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Metabolism of thioridazine by microsomal monooxygenases: relative roles of P450 and flavin-containing monooxygenase

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1. The metabolism of thioridazine by the flavin-containing monooxygenase (FMO) of mouse liver and several P450 isozymes was examined using microsomes, purified FMO, and expressed P450 isozymes. Metabolites were identified by hplc.

2. Thermal inactivation and antibodies to NADPH P450 reductase were used to selectively inactivate FMO and P450 respectively. Inactivation of FMO by heat-treatment reduced the formation of thioridazine-*N*-oxide and northioridazine, whereas inactivation of P450 resulted in decreased amounts of thioridazine-2-sulphoxide, northioridazine, and thioridazine-5-sulphoxide.

3. Liver microsomes from mouse induced with phenobarbital, 3-methylcholanthrene, or acetone were compared with control microsomes. Phenobarbital induction resulted in increased formation of all metabolites except thioridazine-*N*-oxide, while retaining a general metabolic profile similar to that achieved with control microsomes. Neither 3-methylcholanthrene nor acetone induction had any effect on the *in vitro* metabolism of thioridazine.

4. FMO purified from mouse liver produced thioridazine-*N*-oxide as the major metabolite.

5. Preliminary experiments with commercially prepared microsomes made from cells expressing recombinant human liver P450 2D6 and 3A4 suggested that thioridazine is metabolized by 2D6 but not 3A4.

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Introduction

Microsomal monooxygenation is a primary metabolic route whereby xenobiotics are converted to their more polar metabolites. The enzymes responsible for these reactions include the P450 isozymes and the flavin-containing monooxygenases (FMO). Both groups of enzymes are found in hepatic and extrahepatic tissues and catalyze many of the same reactions, including oxidation of secondary and tertiary amines and sulphur and phosphorus heteroatoms. Because of these overlapping substrate specificities, many drugs can, at least in theory, be oxidized by both enzyme systems. We have chosen to focus on phenothiazine derivatives (used as both antipsychotics and as antihistamines) because they represent excellent model substrates for examining the relative roles of both P450 and FMO in the metabolism of pharmacologic agents. The phenothiazine moiety consists of two benzene rings bridged by sulphur (position 5) and nitrogen (position 10) heteroatoms (figure 1). The antipsychotic drug thioridazine (TDZ) (figure 1) is a phenothiazine with a 2-(*N*-methyl-2-piperidyl) ethyl substituent at position 10. In addition, there is a

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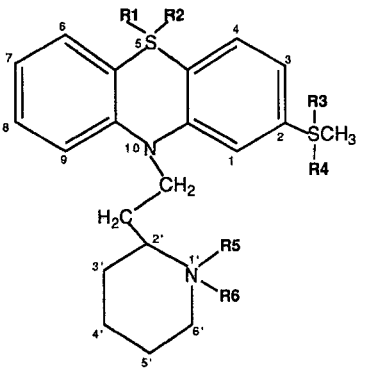

			R1	R2	R3	R4	R5	R6
1	Thioridazine	TDZ	--	--	--	--	CH_3	--
2	Northioridazine	NOR	--	--	--	--	--	--
3	2-Sulphoxide	2SO	--	--	O	--	CH_3	--
4	2-Sulphone	2SO2	--	--	O	O	CH_3	--
5	5-Sulphoxide	5SO	O	--	--	--	CH_3	--
6	N-Oxide	NO	--	--	--	--	CH_3	O
7	2-Sulphoxide-N-Oxide	2SONO	--	--	O	--	CH_3	O
8	Disulphoxide	DiSO	O	--	O	--	CH_3	--
9	Disulphone	DiSO2	O	O	O	O	CH_3	--

Figure 1. Structure of thioridazine and metabolites.

methyl-thioether substituent at position 2. Not only does thioridazine bear several potential sites for monooxygenation by microsomal enzymes, but it is also known that several of its metabolites have biological activity (Kilts *et al.* 1984).

In vivo and *post mortem* studies have shown *S*-oxidation of TDZ to be the primary route of metabolism in humans (Martensson *et al.* 1975, Ng and Crammer 1977, Papadopoulos and Crammer 1986, Lin *et al.* 1993). Significant metabolites include the 2-sulphoxide and 2-sulphone, both of which have increased pharmacologic activity in comparison with the parent compound (Dahl 1982, Kilts *et al.* 1984, Niedzwiecki *et al.* 1984, 1989) and the 5-sulphoxide, which has been linked to the cardiotoxic side effects of TDZ administration (Gottschalk *et al.* 1978, Hale and Poklis 1986). These metabolites, as well as northioridazine (desmethylthioridazine) and the disulphoxide, phenolic and lactam derivatives of TDZ, have been identified in blood and urine of human and rat (Zehnder *et al.* 1962, Muusze and Huber 1974, Papadopoulos and Crammer 1986, Watkins *et al.* 1986, Lin *et al.* 1993). Watkins *et al.* (1986) also noted the presence of TDZ-*N*-oxide in rat urine following a single dose of the parent compound. Because of the thermal lability characteristic of *N*-oxides in general, it was postulated that the spontaneous reduction of this oxidation product may have led to an overestimation of the concentration of TDZ in previous studies (Watkins *et al.* 1986).

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In vitro studies using tissue homogenates have yielded results similar to those found *in vivo*. Traficante *et al.* (1979) examined TDZ metabolism by bovine liver microsomal fractions and noted that TDZ is preferentially sulphoxidized at the 2 position, followed by formation of the 5-sulphoxide and 2-sulphone (Traficante *et al.* 1979).

We are interested in developing the mouse as a model for investigating the relative contributions of P450 and FMO in the metabolism of various compounds. In this study we have used mouse liver microsomes to prepare a semiquantitative metabolic profile for the oxidation of thioridazine. Selective inhibition and induction studies were conducted to distinguish the contribution of the different enzymes. We also purified FMO from mouse liver and examined its specific contribution to the metabolism of this drug. Finally, to complement our findings, we took advantage of the availability of individually expressed human P450s to explore further the role of particular P450 isozymes.

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Materials and methods

Chemicals

Racemic thioridazine-HCl, northioridazine (NOR), thioridazine 2-sulphoxide (2SO), thioridazine 2-sulphone (2SO2), thioridazine 5-sulphoxide (5SO), thioridazine-disulphoxide (DiSO) and thioridazine-disulphone (DiSO2) were supplied by Sandoz Pharmaceuticals (East Hanover, NJ, USA). The purity of these compounds was > 99% as assessed by hplc. Methylamine (hplc grade), 40% solution was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Ammonium hydroxide was purchased from J. T. Baker (Philipsburg, NJ, USA). Methyl-*tert*-butyl ether (hplc grade), dilauroyl phosphatidylcholine, fluorescamine, bovine serum albumin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, tricine, and EDTA disodium salt were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other reagent grade chemicals and hplc grade solvents were from Fisher Scientific (Fair Lawn, NJ, USA).

Preparation of microsomes

Thirteen male CD-1 mice, weighing 19–21 g, were obtained from Charles River Laboratories (Raleigh, NC, USA). Three mice were randomly selected for each treatment group. The remaining four mice were designated as untreated control animals. The animals were housed for 3 days under a 12-h light/dark cycle, fed Prolab Animal Diet: Rat, Mouse, and Hamster 3000 (Agway, Syracuse, NY, USA) and allowed tap water *ad libitum*. Untreated animals were housed under these conditions for an additional 7 days. Acetone induction of mouse liver was accomplished by using 1% (v/v) acetone in drinking water, changing the water each day for 7 days. Phenobarbital and 3-methylcholanthrene inductions were initiated on day 4 in order to synchronize the induction so that all animals could be killed on the same day. Phenobarbital-induced animals were treated with 0·1% (w/v) phenobarbital in the drinking water for 3 days, changing the water daily. Animals induced with 3-methylcholanthrene were treated by intraperitoneal injection with 20 mg/kg in 0·3 ml corn oil for 3 days. On the following day, all animals were killed, their livers were removed, and microsomes were prepared from the pooled livers of each group. Rinsed livers were weighed, and then placed in homogenization buffer (50 mM potassium phosphate, pH 7·5, with 0·1 mM EDTA and 1·15% (w/v) KCl). After homogenizing with a Brinkman Polytron instrument at medium speed, the samples were centrifuged at 10 000*g* at 4°C for 15 min. The supernatant was filtered through glass wool, then centrifuged at 100 000*g* at 4°C for 1 h. Microsomes were washed by resuspending the pellet in homogenization buffer and centrifuging again at 100 000*g* at 4°C for 1 h. The resulting pellet was resuspended in 50 mM potassium phosphate, pH 7·5, containing 0·1 mM EDTA and 0·25 M sucrose. Protein concentrations were measured as the fluorescamine complex (Bolen *et al.* 1973) using bovine serum albumin as the standard. Total microsomal P450 was determined by measuring the CO spectra of dithionite-reduced P450 (Omura and Sato 1964). Specific isozyme induction was confirmed by immunochemical detection (P4501A1, 1A2 and 2B), and by pentoxyresorufin *O*-dealkylation (P4502B) (Lubet *et al.* 1985) and *p*-nitrophenol hydroxylation (P4502E) (Koop 1986) assays. Oxidation of methimazole was measured by the method of Dixit and Roche (1984) to determine FMO activity. Aliquots of microsomes were stored at −80° until used.

Metabolic studies

Incubation and extraction of samples. Microsome preparations were analysed for TDZ metabolism four or more times at each protein concentration and each time point. All steps in the analysis of samples were performed under conditions of subdued light. Microsomes (350 μg protein per assay) were incubated in

a 1-ml reaction mixture containing buffer (100 mM sodium phosphate with 100 mM MgCl, pH 7·6), 60 nmol TDZ (in 10 μl methanol), and an NADPH regenerating system (0·25 mM $NADP^+$, 2·5 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase per ml assay). The reaction was started by the addition of the NADPH regenerating system to prewarmed samples. The mixture was incubated at 37°C in a shaking waterbath for 10 or 30 min (standard assay conditions) or from zero to 60 min for time-course studies. The reaction was stopped by the addition of 2·5 ml methyl-*tert*-butyl ether:hexane (3:1, v/v). The samples were vortexed for approximately 20 s, then centrifuged at 2000*g* for 5 min. The organic layer was removed and retained. The aqueous layer was extracted a second time by sequentially adding 0·75 ml 1 M sodium carbonate, 0·2 ml 2N sodium hydroxide and 2·5 ml of the methyl-*tert*-butyl ether:hexane mixture. The samples were vortexed and centrifuged as before and the organic layer removed and combined with the first extraction. The aqueous layer was extracted a third time by repeating the second step, except that 2·5 ml methylene chloride was added following the addition of the ether:hexane mixture. The combined organic layers were evaporated to dryness under a stream of nitrogen. The dried residue was redissolved in 1 ml mobile phase and injected into the hplc.

Control assays were performed by the exclusion of either the microsomes or purified enzyme. In order to quantify the metabolites that were formed by both enzymatic and non-enzymatic processes, four control assay mixtures were included with each set of incubations. Products formed by autoxidation, if any, were quantified and the mean of the product in the four assays was calculated. This was subtracted from the amount calculated from the enzyme-component, differentially inhibited, and boiled microsome mixtures.

Characterization of specific isozyme involvement. In assays involving boiled microsomes all enzymatic activity was inhibited by boiling microsomes for 2 min, then chilling before adding the microsomes to the assay mixture. For experiments involving heat inactivation of microsomal FMO activity, microsomes were preincubated at 50°C for 90 s, as described previously (Kinsler *et al.* 1988). No FMO activity, as measured by methimazole oxidation (Dixit and Roche 1984), was detected following this treatment.

Antibodies to NADPH P450 reductase were prepared from rabbit and purified as described by Dunbar and Schwoebel (1990). Preliminary studies were performed to determine the amount of antireductase needed to inhibit ≥ 90% of cytochrome c reductase activity in microsomes (Rose and Brindley 1985). In the TDZ study, microsomal P450 activity was inhibited by preincubation of 2 mg antireductase per mg microsomal protein for 10 min at 37°C. Treated microsomes were incubated and extracted as described above for TDZ metabolism assays. Assays involving metabolism of TDZ by P450 2D6 and 3A4 were performed using microsome products purchased from Gentest Corp. (Woburn, MA, USA). These microsomes were prepared from derivatives of the human cell line AHH-1 TK +/−, transfected with cDNA encoding human P4502D6 (*lot no.* 20) or 3A4 (*lot no.* 17). Microsomes from the same cell line containing no insert (*lot no.* 17) were used as negative control. Incubations were performed as described under 'Analysis of samples' except that the amount of microsomes used was adjusted to provide the concentration of P450 desired.

Purified enzyme studies. FMO was purified as described by Sabourin *et al.* (1984), as modified by Venkatesh *et al.* (1991). Purity of the enzyme was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), resulting in a single band with a molecular weight of 58 000 daltons. The purified protein exhibited a high degree of immunoreactivity with antibody to FMO1 as determined by Western blotting. Less intensity was observed with anti-FMO3, and there was no immunoreactivity with either anti-FMO2 of anti-FMO5 (data not shown). K_m and V_{max} of purified FMO for TDZ were determined using an NADPH oxidation assay (Venkatesh *et al.* 1991) and were 50·0 μM and 950 nmol/min/mg respectively (K. Venkatesh, *personal communication*). For assays of TDZ metabolites, purified FMO incubations contained 1·5–6·4 μg FMO (standard assay, 2·5 μg), 60 nmol TDZ, and buffer consisting of 0·2 M tricine and 0·2 M EDTA, pH 8·4. Samples were not prewarmed prior to addition of the NADPH regenerating system to start the reaction. Incubations were performed at 37°C for zero to 60 min.

Hplc

Hplc was performed using a Spectraphysics liquid chromatograph consisting of a model 8700XR solvent delivery system, a model SP8780XR autosampler, and a model SP8440XR UV-VIS detector that was set at a wavelength of 254 nm. Retention times and peak areas were determined with a model SP4200 computing integrator. All components were linked by a Spectraphysics Labnet® communications network. Separation was performed using a Waters RCM 8 × 10 radial compression module equipped with a 5μ silica cartridge (8 mm i.d.). Chromatographic conditions were a modification of those developed by Kilts *et al.* (1982). The mobile phase consisted of a mixture of isooctane–methanol–methylene chloride (8:1:1) containing 0·036% (v/v) methylamine. Degassing was performed by sonication immediately prior to use. An isocratic elution was used at a flow rate of 2 ml/min.

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Identification and quantitation of metabolites

Peaks were identified according to retention time, by spiking the samples after the first run with a small amount of standard and reinjecting, and by comparing R_f's of metabolic products with those of standards using thin layer chromatography (tlc) as previously described (Watkins *et al.* 1986). Because of the high extraction efficiencies of these compounds (> 90% for the metabolites for which standards were available and > 80% for TDZ), the data were not corrected for losses due to extraction.

Quantitation of metabolites was based on a concentration curve of standard mixtures containing known amounts of TDZ and metabolites in 1-ml mobile phase. A calibration curve for each metabolite was determined by plotting the ratio between the peak height of the standard against the amount of compound in the standard samples. Using the slope and intercept of the standard curve, the concentration of each metabolite in an incubation mixture was calculated from its peak height. Where a metabolite standard was not available, quantitation of metabolite concentration was based on the assumption that the unknown metabolite had a similar absorptance to that of 2SO.

Synthesis of N-*oxide.* The *N*-oxide of TDZ was synthesized from the free base of TDZ. Briefly, TDZ-free base was obtained by slowly adding 1 ml 5 M ammonium hydroxide to a solution of 1·3 g TDZ-HCl in water. The resulting precipitate was extracted three times using 1 vol. of methylene chloride. The organic layers were pooled and evaporated to dryness under vacuum to a yellow oil weighing approximately 1·3 g. This oil was resuspended in methylene chloride to a final concentration of 0·1 g/ml. A 30-mg aliquot of TDZ free base was then used to synthesize NO according to the general procedure of Craig and Purushothaman (1970). The product mixture was chromatographed by hplc. A peak with a retention time corresponding to the suspected *N*-oxide metabolic product was collected. The metabolic product and TDZ standard were also collected separately from the hplc. All three samples were submitted for thermospray liquid chromatography-mass spectrometry.

Mass spectral analysis. A mobile phase was prepared consisting of 50% acetonitrile in 0·05 M ammonium acetate. Because a preliminary liquid chromatography analysis showed the synthesis product to be pure, further separation by hplc was not necessary. Mass spectrometry using thermospray chemical ionization was performed in the positive-ion mode. The conditions and instrumentation used were those of Tyczkowska *et al.* (1989).

Statistical analyses

Assays were performed in triplicate using a new aliquot of mouse liver microsomes each day the assay was performed (at least four separate days). Statistical analyses were performed using analysis of variance followed by Scheffe's test for multiple comparisons. Differences were significant when $p < 0.05$.

Results

Chemistry

Standards were available for most of the metabolites observed in the present study, with the exception of two products that were determined to be the putative 2-sulphoxide-*N*-oxide (2SONO) and *N*-oxide (NO) products of TDZ. The mass spectral analysis of the metabolite identified as NO is compared with TDZ and synthetic NO in figure 2. In the metabolic product spectrum (figure 2B), the base peak at m/z 371 differs from the base peak of the synthesis product (m/z 387; figure 2C). This proportional discrepancy was due to contamination of the NO sample with parent TDZ, probably because of reduction during storage of the sample prior to analysis. The NO was collected by hplc from a purified FMO assay 2 days before mass spectral analysis and stored at 4°C, whereas the synthesis product was collected on the day of the analysis. In the spectra of both samples, the highest major mass ion is at m/z 387, corresponding to a monooxgenated derivative of TDZ. A strong ion peak at M^+-30, consistent with the loss of a formaldehyde group from the *N*-oxide (m/z 357), is present in the mass spectrum of the synthesized analyte and in that of the metabolic product. This finding is typical of *N*-oxide rearrangements (see the Discussion).

Tentative identification of 2SONO was based on preliminary mass spectral data suggesting that the compound is a dioxygenated product (data not shown), and by

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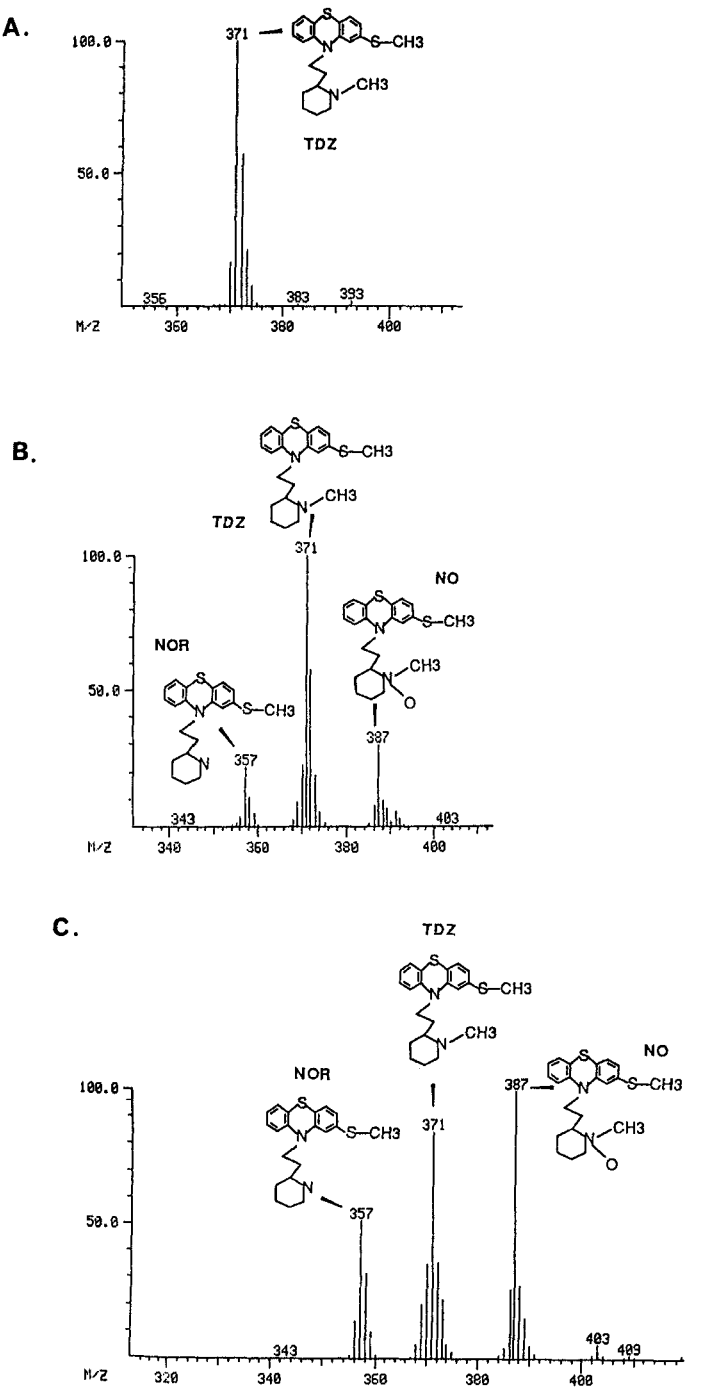

Figure 2. Mass spectra of thioridazine and thioridazine-*N*-oxide. (A) Thioridazine standard; (B) thioridazine-*N*-oxide, FMO oxidation product; and (C) thioridazine-*N*-oxide, synthesis product.

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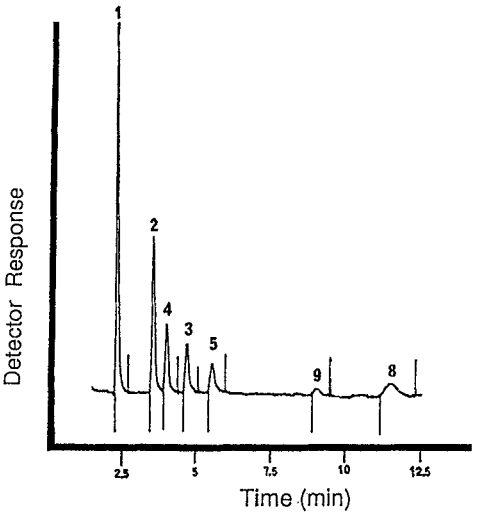

Figure 3. Chromatograph of commercially available TDZ standards, numbered as described in figure 1.

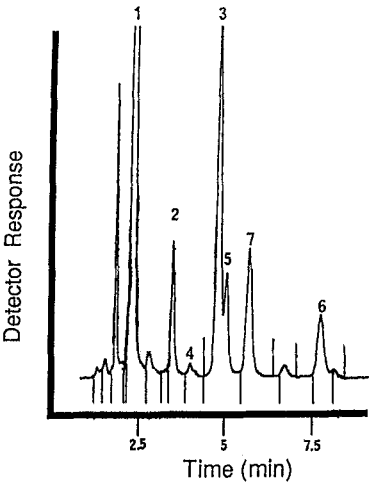

Figure 4. Chromatograph of microsomal TDZ metabolism. Peaks are numbered as described in figure 1.

the fact of its formation by FMO along with 2SO and NO. However, because this product is a minor one, further efforts to identify it were not made.

Figure 3 shows an hplc chromatogram of TDZ and available metabolite standards. The profile of mouse liver microsomal metabolism of TDZ is shown in figure 4. A standard was available for 5SO, but this compound exists as two diastereoisomeric pairs of enantiomers that are separable by the chromatographic method used in this study. Only one stereoisomer was generated in our metabolic studies using racemic TDZ, whereas its diastereomer was supplied as the 5SO standard (Juenge *et al.* 1983). The retention time of a peak corresponding to the metabolically formed stereoisomer was determined by allowing a small amount of the commercial standard to be exposed to sunlight. Exposure of one diastereo-

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isomeric pair of 5SO enantiomers to sunlight results in its inter-transformation to a mixture of both pairs of enantiomers (Eap *et al.* 1991). The resulting products were chromatographed by hplc. The two pairs of diastereomers eluted sequentially and were labelled 5SOFE and 5SOSE (data not shown). Whereas we use a nomenclature similar to Juenge *et al.* (1983) to distinguish the two diastereomeric pairs, we did not determine their stereospecific identity. Therefore, these fast (5SOFE) and slow (5SOSE) eluting pairs may not necessarily correspond to the products of the same name identified in previous work. The UV-VIS spectrum of each pair was identical (data not shown) and allowed a standard curve based on the absorptance of the commercial standard (5SOSE) at 254 nm to be used for quantitation of the metabolite produced enzymatically (5SOFE).

Metabolism

In preliminary studies, the amount of TDZ metabolized (as measured by total product formation) in microsomal incubations was found to increase linearly with the amount of microsomal protein up to approximately 0·36 mg/ml. At protein concentrations $> 0{\cdot}36$ mg/ml, the rate of TDZ metabolism proportional to microsomal protein concentration was slightly lower. The rate of metabolism of TDZ was linear up to 10 min, after which a slight but gradual decrease in the rate of metabolite production was observed (data not shown). The conditions for incubation were therefore standardized using 0·35-mg microsomal protein in a 1-ml reaction mixture, with a 10-min incubation period.

The primary metabolites formed in microsomal incubations were 2SO and 5SO, followed by NOR, and NO. A fifth metabolite is presumed to be 2SONO (table 1). Traces of 2SO2 and an unidentified metabolite were detected, but were not included in the analyses shown in tables 1 and 2. DiSO and DiSO2 were not observed in any of the assays performed in this study. As discussed in the Materials and methods, microsomal assays were prewarmed prior to the addition of the NADPH regenerating system to initiate the reaction. Metabolite profiles of control assays performed without prewarming were not significantly different qualitatively or quantitatively from those of prewarmed samples (data not shown).

Inactivation of microsomes by boiling resulted in reduction in the concentration of all metabolites observed except the supposed 2SONO, which was formed in consistent quantity regardless of the microsome treatment used (table 1). Occasionally trace amounts of 2SO, 5SO and NO were detected as well, although the quantity of these products was much less than detected in the control microsomal assays. One or more of these four products were found to be produced in the absence of enzyme and/or NADPH, and were postulated to be the result of oxidation of the parent compound during incubation or the extraction process. The length of incubation prior to extraction had no effect on the quantity of autoxidation product formed. Quantitation of the enzyme-dependent formation of these products was based on subtraction of the mean amount of product formed in incubations containing no microsomal protein from the amount detected in incubations containing microsomal protein (see Materials and methods).

Preincubation of microsomes with antibody to NADPH-P450 reductase reduced the formation of all metabolites, with NOR and 2SO decreasing most significantly (table 1). This treatment abolished the activity of P450 but had no effect on FMO activity, thus implicating P450 in the formation of all of these metabolites. Heat treatment of microsomes prior to incubation with TDZ reduces FMO activity

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Table 1. Metabolism of thioridazine by control and treated mouse liver microsomes.

Treatment	Metabolites (nmol/mg/min)[a]				
	NOR	2SO	5SO	2SONO[b]	NO
Control microsomes	0·26 ± 0·03	1·49 ± 0·09	0·57 ± 0·04	0·13 ± 0·01	0·23 ± 0·00
Boiled microsomes	0*	0*	0*	0·09 ± 0·03	0*
Heat-treated microsomes	0·13 ± 0·00*	1·15 ± 0·08	0·65 ± 0·05	0·15 ± 0·02	0·03 ± 0·01*
Antireductase-treated microsomes	0*	0·03 ± 0·01*	0·23 ± 0·06	0·09 ± 0·02	0·13 ± 0·02

Incubations were performed at 37°C for 30 min before extraction of metabolites.
* Significantly different from control microsomes, $p < 0.05$.
[a] $n \geq 4$. Numbers represent mean ± SE.
[b] Tentative identification.

Table 2. Effect of microsomal induction by phenobarbital, 3-methylcholanthrene, and acetone on microsomal metabolism of thioridazine.

Induction	Metabolites (nmol/mg/min)[a]				
	NOR	2SO	5SO	2SONO[b]	NO
Control	0·20 ± 0·01	1·80 ± 0·03	0·84 ± 0·07	0·19 ± 0·03	0·29 ± 0·04
Phenobarbital	0·67 ± 0·03*	4·50 ± 0·11*	2·26 ± 0·12*	0·77 ± 0·05*	0·30 ± 0·04
3-Methylcholanthrene	0·25 ± 0·03	1·52 ± 0·20	0·71 ± 0·08	0·18 ± 0·13	0·30 ± 0·05
Acetone	0·18 ± 0·03	1·81 ± 0·09	0·90 ± 0·06	0·32 ± 0·08	0·34 ± 0·07

Incubations were performed at 37°C for 10 min.
* Significantly different from control, $p < 0.05$.
[a] $n \geq 4$. Numbers represent mean ± SE.
[b] Tentative identification.

> 90%, while leaving P450 activity virtually intact (Kinsler *et al* 1988). Heat inactivation of FMO reduced the formation of primarily NO and NOR, whereas 2SO was decreased to a lesser extent (table 1).

Differential induction of P450 isozymes was performed by treating mouse with the appropriate xenobiotic prior to killing and microsome preparation. Phenobarbital induction (of primarily the P4502B family) increased the formation of all metabolites except NO observed in a 10-min incubation (table 2). Induction of P4501A1/1A2 by 3-methylcholanthrene (3-MC) and P4502E1 by acetone had no effect on the profile of metabolite formation by microsomes.

Incubation of TDZ with purified FMO from mouse liver resulted primarily in the production of the *N*-oxide. Trace amounts of 2SO and 2SONO were observed to increase with increasing time of incubation, but not with increasing enzyme concentration. Formation of NO by purified FMO increased with increasing enzyme concentrations up to 6·5 μg (figure 5). The quantity of all three metabolites increased with increasing time of incubation up to 60 min (figure 6). In some assays, as much as 60% of TDZ was converted to NO in 60-min incubations.

In order to investigate the disparity between the amount of NO formation by purified enzyme and that formed by intact microsomes, NO was used as a substrate for microsomes in a 30-min incubation under the same experimental conditions used for the TDZ assays. Approximately 70% of NO was converted to parent TDZ by microsomes. No conversion occurred when either boiled microsomes or purified FMO were incubated with NO (data not shown).

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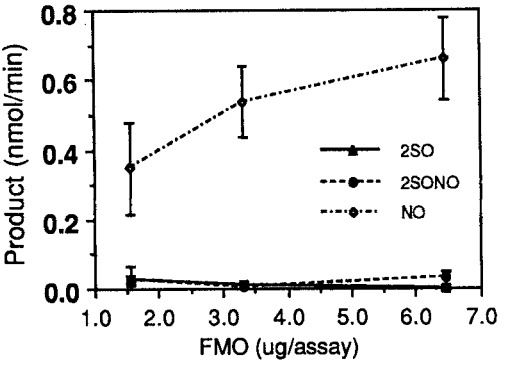

Figure 5. Concentration-dependent formation of TDZ metabolites by purified mouse liver FMO, 30-min incubation. Each data point represents mean of six determinations.

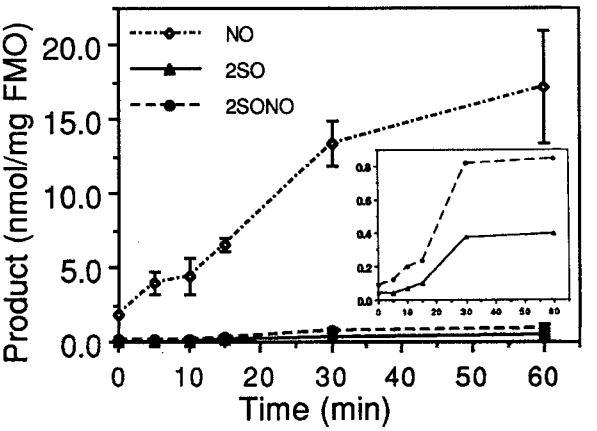

Figure 6. (A) Time-dependent formation of TDZ metabolites by purified mouse liver FMO. Assays were performed as described in the Materials and methods using 2·5-μg FMO protein. Each data point represents a mean of four or more determinations. (B) 2SO and 2SONO as shown in (A). graphed on a smaller scale to show time-depedent increase.

Further exploration of specific P450 isozyme involvement led to preliminary *in vitro* studies, which were performed using microsomes from cells individually expressing human liver P4502D6 or 3A4. The formation of thioridazine metabolites by P4502D6 microsomes increased with respect to enzyme concentration and time. The primary metabolites observed were 2SO, 5SO, and the putative 2SONO. After 30 min of incubation, P4502D6 microsomes had formed 24, 17 and 6 nmol respectively of these metabolites per nmol P450. Small amounts of NOR and NO were occasionally but inconsistently detected. Microsomes from cells expressing P4503A4 and from cells without an insert did not metabolize TDZ.

Discussion

As the principal enzymes mediating microsomal monooxygenation, P450 and FMO are clearly important to the metabolism of thioridazine. As shown here, these enzymes are responsible for the formation of metabolites that have been identified in previous *in vivo* human studies. The aim of this study is to compare semi-quantitatively the metabolites of TDZ generated by P450 and FMO. Findings of this

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nature may then serve in conjunction with *in vivo* data to gain a better understanding of the overall metabolism of TDZ.

Previous studies involving TDZ metabolism have been concerned primarily with identification and measurement of TDZ metabolites found in human biological fluids. In these studies the primary metabolites identified have been 2SO, 2SO2, and 5SO (Martensson *et al.* 1975, Ng and Crammer 1977, Juenge *et al.* 1983, Lin *et al.* 1993). Some studies have also observed NOR and *N*-demethylated TDZ sulphoxides (Muusze and Huber 1974, Dinovo and Gottschalk 1975), as well as phenolic metabolites (Papadopoulos *et al.* 1985), disulphoxides and lactam sulphoxides (Papadopoulos and Crammer 1986, Lin *et al.* 1993). There have been few *in vitro* studies involving oxidative TDZ metabolism (Traficante *et al.* 1979, Watkins *et al.* 1986). Traficante *et al.* (1979) examined chlorpromazine (CPZ) and TDZ metabolism by crude bovine liver microsomal preparations. Their results showed that whereas CPZ was primarily oxidized at the ring sulphur (corresponding to the 5 sulphoxide of TDZ), TDZ was preferentially oxidized at position 2, with 2SO2 and 5SO formed as minor metabolites.

The data presented here are in agreement with the latter findings, except that 2SO2 was infrequently observed in any of the assays performed. This may be due to the short incubation times of microsomal metabolism used in the present study (30 min), or due to species differences in the metabolism of this compound. It is of interest to note that when 2SO was used as a substrate for microsomes in the present study, no further oxidation of this product was observed (data not shown). Traficante *et al.* (1979) demonstrated that 2SO2 was reduced to 2SO in the presence of microsomes at a rate that exceeded the formation of 2SO2 from TDZ, implying that detection of any 2SO2 formed may be masked by the reduction process. If the rate of reduction of 2SO2 exceeds its rate of formation, this may explain why this product was seldom seen in our study.

The metabolite formed in highest abundance by liver microsomes from the male CD-1 mouse was 2SO. Whereas quantitation of this product is complicated by the presence of a small shoulder peak (5SOFE), the relative abundance of 2SO is much greater than 5SOFE, and therefore is not considered to be significantly affected by it. The 2SO product was increased by microsomal induction with phenobarbital and was significantly diminished by inhibition with anti-P450 reductase. Additionally, recombinant human liver P4502D6 was capable of forming this product. It is clear from these results that 2SO is an oxidation product of P450s. Considering the magnitude of the formation of this metabolite by P450 versus autoxidation, it is unlikely that the latter process plays a significant role in the formation of 2SO.

A trace of 2SO, as well as of the product tentatively identified as 2SONO, was also formed in incubations with purified FMO. Following correction of metabolite concentration for autoxidation, measurable amounts of 2SO and the presumed 2SONO were found to increase over time of incubation with FMO. This increase in product formation over time was not observed in incubations lacking enzyme or microsomes, indicating that autoxidation does not occur in a time-dependent manner. The rate of formation of 2SO and the putative 2SONO suggests the involvement of an enzymatic process. There was, however, no increase in formation of these products over the range of enzyme concentrations tested. Accordingly, it is presumed that FMO does not contribute significantly to the formation of these products by microsomes.

The ring-sulphoxidation product (5SO), an important metabolite due to its

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cardiotoxic potential (Gotschalk *et al.* 1978, Hale and Poklis 1986), is also formed by P450. There was a significant increase in its production with phenobarbital induction, and it was also a product of the recombinant P4502D6 microsomes. Heat inactivation of FMO in microsomes did not affect the formation of 5SO, and none of this product was detected in assays using purified FMO.

Previously, both 5SO stereoisomers have been identified in studies measuring the metabolic products of TDZ (Hale and Poklis 1985, Watkins *et al.* 1986, Svensson *et al.* 1990), yet only 5SOFE was observed in any of the assays performed in this study. Since a standard for the 5SOFE stereoisomer was not available, the standard for 5SOSE, which had an identical UV spectrum to the 5SOFE, was used. Identification and quantification of this metabolite was further complicated by the fact that under the chromatographic conditions used in this study, 5SOFE eluted as a shoulder of the 2SO peak.

Although several studies have suggested that phenothiazines are substrates of P450 (Tavoloni and Boyer 1980, Hartman *et al.* 1983, Cashman *et al.* 1993), few have sought to determine the specific P450 isozymes responsible for phenothiazine (PTZ) metabolism. Drug interactions, often due to isozyme induction or inhibition, are sometimes useful for evaluating isozymic involvement. An example is the interaction of TDZ and phenobarbital. Concurrent administration of these drugs results in lower serum levels of both compounds when compared with dosing with either drug alone (Gay and Madsen 1983). FMO activity is not inducible by phenobarbital (Masters and Ziegler 1971). This suggests that P450 isozyme induction is responsible for the increased metabolism of these drugs. Phenobarbital is known to induce at least two specific P450 families in humans: P4502B1/2B2 and the constitutive 3A family (Wrighton and Stevens 1992). Although significant increases in overall hepatic P450 content (including the 3A isozymes) are observed in mouse and rat upon phenobarbital administration, 2B is generally considered to be the primary phenobarbital-inducible isozyme family in rodents (Okey 1990).

Evidence of P4502B involvement in the metabolism of TDZ is apparent in the work of Murray and Reidy (1989). They demonstrated that TDZ was an effective mixed-type inhibitor of 7-pentoxyresorufin *O*-dependentylase activity in phenobarbital-induced rat liver microsomes, implicating the P4502B family as having a role in TDZ interactions in this species. They also examined activity due to P4501A1/1A2 induction by β-naphthoflavone as well as constitutive testosterone hydroxylase activities. Both 7-ethoxyresorufin *O*-dealkylase and testosterone hydroxylase activities were poorly, but measurably, inhibited by TDZ (Murray and Reidy 1989). Likewise, we found that induction by phenobarbital increased the amount of TDZ metabolized in microsomal incubations, whereas induction of P4501A1/1A2 and 2E1 did not.

Our results are consistent with the presumption that the phenobarbital-inducible P450s are at least partially responsible for the metabolism of TDZ. Whether or not this activity is due solely to the P4502B family is uncertain. Although our preliminary results with individually expressed human P4503A4 were negative with respect to thioridazine, this is an area that merits further investigation.

Several studies have implicated P4502D6, the isozyme involved in the polymorphic oxidation of debrisoquine and other important drugs, in the metabolism of TDZ. This implication is based on inhibition of the metabolism of many drugs known to be substrates of P4502D6 by TDZ (Fuller *et al.* 1974, Vincent 1980, von Bahr *et al.* 1985, Syvlahti *et al.* 1986). Subsequently, it has been observed

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that patients expressing the poor metabolizer phenotype of the debrisoquine polymorphism have higher levels of parent TDZ and lower levels of TDZ sulphoxides following dosing than do rapid hydroxylators of debrisoquine (Meyer *et al.* 1990, von Bahr *et al.* 1991). The current findings support our hypothesis that TDZ is a substrate of P4502D6.

Evidence is presented herein that FMO catalyses the oxidation of TDZ, primarily at the basic side-chain nitrogen, but not at the phenothiazine ring sulphur. In an unusual finding, Clement *et al.* (1993) have demonstrated ring sulphoxidation of desmethylpromethazine, but not promethazine (Clement *et al.* 1991), by purified pig liver FMO. This sulphoxidation occurred both with and without concurrent oxidation of the side chain nitrogen. With the exception of Clement *et al.* (1993), investigations using secondary and tertiary amine-containing phenothiazines have consistently shown that the basic nitrogen is the preferred site of oxidation by FMO (Sofer and Ziegler 1978, Ziegler 1988, Nagata *et al* 1990, Cashman *et al.* 1993, Lomri *et al.* 1993a–c).

In previous TDZ studies, detection of NO has been a rare occurrence. Watkins *et al.* (1986) detected trace amounts of this metabolite in rat urine following dosing with TDZ. Lin *et al.* (1993) identified an *N*-oxide of 2SO2 in the urine of volunteers after TDZ dosing, but NO itself was not found.

Several possibilities exist for the elusive nature of the *N*-oxide metabolite. First, species differences may preclude the formation of significant amounts of NO in humans. Several FMO isoforms have been identified, each processing characteristic tissue and substrate specificity (Ziegler 1993, Lawton and Philpot 1995). On the basis of screening and analysis of cDNA libraries, adult human liver FMO consists of primarily FMO3 (1D1) (Lomri *et al.* 1992) with relatively minor amounts of FMO1 (1A1) and FMO4 (1E1) (Dolphin *et al* 1991, 1992). Likewise, Northern blotting has demonstrated that hepatic expression of FMO3 in the mouse is high compared with its expression in the livers of other mammalian species (Burnett *et al.* 1994). FMO5 (1C1) and FMO1 mRNAs have also been detected in mouse liver (Atta-Asafo-Adjei *et al.* 1993). Despite the fact that FMO3 and FMO1 transcripts are found in the hepatic tissues of both human and mouse, minor differences in relative isozyme protein levels (which have yet to be fully characterized) may account for a lack of NO formation in humans. Both FMO1 (Sofer and Ziegler 1978, Ziegler 1988, Nagata *et al.* 1990, Clement *et al.* 1991, Lomri *et al.* 1993a) and FMO3 (Cashman *et al.* 1993, Lomri *et al.* 1993b,c) have been shown to catalyse the *N*-oxidation of the tertiary amine centres of a variety of phenothiazines. It is likely that both forms are capable of generating the *N*-oxide product of TDZ. However, the relative affinities of these two isozymes for thioridazine are unknown.

A second reason for the previous inability to detect NO may be that reduction of the product can occur during analysis. This phenomenon has been observed for CPZ-*N*-oxide, due both to *N*-oxide decomposition during gas chromatography (Craig *et al.* 1964, Essien *et al.* 1975, Curry and Evans 1976), and to problems with the extraction technique (Hubbard *et al.* 1985). Third, reductive processes are known to occur *in vivo*, and in the microsomal preparations themselves (Bickel 1969, Lewis *et al.* 1983, Ziegler 1988). In the present study it was noted that NO is reduced by microsomes to parent TDZ. There is no reason to assume that this would not also occur *in vivo*. If this is the case, the significance of NO formation may lie in its ability to act as a reservoir of parent TDZ, continually being oxidized and reduced, while other metabolites are excreted.

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Finally, a demethylation reaction may be occurring in which NO is converted to NOR and formaldehyde. Some tertiary amine oxide elimination reactions occur readily by chemical and enzymatic mechanisms (Craig *et al.* 1961, Bickel 1969, Lewis *et al.* 1983, Baba *et al.* 1987, Gut and Conney 1993). Whereas NOR is not generated by purified mouse liver FMO, the formation of NOR is decreased by heat inactivation of microsomal FMO. This suggests that elimination of formaldehyde from NO may have occurred in the *in vitro* microsomal assays leaving NOR. Consistent with our results, reducing the amount of NO generated by microsomal FMO (i.e. heat treatment) would decrease the formation of NOR. P450 mediates direct *N*-demethylation of tertiary amines, and on the basis of our results, is the primary agent of NOR formation from TDZ. However, the *N*-oxide is not an expected intermediate in this type of demethylation reaction (Bickel 1969).

In summary, P450 is the enzyme shown to be responsible for the formation of the pharmacologically active 2SO metabolite of TDZ, as well as the potentially cardiotoxic ring-sulphoxide (5SO). Moreover, P450 monooxygenases are at least partialy responsible for the formation of the demethylated product NOR and the *N*-oxide. The phenobarbital-inducible isozymes of mouse liver, and probably human liver P4502D6 are active P450 isozymes with respect to TDZ metabolism. The flavin-containing monooxygenase forms primarily the *N*-oxide product of TDZ that can be further demethylated (non-enzymatically) to NOR. The *N*-oxide is also reducible to its parent, thus to some degree prolonging the half-life of TDZ. These data provide a means for understanding the role pharmacokinetics plays in TDZ pharmacodynamics, and may also be relevant to other drugs of similar structure.

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