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IN VITRO INHIBITION OF HEPATIC DRUG OXIDATION BY THIORIDAZINE

KINETIC ANALYSIS OF THE INHIBITION OF CYTOCHROME P-450 ISOFORM-SPECIFIC REACTIONS

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Abstract—The phenothiazine tranquilizer thioridazine has been associated with drug interactions in man. This study investigated the capacity of the drug to inhibit hepatic drug oxidations mediated by cytochromes P-450 (P-450) in microsomes *in vitro*. Thioridazine was a potent linear mixed-type inhibitor of P-450b-dependent 7-pentoxyresorufin *O*-depentylase activity in phenobarbital-induced rat liver. The kinetic analysis revealed the enzyme-substrate dissociation constant (K_s) to be 1.6 μM whereas the dissociation constant of the enzyme–inhibitor complex (K_i) was 0.11 μM. In contrast, 7-ethoxyresorufin *O*-deethylase activity (mediated by P-450c) in β-naphthoflavone-induced rat hepatic microsomes was inhibited to a lesser extent (K_i = 2.4 μM) in relation to the K_s value (0.5 μM). Spectral studies indicated that the efficiency of thioridazine binding in phenobarbital-induced microsomes was about 25-fold greater than in microsomes from β-naphthoflavone-induced rat liver. This finding is consistent with the relative capacity of thioridazine to inhibit oxidase activities catalysed by P-450b and P-450c. Mixed-function oxidase activities catalysed by other P-450s were also inhibited by thioridazine, although to a lesser extent than those catalysed by forms b and c. Thus, the 6β- and 16β-hydroxylations of androst-4-ene-3,17-dione in hepatic microsomes from untreated rats were inhibited to a similar extent (I_{50}s = 52 and 43 μM, respectively). The 7α- and 16α-hydroxylase pathways were approximately only half as susceptible to inhibition by thioridazine. These findings demonstrate the capacity of thioridazine to inhibit a range of P-450-dependent drug oxidations, with those catalysed by forms b and c most susceptible. The present study strongly suggests that drug interactions elicited by thioridazine are most likely a consequence of inhibitory interactions with P-450 enzymes.

The phenothiazine tranquilizers are important drugs in the treatment of schizophrenia and other psychoses. A major problem associated with the use of these agents, however, is the incidence of adverse side-effects. Although many such effects are related to the pharmacological properties of the drugs it is apparent that additional side-effects occur. Thus, a number of reports have appeared in regard to the capacity of phenothiazines to interfere with the hepatic elimination of coadministered drugs.

Numerous studies have demonstrated that the principal group of enzymes involved in hepatic drug metabolism is the cytochrome P-450 (P-450)-mediated mixed-function oxidase (MFO) system. The P-450 system in mammalian liver consists of a number of isoenzymic forms with distinct, but overlapping, substrate specificities [1–4]. Certain drug oxidations appear to be more susceptible than others to the inhibitory actions of coadministered phenothiazines and this raises the possibility that the tranquilizers possess different degrees of inhibitory potency toward different P-450s [5–7].

The piperidinyl derivative thioridazine (Fig. 1) was selected for the present study since it is an antipsychotic agent in common use that is reputed to produce fewer side-effects than other phenothiazines. Despite this point, thioridazine has been implicated in phenytoin intoxication during concurrent drug therapy [8]. The capacity of thioridazine to elicit P-450 isozyme-specific inhibition of drug oxidase activities was investigated in this study. MFO substrates were selected as specific catalytic probes of six forms of P-450 and further experiments assessed the kinetic mechanism of inhibition of the activities of two P-450s by thioridazine.

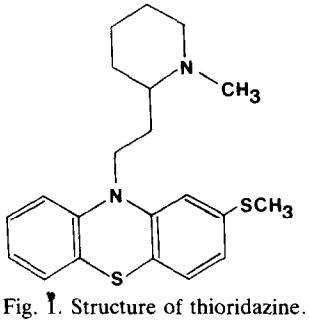

Fig. 1. Structure of thioridazine.

MATERIALS AND METHODS

Chemicals. Thioridazine hydrochloride (TRZ) and biochemicals were purchased from Sigma Chemical Co. (St Louis, MO). 7-Ethoxyresorufin and 7-pentoxyresorufin were obtained from Pierce Chemicals (Rockford, IL), and resorufin, isoniazid, and *N*-nitrosodimethylamine were from Aldrich Chemicals (Milwaukee, WI).

[^{14}C]Androst-4-ene-3,17-dione (androstenedione; sp. act. 59 mCi/mmol) was purchased from Amersham Australia (Sydney, NSW). Testosterone, unlabeled androstenedione, and its 6β- and 16α-hydroxy metabolites were from Sigma. 7α-Hydroxyandrostenedione was generously provided by the MRC Steroid Collection (Queen Mary's College, London, U.K.), whereas 16β-hydroxyandrostenedione was prepared as before [9].

Solvents and miscellaneous reagents were from Ajax Chemicals (Sydney, Australia), and were at least analytical reagent grade.

Animals. Male Wistar rats (approx. 300 g) were used in all experiments and were either untreated, or induced with phenobarbital (PB, 100 mg/kg i.p. in saline daily for 3 days), β-naphthoflavone (BNF, 40 mg/kg i.p. in corn oil daily for 3 days) or isoniazid (0.1% w/v in drinking water for 10 days). Hepatic microsomes were prepared 48 hr after the final exposure to inducer and were stored as frozen aliquots (−70°) in 20% glycerol/50 mM potassium phosphate buffer (pH 7.4, containing 1 mM EDTA) until required for experiments.

Androstenedione hydroxylase activity. The assay of androstenedione hydroxylation was performed as outlined previously [10] except that the microsomal protein concentration [11] was 0.5 mg/ml and the substrate concentration was 100 μM. Metabolites were resolved by TLC (silcia gel 60 F_{254} type, heated at 100° for 15 min before use; E. Merck, Darmstadt, F.R.G.) in the solvent system of Waxman *et al.* [12]. Zones that migrated with authentic standards were scraped into vials for scintillation spectrometry (Econofluor, New England Nuclear Corp., Boston, MA).

Other mixed-function oxidase assays. 7-Pentoxy- and 7-ethoxyresorufin O-dealkylations were assayed in PB- and BNF-induced rat liver, respectively, by the procedure of Prough *et al.* [13]. Substrate and protein concentrations were 1–10 μM and 100 μg/ml, respectively, in the case of 7-pentoxyresorufin oxidation, and 0.1–5.0 μM and 50 μg/ml for 7-ethoxyresorufin metabolism. *N*-Nitrosodimethylamine *N*-demethylase activity was measured in microsomes from isoniazid-induced rat liver using substrate and protein concentrations of 4 mM and 1.7 mg/ml, respectively. Formaldehyde production was quantified by the Nash procedure [14]. 7-Ethoxycoumarin *O*-deethylase activity was measured in hepatic microsomes from PB- and BNF-induced rats by a literature procedure [13] with a substrate concentration of 0.5 mM and a protein concentration of 200 μg/ml.

TRZ was included in microsomal incubations in potassium phosphate buffer (0.1 M, pH 7.4) at several different concentrations. I_{50} values were determined where appropriate, by regression analysis, from plots of log inhibitor concentration vs percent inhibition, using at least four different inhibitor concentrations and at least in duplicate ($r > 0.95$ in all cases).

Binding studies. Optical difference spectroscopy was used to monitor the spectral perturbations produced by thioridazine in hepatic microsomes *in vitro*. Double reciprocal plots of Δ absorbance (peak to trough of the TRZ type I spectrum) as a function of ligand concentration were constructed. Spectral dissociation constants (K_s) and maximal absorbance changes (ΔA_{max}) were calculated from the x- and y-axis intercepts of the double reciprocal plots [15]. Pooled data from three separate optical titrations, each using at least five ligand concentrations, were employed in these calculations.

Cytochrome P-450 was determined by the procedure of Omura and Sato [16] using an extinction coefficient of 91/mM/cm for the reduced-carbonyl spectrum of P-450.

Graphical methods. Inhibitory data were plotted according to the method of Lineweaver and Burk [17] and, in some instances, by the Dixon method [18]. Replots of double reciprocal plot slopes and intercepts vs substrate or inhibitor concentration were also constructed in order to assess further the apparent inhibition kinetics [19]. Correlation coefficients were determined for the regression of each series of data points and were at least 0.9 in all cases.

The data obtained for the inhibition of 7-ethoxyresorufin *O*-deethylase activity from BNF-induced hepatic microsomes was also subjected to secondary replot analysis. Reciprocal Δ slope and Δ intercept data were plotted as functions of reciprocal inhibitor concentration. Δ Slope is defined as the difference

Table 1. Inhibition of hepatic microsomal mixed function oxidase activities by thioridazine

Animal pretreatment	Oxidase activity	P-450 form*	I_{50} (μM)	Substrate concentration
None (control)	Androstenedione 6β-hydroxylase	2a	52	100 μM
	Androstenedione 7α-hydroxylase	a	95	
	Androstenedione 16α-hydroxylase	h	100	
	Androstenedione 16β-hydroxylase	—‡	43	
Phenobarbital (100 mg/kg × 3 days)	7-Pentoxyresorufin *O*-depentylase	b	0.37	10 μM
	7-Ethoxycoumarin *O*-deethylase	b (likely)	5.8	500 μM
β-Naphthoflavone (40 mg/kg × 3 days)	7-Ethoxyresorufin *O*-deethylase	c	8.6	2.5 μM
	7-Ethoxycoumarin *O*-deethylase	c (likely)	22	500 μM
Isoniazid (0.5% in drinking water × 10 days)	*N*-Nitrosodimethylamine *N*-Demethylase	j	NI†	4 mM

* Form of cytochrome P-450 that is involved in the oxidase activity stated (refer to Materials and Methods for comparative nomenclature of P-450s).

† NI, no inhibition observed at concentrations up to 100 μM.

‡ P-450 catalysing reaction not yet identified.

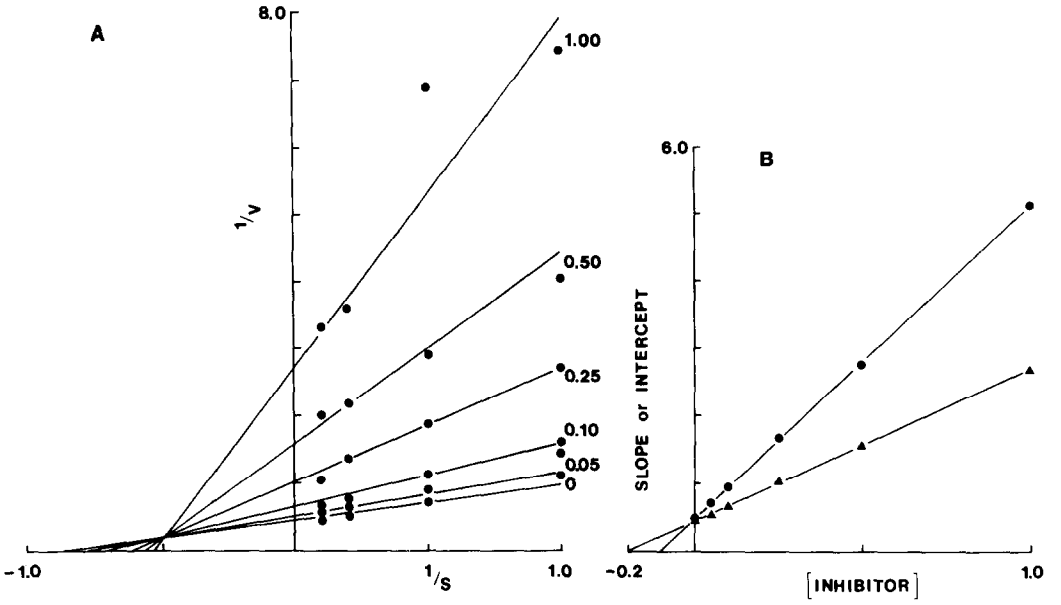

Fig. 2. Inhibition of 7-pentoxyresorufin *O*-depentylase activity in hepatic microsomes from phenobarbital-induced rats by thioridazine. (Panel A): Lineweaver–Burk plot; substrate concentration is micromolar and reaction velocity is in nmol product/min/mg protein. Inhibitor concentrations are indicated beside each line. (Panel B): primary replot of inhibitor concentration (μM) vs Lineweaver–Burk slope (●) or intercept (▲) data.

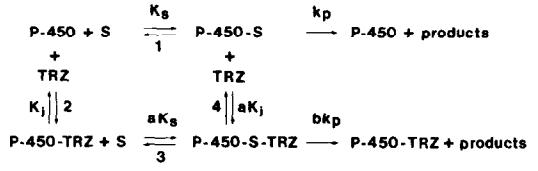

Fig. 3. General scheme of the equilibria between P-450, substrate (S), and inhibitor (TRZ). The appropriate equations are:

$$\text{step (1)}\quad K_s = \frac{[\text{P-450-}][\text{S}];}{[\text{P-450-S}]}$$

$$\text{step (2)}\quad K_i = \frac{[\text{P-450}][\text{TRZ}];}{[\text{P-450-TRZ}]}$$

$$\text{step (3)}\quad aK_s = \frac{[\text{P-450-TRZ}][\text{S}];}{[\text{P-450-S-TRZ}]}$$

and

$$\text{Step (4)}\quad aK_i = \frac{[\text{P-450-S}][\text{TRZ}]}{[\text{P-450-S-TRZ}]}$$

a is the factor by which K_s and K_i alter when the inhibitor and substrate, respectively, occupy the enzyme. *b* is the factor by which the rate of product formation is altered by the inhibitor.

in slopes of two lines from the initial Lineweaver–Burk plot e.g. Δ slope when the TRZ concentration is 1 μM *minus* the slope of the plot when TRZ is 0 μM. Δ Intercept was derived in analogous fashion using the intercept values from the initial Lineweaver–Burk plot. Secondary replots were used to determine the variables *a* and *b* and the inhibitor equilibrium contant (K_i) by methods outlined by Segel [19]. K_s and V_{max} were obtained from the Lineweaver–Burk plot in the absence of inhibitor.

P-450 *Nomenclature*. Equivalent forms of P-450 mentioned in this study: P-450 a [20] is apparently identical with P-450 3 [4] and P-450 *UT-F* [1]: gene designation P-450 IIA1 [21]; P-450 b [20] is P-450 *PB-B* [1] and P-450 PB-4 [4]: gene designation P-450 IIB1 [21]; P-450c [20] is P-450 *BNF-B* [1]: gene designation P-450 IA1 [21]; P-450 h [2] is P-450 *UT-A* [1], P-450 2c [4], P-450 RLM5 [22] and P-450$_{16\alpha}$ [23]: gene designation P-450 IIC11 [21]; P-450 j [3] is P-450 ac [24] and P-450 RLM6 [25]: gene designation P-450 IIE1 [21]; P-450 p [26] is a member of the P-450 IIIA subfamily [21] as are P-450 *PCN-E* [1] and P-450 2a [4].

RESULTS

Inhibition of hepatic microsomal P-450 isoform-specific oxidation by thioridazine

Preliminary experiments demonstrated that TRZ is a phenothiazine derivative with the capacity to elicit quite potent inhibition of microsomal P-450-dependent oxidation reactions. As shown in Table 1, the lowest I_{50} value (0.37 μM) measured for TRZ was toward P-450 b-mediated 7-pentoxyresorufin *O*-depentylase activity (in PB-induced liver fractions). BNF-induced 7-ethoxyresorufin *O*-deethylase (P-450 c-mediated) activity was somewhat less susceptible to inhibition (I_{50} = 8.6 μM). Although the substrate 7-ethoxycoumarin is a less specific probe for individual P-450s, it is readily dealkylated by both P-450 b and P-450 c (isoforms that predominate in PB- and BNF-induced microsomal fractions, respectively). TRZ was approximately four times more potent against PB-inducible ethoxycoumarin dealkylation (I_{50} = 5.8 μM) than against the BNF-inducible activity (I_{50} = 22 μM; Table 1).

Additional studies evaluated the capacity of TRZ to inhibit the regioselective and stereoselective

Table 2. Kinetic parameters of the inhibition of 7-ethoxy- and 7-pentoxyresorufin O-dealkylations by thioridazine*

	7-Ethoxyresorufin *O*-deethylase	7-Pentoxyresorufin *O*-depentylase
K_s	0.5 μM	1.6 μM
V_{max}	2.6 nmol resorufin/min/mg protein	2.5 nmol resorufin/min/mg protein
K_i	2.4 μM	0.11 μM
a	3.1	1.8
b	0.17	0

* The kinetic parameters were determined from replots of the inhibition data as described in Materials and Methods.

hydroxylation of androstenedione in hepatic microsomes from untreated male rats. I_{50} values were determined for TRZ toward each of the four hydroxylation pathways but P-450s involved in the formation of the 6β- (P-450 2a [4]) and 16β- (an as yet unassigned P-450 [27]) alcohols were most susceptible to inhibition (Table 1).

TRZ, even when tested at concentrations of up to 100 μM, proved to be non-inhibitory toward *N*-nitrosodimethylamine *N*-demethylase activity from isoniazid induced rat liver (data not shown).

Kinetics of inhibition of 7-pentoxyresorufin O-depentylase activity by thioridazine

The kinetics of inhibition of 7-pentoxyresorufin *O*-depentylase activity by TRZ was studied in microsomal fractions from PB-induced rat liver. As shown in the Lineweaver–Burk plot (Fig. 2A) of the data, the inhibition type was initially characterized as mixed since the point of intersection of the lines is to the left of the $1/V$ axis and is above the $1/S$ axis. The replot of the slopes and y-intercepts (of the family of lines shown in Fig. 2A) is depicted in Fig. 2B. Since the replot lines intersect on the y-axis the inhibition is characterized as linear-mixed type and from this plot, a value of $K_i = 0.11$ μM was determined. In the general scheme (Fig. 3) inhibition of 7-pentoxyresorufin *O*-depentylase activity by TRZ is characterized as follows: $K_s = 1.6$ μM, $K_i = 0.11$ μM, $a = 1.8$ and $b = 0$ (Table 2).

Kinetics of inhibition of 7-ethoxyresorufin O-deethylase activity by thioridazine

A kinetic analysis of the inhibition by TRZ of 7-ethoxyresorufin *O*-deethylase activity in hepatic microsomes from BNF-induced microsomes was performed. The appearance of the initial Lineweaver–Burk plot of the data (Fig. 4A) suggested that the inhibition kinetics may well be competitive. This possibility was also supported by the Dixon plot (Fig. 5A) but, importantly, the Dixon replot (reciprocal substrate concentration vs the slopes of the family of lines in the Dixon plot) shown in Fig. 5B was non-linear. Thus, a simple competitive mechanism of inhibition was ruled out. Instead, a replot of reciprocal Δ inhibitor concentration vs reciprocal Δ slope of the initial Lineweaver–Burk plot (refer to Materials and Methods for definitions), was linear and therefore indicative of hyperbolic mixed inhibition (Fig. 4B). Again, in relation to the general scheme outlined in Fig. 3, the following parameter values were calculated from the kinetic analysis: $K_s = 0.5$ μM; $K_i = 2.4$ μM; $a = 3.1$, and $b = 0.17$ (Table 2).

Spectral studies of the binding of thioridazine to microsomal cytochrome P-450

The addition of TRZ to hepatic microsomes from PB- and BNF-induced male rats resulted in the appearance of a type I optical difference spectrum ($\lambda_{max} \sim 386$ nm, $\lambda_{min} \sim 417$ nm; not shown). Double reciprocal analysis of ligand concentration vs absorbance change data was performed and values for the spectral dissociation constant (K_s) and maximal spectral change (ΔA_{max}) were obtained. In PB-induced microsomal fractions the K_s was 1.2 μM and 80 μM in BNF-induced microsomes; ΔA_{max} values were 4×10^{-3} and 10×10^{-3} absorbance units/nmol P-450 in PB- and BNF-induced rat hepatic fractions, respectively (Table 3). Calculation of the parameter $\Delta A_{max}/K_s$, which reflects the efficiency of the binding interaction, yielded values of 3300 and 130 for TRZ binding in PB- and BNF-microsomes, respectively (Table 3). Thus, an approximate 25-fold greater binding efficiency was observed for TRZ binding in PB-induced rat liver.

DISCUSSION

Initial experiments revealed that TRZ appeared to be an exceptionally potent inhibitor of 7-pentoxyresorufin *O*-depentylase activity in hepatic microsomes from PB-induced rats. The kinetics of TRZ inhibition of this activity, which has been ascribed almost exclusively to the P-450 isoform b [28], was studied in detail and in relation to the inhibition of P-450 c-mediated 7-ethoxyresorufin *O*-deethylase activity. In the case of the inhibition of 7-pentoxyresorufin oxidation, a value of 0.11 μM was determined for the K_i of TRZ whereas the dissociation constant of the enzyme–substrate complex (K_s) was determined to be 1.6 μM; that is, the inhibitor has an approximate 15-fold greater affinity than the substrate for the enzyme P-450 b. Since the parameter b (Fig. 3) in Table 2 is equal to zero, it is clear that the ternary 7-pentoxyresorufin:TRZ:P-450 b complex is non-productive and that the kinetics are linear mixed-type with a pronounced non-competitive component (in simple competitive inhibition the parameters $a = 0$ and $b = 0$ and, in simple non-competitive inhibition, $a = 1$ and $b = 0$). In contrast, TRZ was a hyperbolic non-competitive (mixed) inhibitor of 7-ethoxyresorufin oxidation using the criteria described by Segel [19]. In this case $b =$

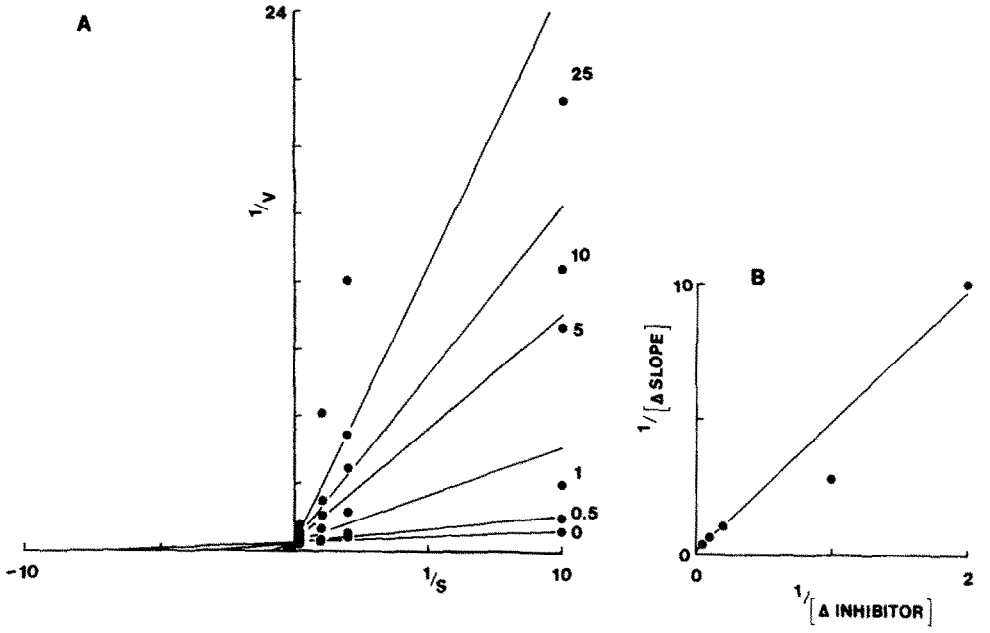

Fig. 4. Inhibition of 7-ethoxyresorufin *O*-deethylase activity in hepatic microsomes from β-naphthoflavone-induced rats by thioridazine. (Panel A): Lineweaver–Burk plot; substrate concentration is micromolar and reaction velocity is in nmol product/min/mg protein. (Panel B): Secondary replot of reciprocal Δ inhibitor concentration vs reciprocal Δ slope from the lines in Panel A. Refer to Materials and Methods section for definitions of Δ inhibitor and Δ slope.

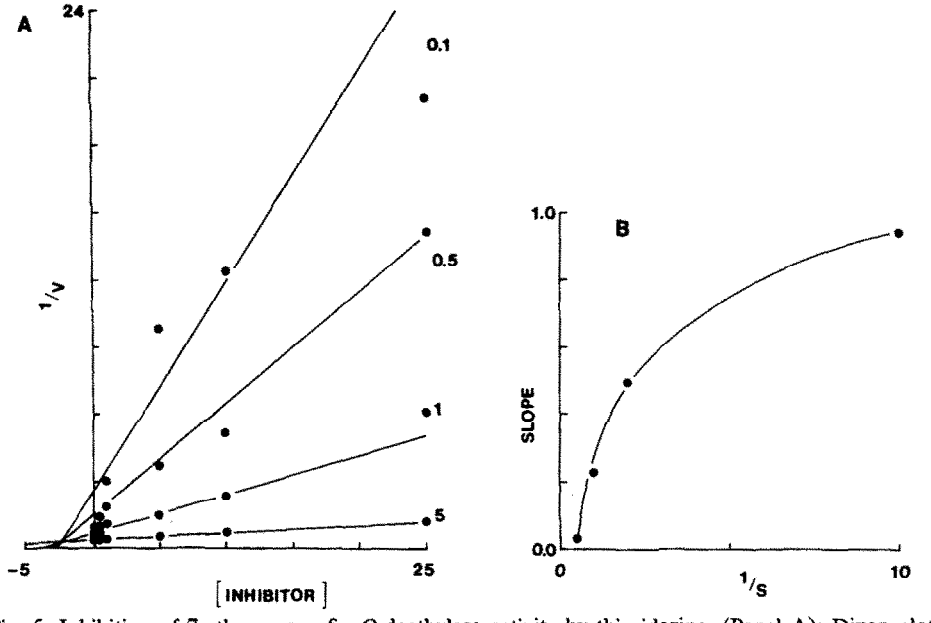

Fig. 5. Inhibition of 7-ethoxyresorufin *O*-deethylase activity by thioridazine. (Panel A): Dixon plot; inhibitor concentration is micromolar. (Panel B): Dixon plot slope replot as a function of reciprocal substrate (7-ethoxyresorufin) concentration.

0.17 so that the ternary complex comprised of 7-ethoxyresorufin:TRZ:P-450 c yields product (resorufin) but at only 17% of the rate of product formation from the enzyme–substrate complex. Another important point to emerge from this kinetic analysis is that the affinity of TRZ for P-450 c (K_i = 2.5 μM) is actually 5-fold lower than the affinity of the substrate for the enzyme (K_s = 0.5 μM). It is perhaps for this reason that TRZ is a far less potent inhibitor of P-450 c activity than P-450 b activity. These findings from catalytic studies are complemented by the results of the spectral binding studies that defined the much lower binding affinity and efficiency that TRZ has for P-450 c than for P-450 b. Apparent spectral dissociation constants were found to be one to two orders of magnitude greater than

Table 3. Parameters of the type I spectral binding interaction of thioridazine in microsomes from phenobarbital- and β-naphthoflavone-induced rat liver

Animal pretreatment	K_s (μM)	ΔA_{max} (absorbance units/nmol P-450)	$\Delta A_{max}/K_s$ (absorbance units/nmol P-450/M)
Phenobarbital	1.2	4.0×10^{-3}	3300
β-Naphthoflavone	80	1.0×10^{-2}	130

the analogous kinetic constants describing the affinity of the enzyme–inhibitor complex (K_i). One explanation is that the effective concentration of ligand at the P-450 active site is not necessarily equivalent to that introduced into the cuvette. Indeed, due to partitioning between phases, it is more likely that the concentration of TRZ that reaches the P-450 binding region is somewhat lower than expected.

There is evidence that certain phenothiazine derivatives are substrates [29, 30] and inhibitors [5, 6] of the microsomal P-450-mediated MFO system. It may well be for this reason that the present inhibition studies of 7-alkylresorufin O-dealkylations by TRZ revealed a competitive component (since they were mixed-type inhibitors). Most studies of the inhibition of oxidative drug metabolism by phenothiazines have been restricted to chlorpromazine [5–7] but the present study establishes that TRZ is a potent MFO inhibitor, especially of the P-450 b-mediated O-dealkylation of 7-pentoxyresorufin. There have been reports in the literature that have alluded to the capacity of TRZ to inhibit drug metabolism by an interaction with P-450. Thus, TRZ has been shown to interfere with the kinetics of imipramine elimination from the isolated perfused rat liver [31] and to be involved in a drug interaction during phenytoin therapy [8]. TRZ has also been reported to decrease serum testosterone levels in men receiving the drug [32]. Although the mechanism of this effect was not elucidated it has been noted by others that phenothiazines influence endocrine function without lutenizing hormone suppression [33]. Since it is now clear that a testicular P-450 plays a central role as the progesterone 17α-hydroxylase and 17,20-lyase [34, 35], it is a possibility that TRZ perturbs testicular testosterone production via a direct inhibitory effect on this steroidogenic P-450.

The development of potent inhibitors of individual P-450 reactions will ultimately provide useful tools for the understanding of the active site differences between P-450s. The present study notes that TRZ is a very potent inhibitor of P-450 b-dependent 7-pentoxyresorufin *O*-depentylase, with reactions catalysed by other isoforms of the cytochrome somewhat less susceptible to inhibition. It is now important that the capacity of TRZ to inhibit P-450s that have physiological roles, such as in testicular steroidogenesis, should be assessed.

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