide 5′-CGG-CAT-GTA-TTA-GCT-CTA-GAA-TTA-CCA-CAG-3′ [16] was obtained from Pac-Bio Pty Ltd. (Rushcutters Bay, NSW, Australia).

Animals and Preparation of Hepatic Microsomal Fractions

The experimental procedures were approved by the Western Sydney Area Health Service Animal Ethics Committee. Male Wistar rats (~250 g) were obtained from the Department of Animal Care at Westmead Hospital and were held in cages at constant temperature and lighting (12 hr day/night cycle).

Animals received a single dose of DS (100 mg/kg, i.p., in corn oil) and were killed 24 hr later. Livers were removed, perfused with cold saline, and snap frozen for storage at −70°, until required for RNA isolation or the preparation of microsomal fractions. The final (washed) microsomal pellets were suspended in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA, and were frozen in liquid nitrogen for storage at −70° until used in experiments [17]. Microsomal protein was estimated by the procedure of Lowry *et al.* [18] and total holo-P450 by the spectrophotometric procedure of Omura and Sato [19].

Assays of Microsomal Testosterone Hydroxylation

[^{14}C]Testosterone (50 μM; 0.18 μCi/0.4 mL incubation) hydroxylation reactions (0.15 mg microsomal protein; 37°) were conducted in potassium phosphate buffer (0.1 M, pH 7.4; 1 mM EDTA) [9]. NADPH (1 mM) was used to start incubations, which were terminated after 2.5 min by the addition of chloroform (5 mL) and removal to an ice bath. After extraction, the organic phase was evaporated to dryness, and the residue was dissolved in a small volume of chloroform and applied to TLC plates (Merck silica gel 60 F_{254} type; Darmstadt, Germany) that were developed in dichloromethane:acetone (4:1) and then in chloroform: ethyl acetate:ethanol (4:1:0.7), with air drying in between [20]. Radioactive products were detected by autoradiography (Hyperfilm-MP; Amersham) over 40–60 hr and quantified by β-scintillation spectrometry (ACS II; Amersham).

In some experiments, DS was added to microsomal incubations for the determination of P450 inactivation. Thus, DS was preincubated at 37° in NADPH-supplemented microsomes for 20 min prior to transfer to tubes containing the steroid substrate; control incubations, from which DS was omitted, were run in parallel. Steroid hydroxylation reactions were conducted and activities were determined as described above.

Assay of Microsomal Aniline 4-Hydroxylation

Aniline 4-hydroxylase activity was monitored by the determination of 4-aminophenol formation as described previously [21]. Reactions (0.6 mL) contained 1.6 mg microsomal protein and 5 mM aniline. In inactivation experiments, components with the exception of substrate were incubated (at 37°) with NADPH-supplemented microsomes for 20 min and then transferred to tubes containing aniline. After 12 min, reactions were stopped with 10% trichloroacetic acid, and metabolite formation was quantified colorimetrically.

Detection of CYPs in Rat Liver Microsomes by Immunoblotting

Microsomal fractions (6 μg protein each lane, with the exception of blots for CYP2E1 in which 10 μg was loaded; response rates around these protein loadings were in the linear range) were heated for 5 min at 100° with 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and loaded onto vertical polyacrylamide gels (7.5%; 15 lanes per side). Gels were electrophoresed overnight at ~10 mA per side, and then proteins were transferred electrophoretically to nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany). Nitrocellulose sheets were then washed sequentially in the solutions described previously [22].

Several primary antibodies were available for these studies (dilutions used in immunoblotting are indicated in parentheses). The anti-CYP2C11 IgG (3.7 μg protein/mL) and anti-CYP2E1 IgG (4.1 μg protein/mL) have been described previously [9, 23], and anti-CYP3A (3.7 μg protein/mL) was provided by Dr. A. Astrom, Karolinska Institutet. CYP2A1 was isolated from female rat liver and was used to immunize female NZ rabbits; the anti-CYP2A1

TABLE 1. Effect of disulfiram treatment on cytochrome P450, aniline 4-hydroxylation, and testosterone hydroxylation in rat hepatic microsomes

Treatment	holo-P450 (nmol/mg protein)	Aniline 4-hydroxylation (nmol/mg protein/min)	Hydroxytestosterone metabolite (nmol/mg protein/min)			
			2α-	6β-	7α-	16α-
None (control)	1.06 ± 0.04	0.97 ± 0.05	2.70 ± 0.17	4.31 ± 0.38	0.48 ± 0.04	2.82 ± 0.20
Disulfiram (100 mg/kg)	0.77 ± 0.06*	0.47 ± 0.07†	1.68 ± 0.17*	1.47 ± 0.14†	0.28 ± 0.02*	2.06 ± 0.19‡
% of Control	73	48	62	34	58	73

Data are means ± SEM from four individual rats in each group.
*–‡ Significantly different from control: *$P < 0.01$, †$P < 0.001$, and ‡$P < 0.05$.

IgG selectively inhibited CYP2A1-mediated steroid 7α-hydroxylation (0.08 μg protein/mL, after cross-adsorption against CYP2A1 immobilized on CNBr-activated Sepharose 4B; not shown). NADPH-CYP-reductase was purified from male rat liver, and an anti-NADPH-CYP-reductase IgG was raised in female NZ rabbits (4.6 μg protein/mL). CYP and NADPH-CYP-reductase immunoreactive proteins were detected on Hyperfilm-MP (Amersham) by enhanced chemiluminescence and autoradiography. The resultant signals were analyzed by laser densitometry (LKB Ultroscan-XL, Bromma, Sweden).

Extraction of Total RNA from Rat Liver and Analysis of CYP mRNAs

Total RNA was extracted from male rat liver by the RNA-easy method (Qiagen, Clifton Hill, Australia). For analysis of CYP3A2 mRNA, the synthetic 30-mer oligonucleotide described previously [24], and complementary to nucleotides 1594–1623 of the published cDNA sequence [25], was employed. For CYP2E1 mRNA, the synthetic 24-mer oligonucleotide described by Sundseth and Waxman [24], and complementary to nucleotides 930–953 of the published cDNA sequence [26] was employed. The oligonucleotides were labeled using [γ-^{32}P]ATP and polynucleotide kinase. In northern analysis, total RNA (10 μg) was electrophoresed on 1.2% agarose in the presence of 2.2 M formaldehyde and then transferred to Hybond-N$^+$ nylon filters (0.45 μm, Amersham) [27]. Hybridization and washing conditions were as described previously [28], and signals corresponding to CYP mRNAs were quantified using a Molecular Dynamics Phosphorimager. To demonstrate equivalence of RNA loading between samples, filters were stripped and rehybridized to a γ-^{32}P-labeled probe to 18S ribosomal RNA.

Statistics

Data are presented as means ± SEM. Differences between means were detected using Student's *t*-test.

RESULTS

Effects of DS Administration on Microsomal CYP Function and Expression

A single injection of DS 24 hr before death elicited a 27% decrease in the microsomal content of total holo-P450 in rat liver (0.77 ± 0.06 vs 1.06 ± 0.04 nmol/mg protein in control; Table 1; $P < 0.01$). DS has been proposed as an inhibitor of CYP2E1 [3], but the observed 27% decrease in holo-P450 was not consistent with (lower) literature estimates of 2E1 expression in liver [15]. The selectivity of CYP loss produced by DS was investigated further. From Table 1 it is apparent that aniline 4-hydroxylation, an activity mediated primarily by CYP2E1, was indeed decreased after DS administration (to 48% of control; $P < 0.001$). However, significant decreases in the activities of several pathways of testosterone hydroxylation mediated by other CYPs suggested that the expression of CYP3A2 and, to a lesser extent, CYP2C11 was also impaired by DS administration (Table 1). The effect of DS administration on CYP3A2-dependent steroid 6β-hydroxylation was most pronounced (to 34% of control activity: 1.47 ± 0.14 vs 4.31 ± 0.38 nmol/mg protein/min). The 2α-/16α-hydroxylations of the steroid are mediated in male rat liver by CYP2C11 and were decreased to 62% ($P < 0.01$) and 73% ($P < 0.05$) of the respective control. A decrease in testosterone 7α-hydroxylation activity was apparent, but it is noteworthy that the 15β-hydroxy metabolite (which is formed by CYPs 3A) is not resolved completely in this chromatographic system [20].

Effects of DS Administration on CYP Apoprotein and mRNA Expression

The observed effects of *in vivo* DS administration on the activities of major constitutive CYP enzymes were investigated further. A series of immunoblot analyses was performed to determine the effects of DS administration on the microsomal contents of specific CYP apoproteins (Fig. 1). There was close correspondence between the decreases in CYP activities (Table 1) and CYP apoproteins. Thus, CYP2E1 and CYP3A immunoreactive proteins were decreased to 38 ± 4 and 37 ± 8% of the respective control. Less pronounced effects on CYPs 2C11 and 2A1 were observed. The relative expression of CYP2C11 apoprotein was decreased after DS administration to 66 ± 4% of control, and there was a trend toward a decline in the relative expression of CYP2A1 apoprotein, but this did not attain statistical significance. The apparent decrease in CYP2A1-dependent 7α-hydroxylation to 58% of control may include a contribution from CYP3A-mediated steroid

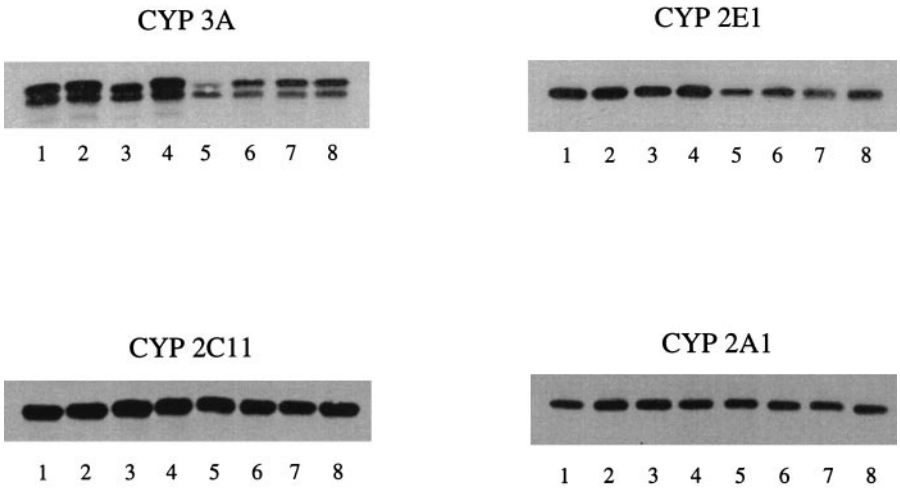

FIG. 1. Western immunoblots of CYP3A, CYP2E1, CYP2C11, and CYP2A1 immunoreactive protein in hepatic microsomes from untreated (lanes 1–4) and DS-treated (lanes 5–8) male rats. The appearance of at least two proteins immunoreactive with the anti-CYP3A IgG is typical in our Wistar rat strain.

15β-hydroxylation. In contrast to these findings, microsomal NADPH-CYP-reductase immunoreactive protein was not affected significantly by DS administration.

Because the effects of DS administration on CYPs 3A2 and 2E1 expression were so pronounced, the level of the regulatory impairment was investigated further by northern analysis. From Fig. 2 it is apparent that the mRNAs for both hepatic CYPs were decreased markedly 24 hr after administration of a single dose of DS to rats; these decreases were to 10 ± 1 and 38 ± 4% of respective control values.

In Vitro Inactivation of CYP-Dependent Microsomal Oxidation Activities by DS

In vivo experiments in rats established that DS administration decreases the expression of several CYPs in rat hepatic microsomes. However, DS is also known to modulate CYP2E1 function *in vitro* by inhibition and mechanism-based inactivation. Therefore, the capacity of DS to inhibit constitutive CYP enzymes directly and in a time-dependent fashion was assessed in this study. As indicated in Fig. 3, DS was relatively nonpotent as an inhibitor of CYP2E1-dependent aniline 4-hydroxylation (only ~30% inhibition at 250 μM DS), although preincubation of DS with NADPH-supplemented microsomes for 20 min enhanced the observed inhibition about 2-fold. In contrast, constitutive steroid hydroxylases appeared more susceptible than CYP2E1 to inhibition by DS. Thus, 20 μM DS elicited marked direct inhibition (decreases of the order of 40–60%) of all four principal pathways of testosterone hydroxylation (Fig. 3). Preincubation enhanced the apparent inhibition of pathways mediated by CYPs 2C11 and 3A2 (2α-/16α- and 6β-hydroxylation, respectively), but not CYP2A1-dependent 7α-hydroxylation, at quite low concentrations of DS (5–10 μM). Thus, CYPs 2C11 and 3A2 appeared much more susceptible than CYP2E1 to mechanism-based inactivation during DS oxidation.

DISCUSSION

CYP2E1 catalyzes the oxidation of numerous small molecules, such as benzene and nitrosamines, to toxic metabolites. Thus, CYP2E1 inhibition may be a viable mechanism for the protection of the liver and other organs from toxic metabolites. DS and its reduced metabolite, diethyldithiocarbamate, have been considered as CYP2E1 inhibitors. However, from the present study, DS clearly modulates the activities of CYPs other than 2E1, and similar findings have been reported for diethyldithiocarbamate [29]. Indeed, DS was a more potent inhibitor of the major rat hepatic constitutive CYPs 2C11 and 3A2 than of CYP2E1. Similarly, diethyldithiocarbamate was found to inhibit the activities of human hepatic CYPs 2A6, 2B6, 2C8, and 3A3 somewhat more effectively than that of CYP2E1 [29]. At low concentrations of diethyldithiocarbamate, CYPs 2A6 and 2B6 were inhibited markedly even though inhibition of CYP2E1 was minimal. This is reminiscent of the present

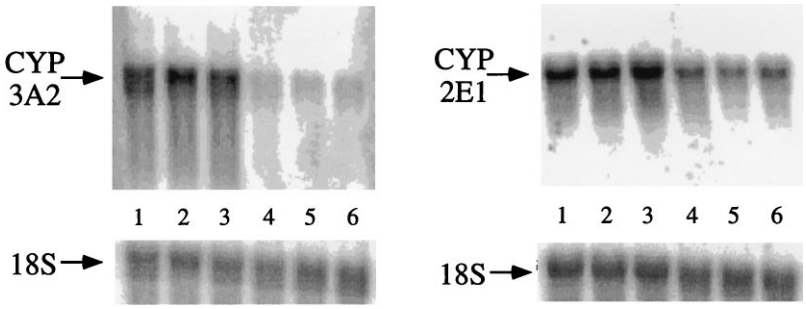

FIG. 2. Northern analysis for CYP3A2 and CYP2E1 mRNAs in liver from untreated (lanes 1–3) and DS-treated (lanes 4–6) male rats. The 18S signal was used to correct for differences in RNA loading prior to quantification.

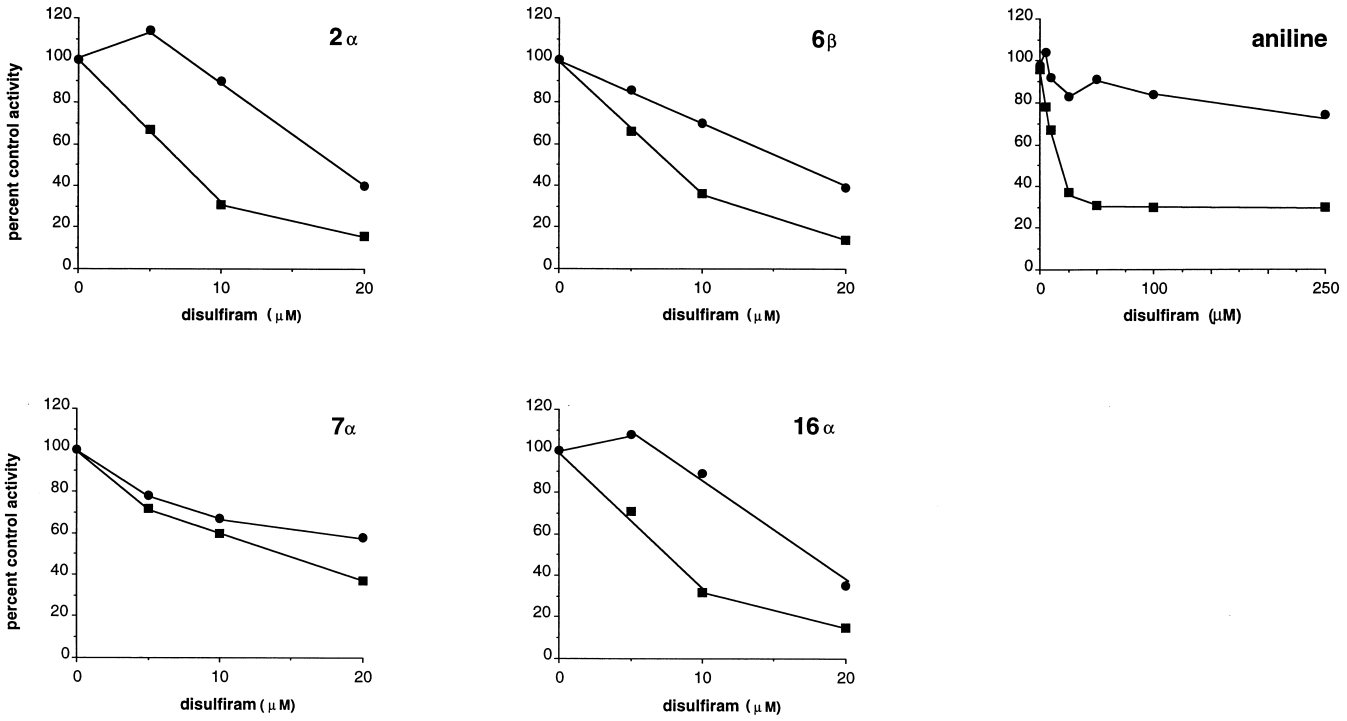

FIG. 3. Effect of DS on microsomal testosterone 2α-, 6β-, 7α-, and 16α-hydroxylations and aniline 4-hydroxylation *in vitro*. Key: (■) activity determined after a 20-min preincubation step between NADPH, microsomes, and DS, or (●) in the absence of preincubation. Control reactions were run in parallel. Control activities were: 1.94, 2.87, 0.47, and 2.01 nmol metabolite/mg protein/min for testosterone 2α-, 6β-, 7α-, and 16α-hydroxylation, respectively, and 0.47 nmol 4-aminophenol/mg protein/min. Each value is the mean derived from duplicate experiments that varied by less than 10%.

observations that constitutive steroid hydroxylases (CYPs 2A1, 2C11, and 3A2) were inhibited at much lower concentrations than those required for CYP2E1 inhibition. Studies in humans and rodents indicate that DS can influence the elimination of a number of coadministered drugs [10–13, 30, 31]. Thus, observations regarding the lack of specificity of DS and diethyldithiocarbamate as CYP2E1 inhibitors are consistent with clinical experience.

Selective inhibitors of particular CYPs are potential tools in the study of microsomal drug oxidations, but few such chemicals have been identified. Mechanism-based inactivation by suicide processing or metabolite complexation is one approach to enhance the selectivity of CYP inhibition. Thiono-sulfur compounds, exemplified by DS and the pesticide parathion, constitute an important chemical class of mechanism-based inactivators of CYPs. Inactivation of CYPs by thiono-sulfur compounds occurs during their oxidative biotransformation and results in the binding of atomic sulfur to the CYP apoprotein. Most of the studies describing such mechanisms have been done with parathion and carbon disulfide [6–9], but this process is likely to be applicable to other sulfur-containing agents.

Although *in vitro* inhibition of CYPs by DS clearly involves inactivation, it is apparent from the present study that DS exerts effects on CYPs at multiple levels. Thus, CYPs 2E1 and 3A2 were down-regulated at a pretranslational level in liver from DS-treated male rats. Similar findings have been reported with other drugs and chemicals, such as chlormethiazole [32] and YH439 [33], which impair CYP2E1 transcription in rat liver. Diallyl sulfide and phenethyl isothiocyanate also appeared to produce small decreases in CYP2E1 mRNA levels when administered to rats in experimental diets [34], although acute administration of diallyl sulfide by intraperitoneal injection did not affect CYP2E1 mRNA levels [35]. Thus, the route of administration of certain chemicals may exert differential effects on CYP2E1 expression and function. In the case of chlormethiazole, CYP2E1 was a preferred target in that CYPs 1A1, 2B1, and 3A1/2 were refractory to altered regulation [32]. DS is less selective than chlormethiazole because several CYPs are down-regulated after *in vivo* administration. High concentrations of DS in *in vitro* incubations were required for inactivation, whereas a single dose of the drug produced substantial pretranslational down-regulation *in vivo*. Thus, pretranslational down-regulation and not posttranslational modification may be the predominant mechanism that influences CYP2E1 expression and function after *in vivo* administration of DS. It remains to be established whether DS decreases transcription rates of the *CYP2E1* and *CYP3A2* genes or decreases mRNA stability. Chlormethiazole administration to rats decreased *CYP2E1* run-on transcription rates in hepatic nuclei [32]. However, analogous run-on experiments with *CYP3A* genes would be difficult because there are at least four members of this subfamily in rat liver that exhibit

considerable nucleotide sequence similarity at the 5′-end of their cDNAs [36].

The use of chemical agents to inhibit toxic processes, such as CYP2E1-mediated drug and carcinogen activation, is an attractive possibility. Strategies of this type may have utility in the management of individuals after acute intoxication by CYP2E1 substrates, such as the prevention of CYP2E1-mediated free radical generation after ingestion of ethanol. This can be considered analogous to the use of supportive therapy after poisoning with organophosphorus pesticides [37]. However, it would be desirable that such agents elicit short-term and highly specific effects on CYPs or other protein targets. The present findings with DS, and earlier work with chlormethiazole [32], indicate that complex multifactorial effects on CYPs are possible and may not be restricted to transient inhibition. Thus, effects on *CYP2E1* gene transcription and mRNA concentrations are apparent with these drugs. The possibility of low selectivity for individual CYPs, as highlighted in the present study with DS, would decrease their value as hepatoprotectants.

In conclusion, the present findings that DS inhibits metabolic oxidations by CYPs 2A1/2, 2C11, and 3A, as well as CYP2E1, are consistent with clinical studies suggesting that DS has significant inhibitory activity against multiple CYPs. Indeed these CYPs comprise most of the total holo-CYP in male rat liver. If DS exerted effects on human CYP gene regulation similar to those observed in rat liver, then it is likely that effects on the pharmacokinetics of coadministered drugs would be protracted.

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