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Stereoselective S-oxygenation of an aryl-trifluoromethyl sulfoxide to the corresponding sulfone by rat liver cytochromes P450

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Abstract—Toltrazuril sulfoxide (TZR.SO) is the metabolite of the antiparasitic drug toltrazuril (TZR; 1-methyl-3-[3-methyl-4-[4-[trifluoromethyl]thio]phenoxy]phenyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione). The results of the present paper demonstrate that TZR.SO was metabolized by rat liver microsomes to the corresponding sulfone ($TZR.SO_2$). The reaction was mediated almost exclusively by different cytochromes P450, the most active being cytochromes P450 3A. TZR.SO exists as a racemic mixture; when each enantiomer was incubated separately in the presence of untreated rat liver microsomes, a 7.3-fold difference in the rate of S-oxygenation was found, indicating a marked substrate enantioselectivity for the reaction.

In rodent liver, many sulfur-containing xenobiotics are metabolized to sulfoxides by two major enzyme systems, the cytochrome P450-dependent monooxygenases (P450*) and the flavin-containing monooxygenases (FMO) [1, 2]. The two enantiomers formed from the achiral sulfur-containing compound [3, 4] are further metabolized to an achiral sulfone, but very little is known about the enzymatic mechanisms of this latter metabolic step, although the involvement of cytochromes P450 is generally assumed [5–10].

In a previous study, we have shown that the *in vitro* sulfoxidation of the antiparasitic drug toltrazuril (TZR; 1-methyl-3-[3-methyl-4-[4-[trifluoromethyl]thio]phenoxy]phenyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione) is catalyzed by several cytochromes P450, with enantioselectivity of the reaction differing widely among these isoenzymes [11]. The present paper reports on the relative contribution of cytochromes P450 to the S-oxygenation of TZR sulfoxide to the corresponding sulfone by rat liver microsomes, and the substrate stereoselectivity of the reaction.

Materials and Methods

TZR (purity: 99.9%), toltrazuril sulfoxide (TZR.SO; purity: 99.7%) and toltrazuril sulfone ($TZR.SO_2$; purity: 97.8%) were a gift from Bayer AG (Leverkusen, Germany). Clotrimazole, orphenadrine HCl, dexamethasone (monosodium phosphate salt), triacetyloleandomycin and erythromycin were obtained from the Sigma Chemical Co. (St Quentin-Fallavier, France). Pentylresorufin was purchased from Boehringer (France). All other chemicals were obtained from commercial sources.

Male and female Sprague–Dawley rats (IOPS–OFA) were purchased from IFFA-Credo (L'Arbresle, France). Adult male animals (four groups of four animals; 180–200 g; 8 weeks of age) were induced with either phenobarbital (PB) in saline, 80 mg/kg i.p. daily for 3 days, 3-methylcholanthrene (3-MC) in corn oil, 20 mg/kg i.p. daily for 3 days, dexamethasone (DEX) 100 mg/kg i.p. daily for 3 days, or triacetyloleandomycin (TAO) 100 mg/kg i.p. daily for 4 days. All animals were killed 24 hr after the last administration of the inducer. A group of four male rats was also killed at 4 weeks of age.

The livers were removed and homogenized in a volume of ice-cold buffered KCl (0.15 M KCl, sodium–potassium phosphate buffer 50 mM, pH 7.4) corresponding to three times the weight of the tissue. Microsomes were prepared by centrifugation, resuspended in 0.1 M sodium–potassium phosphate buffer, pH 7.4 containing 1 mM EDTA and 20% (v/v) glycerol, and stored in small aliquots at −70°.

The microsomal fractions were incubated with the various substrates at 37° in a final volume of 1 mL containing 100 mM sodium–potassium phosphate buffer (pH 7.4), 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 1 U glucose 6-phosphate dehydrogenase. The TZR.SO (0.2 mM) was added to the incubation medium in 5 μL of methanol. After incubation for 5–20 min, TZR.SO and metabolites were extracted from the incubation mixture with diethyl ether. The organic phase was evaporated under a nitrogen stream, the residue redissolved in a small volume of methanol:water (1:4), then concentrated on a C_8 precolumn with an AASP® system (Varian Instruments, Les Ulis, France). Products were eluted on a Kromasil C_{18} column (15 cm × 0.4 cm i.d.) with an acetonitrile:water (containing monopotassium phosphate 10 mM) linear gradient (20–70% acetonitrile in 15 min at a flow rate of 2 mL/min) and monitored by UV absorption at 260 nm. The chromatographic method allowed separation of TZR, TZR.SO and $TZR.SO_2$ (retention times: 8.0 min and 10.2 min for TZR.SO and $TZR.SO_2$, respectively). With microsomes from TAO-pretreated animals, metabolism of TZR.SO was also measured after pretreatment of the microsomes for 30 min with 75 μM potassium ferricyanide. Microsomes were preincubated for 8 min with 20 μM orphenadrine in presence of NADPH before addition of the substrate.

The resolution of TZR.SO enantiomers was achieved by HPLC on a α-glycoprotein chiral stationary phase (ChromTech, Sweden) and isocratic elution [8 mM Na_2HPO_4, 1.5% (v/v) isopropanol in water], and monitored by UV absorption at 260 nm; about 1 mg of each enantiomer of TZR.SO was prepared. Absolute configuration and rotatory power of the two enantiomers are not known, so they are referred to as (A)- and (B)-TZR.SO (in the order of elution from the chiral column).

For studying the stereospecifity of the oxygenation rate, 20 μg/mL of each pure enantiomer of TZR.SO was added to the reaction medium and the formation of the sulfone was measured.

Microsomal metabolism of pentylresorufin was assessed fluorometrically [12]. Erythromycin *N*-demethylase activity was detected by formaldehyde formation [13]. After

* Abbreviations: P450, cytochrome P450-dependent monooxygenases; FMO, flavin-containing monooxygenases; TZR, toltrazuril 1-methyl-3-[3-methyl-4-[4-[trifluoromethyl]thio]phenoxy]phenyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione); TZR.SO, toltrazuril sulfoxide; $TZR.SO_2$, toltrazuril sulfone; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PB, phenobarbital, 3-MC, 3-methylcholanthrene; DEX, dexamethasone; TAO, triacetyloleandomycin.

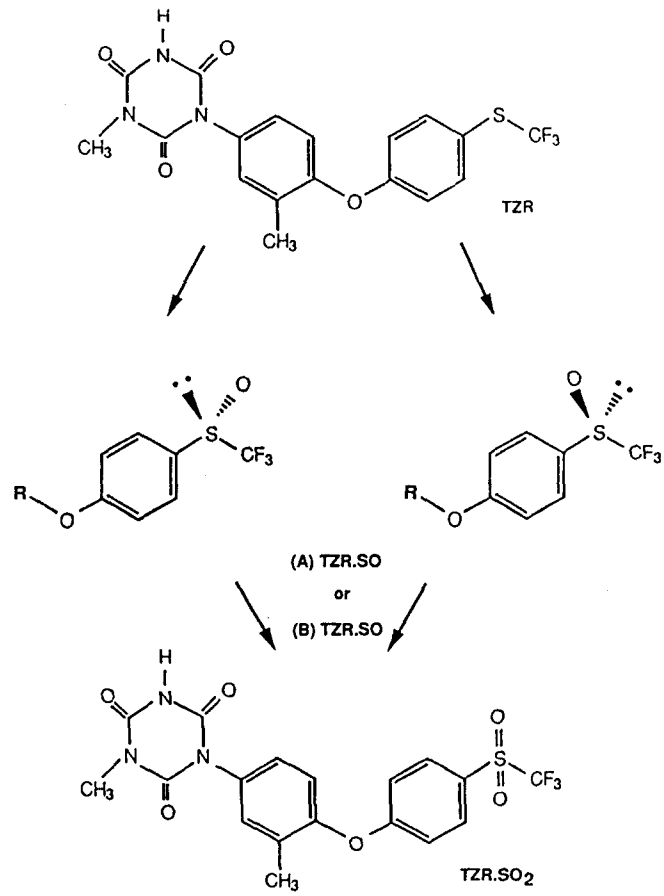

Fig. 1. Structure of TZR, enantiomers of TZR.SO, (A) and (B), and TZR.SO_2.

incubation of testosterone (250 μM), the hydroxylated products were resolved and quantified by HPLC [14].

Protein was determined by a modified Lowry's method [15] with bovine serum albumin as a standard.

All analyses were performed in duplicate.

Results

The only metabolite detected after incubation of TZR.SO with microsomes and NADPH was TZR.SO_2 (Fig. 1). In the absence of NADPH, the activity was reduced by 94%. Under the conditions of the assay, the reaction rate was linear for 5–60 min following addition of TZR.SO with 0.25–1 mg of protein in the incubation medium. The reaction was strongly inhibited by clotrimazole (50 and 85% inhibition in the presence of 5 and 10 μM of clotrimazole, respectively). The kinetic constants for adult male rats (N = 4) were $K_m = 210 \pm 22$ μM and $V_m = 1.51 \pm 0.67$ nmol/min/mg (Fig. 2). The specific activity in standard conditions (i.e. 0.2 mM of TZR.SO in the incubation medium) was 0.75 nmol/min/mg (Table 1). Incubation of TZR.SO_2 (0.2 mM) with microsomes and NADPH did not lead to the reductive formation of the parent compound TZR.SO.

S-Oxygenation of TZR.SO, 6β-hydroxylation of testosterone and 16α-hydroxylation of testosterone were measured with microsomes from 4-week (immature) and 8-week (mature) old male rats. The S-oxygenation of TZR.SO was slightly modified by aging, 6β-hydroxylation of testosterone was not significantly modified (×0.96), but 16α-hydroxylation of testosterone was dramatically increased (×9.1). In adult female animals, the S-oxygenation rate was only 15% of that in male animals.

The effects of inducers on oxidation are shown in Table 1. When male rats were pretreated with PB and DEX, the reaction rate was increased 4.3- and 11.7-fold, respectively, while no modification was observed after pretreatment with 3-MC. The value of K_m was not modified after induction by DEX (Fig. 2). Pretreatment of rats by TAO resulted in a 35% reduction of the S-oxygenation rate, but the *in vitro* treatment of microsomes with 75 μM potassium ferricyanide led to a 4.5-fold increase in S-oxygenation rate and a 2.2-fold increase in erythromycin *N*-demethylase activity.

Selective inhibitors of cytochrome P450-dependent activities were able to modify the rate of S-oxygenation. In control and DEX-pretreated rats, the reaction was inhibited by 50% by erythromycin (400 μM). In microsomes from PB-pretreated animals, S-oxygenation was inhibited by 30 and 50% by orphenadrine (20 μM) and erythromycin (400 μM), respectively. However, 95% of pentylresorufin *O*-dealkylase activity was inhibited by orphenadrine under the same conditions.

When incubating microsomes from untreated rats in the presence of racemic sulfoxide, the rate of disappearance of the (A)-enantiomer was 4.5-fold higher than that of the (B)-enantiomer, clearly indicating that the rate of S-oxygenation depends on the stereoisomeric configuration of the sulfoxide. The kinetic constants, determined by incubation of pure enantiomer in the presence of microsomes from untreated animals, were $V_{max} = 1.54$ and

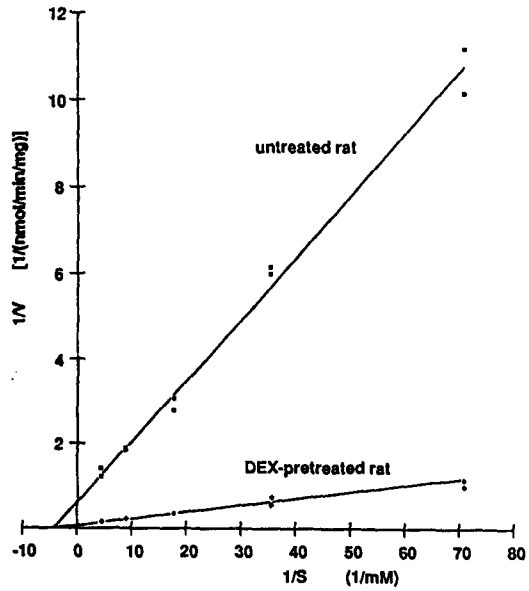

Fig. 2. Double reciprocal plot of S-oxygenation of TZR.SO by liver microsomes from both untreated and DEX-pretreated rats.

0.21 nmol/min/mg, and K_m = 112 and 66 μM, for (A)- and (B)-enantiomers, respectively (Fig. 3). These results confirm the enantioselectivity observed after incubation of the racemic compound.

Discussion

Very few reports have dealt with the mechanisms and substrate specificity of S-oxygenation of sulfoxide to sulfone. In an earlier report, Levi and Hodgson [10] have shown that some cytochromes P450 purified from livers of control and PB-induced mice catalyse the metabolism of the insecticide phorate sulfoxide to phorate sulfone. The most active isoenzyme was the cytochrome P450 purified from PB-induced animals and the (+)-isomer was the preferred substrate for all isoenzymes.

The above results clearly show that the microsomal metabolism of TZR.SO leads to the formation of the sulfone, as unique metabolite. The almost exclusive involvement of the cytochrome P450 system in S-oxygenation was demonstrated by the lack of metabolism in (a) the absence of NADPH and (b) the presence of known inhibitors of cytochrome P450-dependent monooxygenase activities, such as clotrimazole [16]. This conclusion is also supported by the large increases observed after pretreatment of rats with various inducers of cytochromes P450.

The role of different isoenzymes of hepatic cytochromes P450 was estimated by using microsomes obtained from

Table 1. Rate of S-oxygenation of TZR.SO by rat liver microsomes from different sources

Rats	Treatment	S-oxygenation of TZR.SO nmol/min/mg mean (SEM)
8-Week males	Untreated	0.75 (0.13)
	TAO-pretreated	0.49 (0.11)
	TAO-pretreated (ferricyanide activated)	2.2 (0.25)
	PB-pretreated	3.22 (0.31)
	DEX-pretreated	8.77 (0.55)
	3-MC-pretreated	0.71 (0.05)
8-Week females	Untreated	0.11 (0.06)
4-Week males	Untreated	0.54 (0.08)

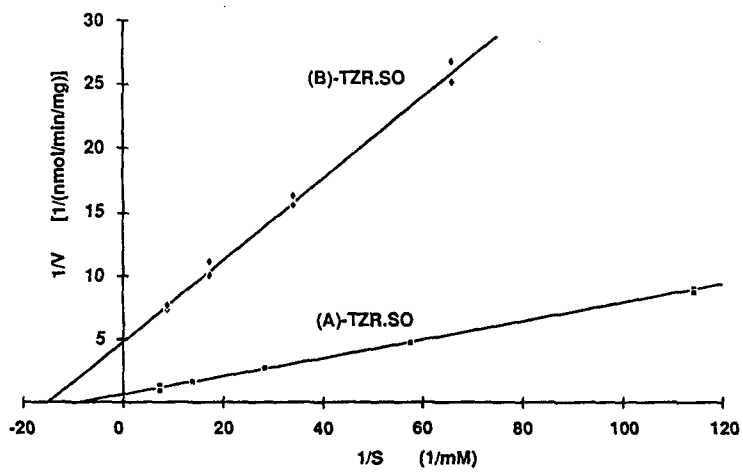

Fig. 3. Double reciprocal plot of S-oxygenation of (A)- and (B)-enantiomers of TZR.SO by liver microsomes from untreated rats.

rats pretreated with inducers, thus having variable isoenzyme composition. The results suggest that several isoenzymes of cytochrome P450 could be involved in the metabolism of TZR.SO. The low activity observed in microsomes from female rats suggests a minor influence of the most important female constitutive isoenzymes of cytochrome P450, 2C12, 2A1 and 2C6, and conversely, the involvement of the two major male-specific isoenzymes of cytochrome P450, namely 2C11 and 3A2 [17]. The two latter isoenzymes have different patterns of evolution with aging, the level of cytochrome 2C11 dramatically increasing in adult mature animals, but that of cytochrome P450 3A2 remaining unchanged [17]. By the use of testosterone 16α- and 6β-hydroxylase activities as specific probes for cytochromes P450 2C11 and 3A2, respectively, we show here the parallel evolution of S-oxygenation and testosterone 6β-, but not 16α-hydroxylase activity as a function of age, thus demonstrating the involvement of cytochrome P450 3A2 rather than 2C11 in S-oxygenation.

Erythromycin is considered to be a specific substrate for cytochromes P450 3A. The major isoenzyme of P450 induced by pretreatment with DEX is cytochrome P450 3A1 [18]. S-Oxygenation is strongly induced by DEX and inhibited by erythromycin in microsomes from DEX-pretreated rats, suggesting the participation of cytochrome P450 3A1. Additional evidence is provided by the dramatic increase which was observed when microsomes from TAO-pretreated animals were pre-incubated in the presence of potassium ferricyanide [18] leading to the concomitant increase in erythromycin *N*-demethylase activities and S-oxygenation.

The pretreatment of animals with PB leads to an increase in cytochromes 2B1, 2B2, 2C6, 2A1, 3A1 and 3A2 [19]. Previous reports have shown that orphenadrine is a potent inhibitor of pentylresorufin *O*-dealkylase (the most specific probe for cytochromes P450 2B1, 2B2 and 2C6 [20, 21]). Under our experimental conditions, with a concentration of orphenadrine leading to a near complete inhibition of pentylresorufin *O*-dealkylase activity, the partial inhibition of S-oxygenation suggests the involvement of the 3A isoenzymes in the reaction. Cytochrome P450 2A1 does not participate in the reaction, as discussed above, but the potent inhibition of S-oxygenation by erythromycin clearly confirms the role of cytochromes 3A in the reaction.

The lack of effect of 3-MC on S-oxygenation excludes the participation of cytochromes P450 1A in the reaction. However, it was recently demonstrated that the *in vitro* S-oxygenation of albendazole sulfoxide, another anti-parasitic drug, is catalysed by rat liver cytochromes P450 1A rather than cytochromes P450 2B [9].

In our previous work, the sulfoxidation of TZR was found to depend on several cytochromes P450 and the product stereospecificity of the sulfoxidase activity to be highly dependent on the isoenzyme of cytochrome P450. The present results show that the further oxygenation to the sulfone is highly enantioselective. According to the values of V_{max}, the (B)-enantiomer was slowly metabolized and accounted for less than 15% of the total metabolism. The K_m value for racemic mixture which is twice that calculated for the (A)-enantiomer, is an additional evidence for the weak participation of (B)-enantiomer in the oxygenation of the racemic compound. Also, we can conclude that the S-oxygenation of the (A)-enantiomer is mainly catalysed by cytochromes P450 3A, but we have no indication that the S-oxygenation of the (B)-enantiomer is catalysed by the same enzymes.

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