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CHARACTERIZATION OF SPECIFIC CYTOCHROME P450 ENZYMES RESPONSIBLE FOR THE METABOLISM OF DIAZEPAM IN HEPATIC MICROSOMES OF ADULT MALE RATS

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Abstract—The role of several P450 enzymes in the metabolism of diazepam (DZ) has been investigated. Hepatic microsomes of adult male rats were pretreated with antisera raised against the P450s CYP3A2, 2B1, 2C6, 2C11, 2D1 and 2E1, and their influence on the subsequent metabolism of DZ was determined by simultaneously measuring the changes in the relative rates of formation of its metabolites. Several forms of P450 were found to be positively involved in DZ metabolism. Antisera of the "male-specific" P450 enzyme CYP2C11 partially inhibited both DZ *N*-demethylase and C_3 hydroxylase activities (60%) which resulted in decreased formations of *N*-desmethyl-DZ (NDZ) and 3-hydroxy-DZ (3HDZ), respectively. In a reconstitution experiment with the purified enzyme, CYP2C11 predominantly catalysed the formation of NDZ from DZ. Antisera of a further male-specific P450 CYP3A2 strongly inhibited (95%) the C_3 hydroxylase of DZ and thus 3HDZ formation. A corresponding reconstitution experiment with this same P450 enzyme gave 3HDZ as principal product. CYP2D1 antisera inhibited the aromatic hydroxylation of DZ (98%) and subsequent formation of 4′-hydroxy-DZ (4′HDZ). This enzyme was also observed to inhibit DZ *N*-demethylase activity (60%). A reconstitution experiment with pure CYP2D1 catalysed the formation of both 4′HDZ and NDZ.

Diazepam (DZ§) is a representative member of the benzodiazepienes, a group of drugs which are widely used clinically as muscle relaxants and anticonvulsants. As their hepatic metabolism produces derivatives which also often have significant pharmacological activity, the metabolic fate of the parent drugs has been of continued interest. The metabolism of DZ, summarized in Fig. 1, has been demonstrated [2–7] to be species, age and sex dependent. *N*-Desmethyl-DZ (NDZ) and 3-hydroxy-DZ (3HDZ) however are two principal metabolites of DZ that are common to all species including man. They arise due to N^1-demethylation and C_3 hydroxylation of the drug as exemplified by routes DZ→NDZ and DZ→3HDZ, respectively, in Fig. 1. Relative rates of formation of these metabolites in rats have been shown to be both age and sex dependent. Hence, in young immature animals 3HDZ is formed [8, 9] to a much higher extent than NDZ. In contrast, it was shown [4, 10] that as a consequence of ageing (senescence), there is a dramatic decrease in the rate of formation of 3HDZ in older rats, a phenomenon which showed sex differences and was thus most pronounced in the microsomes of adult males.

The aromatic C-4′ hydroxylated metabolites of DZ, which include 4′hydroxy-DZ (4′HDZ) and 4′-hydroxy-*N*-desmethyl-DZ (4′HNDZ) (Fig. 1), occur only in rats and rabbits [6, 11–14]. They presumably arise due to the hydroxylation of DZ itself or of its resulting primary metabolites such as NDZ, as summarized by the routes DZ→4′HDZ and NDZ→4′HNDZ, respectively, in Fig. 1. In phase II reactions, they ultimately may become conjugated in the form of glucuronides.

Sex and ageing differences are frequently encountered during drug metabolism in rats. It is now currently accepted that these sex and ageing differences arise due to changes in the relative composition of the various enzymes comprising the drug-metabolizing cytochrome P450 family [15, 16] and are apparently influenced by hormones [17, 18]. Numerous laboratories [15, 16, 19–24] have now isolated and purified several sex-specific forms of cytochrome P450, usually from rat microsomes. The pure enzymes were subsequently used in reconstitution studies and to raise corresponding antibodies [10, 19, 23, 25] that could then be used in conjunction with hepatic liver microsomes to characterize, compare and contrast their metabolic activities with various drug substrates.

Only a few reports [10, 26, 27] have attempted to identify the P450 enzymes that are responsible for the metabolism of DZ. In these studies we have

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§ Abbreviations: DZ, diazepam; NDZ, *N*-desmethyl-DZ; 3HDZ, 3-hydroxy-DZ; 3HNDZ, 3-hydroxy-*N*-desmethyl-DZ; 4′HDZ, 4′-hydroxy-DZ; 4′HNDZ, 4′-hydroxy-*N*-desmethyl-DZ; DLPC, dilauroylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; PB, phenobarbital. Specific cytochrome P450 enzymes are assigned using the gene coding system designated by Nebert *et al.* [1].

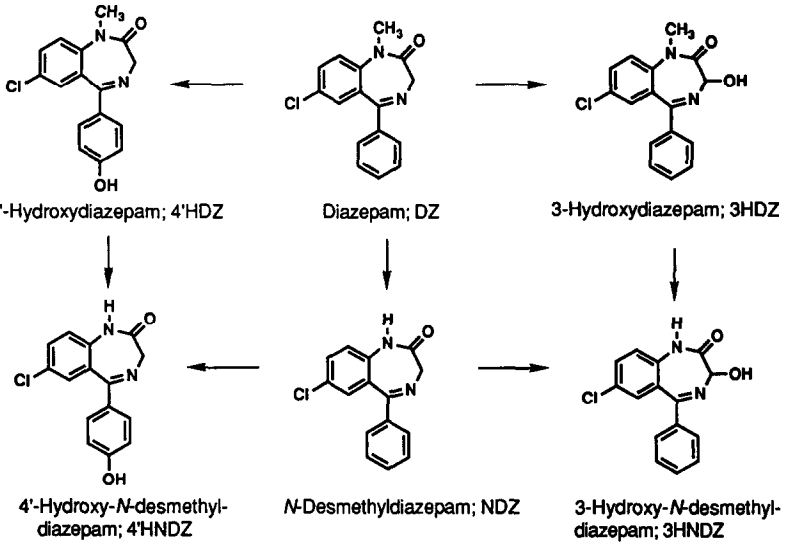

Fig. 1. Metabolic fate of DZ in rat microsomes.

investigated more closely the role of several P450s in the microsomal metabolism of this drug. In preliminary experiments, microsomes of young adult male rats were pretreated with antisera raised in goats or rabbits against the P450s CYP3A2, 2B1, 2C6, 2C11, 2D1 and 2E1. Their effect on DZ metabolism was then compared with control samples which were pretreated with non-immunized sera. After identifying antisera which influenced the metabolic profile of the drug, metabolism of the drug was also then studied in reconstituted systems using the corresponding pure P450 enzymes that were initially used to raise the antisera. Results from these studies may be subsequently correlated to explain the sex, species and aging differences apparent with DZ metabolism.

MATERIALS AND METHODS

Chemicals and reagents. [2-^{14}C]DZ, supplied by Amersham International (Amersham, U.K.), had a specific activity of 2.00 GBq/mmol. Authentic samples of NDZ, 3HDZ and 3-hydroxy-*N*-desmethyl-DZ (3HNDZ) were synthesized in our laboratories. Dilauroylphosphatidylcholine (DLPC) and dioleoylphosphatidylcholine (DOPC) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Phosphatidylserine (bovine, PS) was supplied by P-L Biochemicals (Milwaukee, WI, U.S.A.) and NADPH by the Oriental Yeast Co. (Tokyo, Japan). All other reagents and solvents were obtained from Nacalai tesque (Kyoto, Japan) and were of analytical grade or better.

Microsomes, purified P450s and P450 antibodies. Adult male rats, 7 weeks old, of the Wistar Imanichi strain (weighing 200–250 g), were obtained from Dobutsu Hanshoku Kenkyujo (Saitama, Japan). Livers were excised and immediately perfused with ice-cold physiological saline. Microsomes were then prepared in 50 mM Tris–HCl buffer, containing 0.15 M KCl, pH 7.4, by differential centrifugation of a 25% (w/v) homogenate. Cytochrome P450 was assayed by the method of Omura and Sato [28].

Cytochrome P450s were purified by the methods reported previously [22, 24]. Antisera against the purified P450s were raised in female Japanese white rabbits, obtained from Biotech (Saga, Japan) as described previously [23]. They were characterized and their specificity defined as reported earlier [29]. Cytochrome P450 reductase (fp_2) and cytochrome b_5 were also purified as described earlier [22]. They had a specific activity of 47 U/mg protein and a specific content of 28 nmol/mg protein.

Incubations of microsomes with P450 antisera. Microsomal suspensions (50 μL) containing 50–70 pmol of P450 were preincubated for 25 min at 37° with the appropriate diluted titer series of antisera (50 μL). Titer series and controls were prepared with non-immunized goat or rabbit serum as diluent. The following reactants were then added: NAD (1 mM), NADP (1 mM), $MgCl_2$ (37 mM), glucose-6-phosphate (10 mM), reduced glutathione (1 mM), glucose-6-phosphate dehydrogenase (10 IU/mL) and the labelled substrate 2-[^{14}C]DZ (5–8 μM in 10% ethanol–saline) in 0.5 M Tris–HCl (pH 7.4) to give a final volume of 200 μL. The microsomal suspensions were then incubated at 37° for a further 3 hr. Incubations were terminated by the addition of ice-cold methanol (0.5 mL) and the mixtures were centrifuged at 3000 *g* for 10 min to precipitate proteins. The supernatants were then analysed by TLC, as described later under "Analysis of metabolites".

Reconstitution studies with purified P450s. Suspensions of the relevant purified P450s (30–35 pmol), fp_2 (0.3 U) and DLPC (5 μg) were thoroughly mixed by Vortex. For the assay of CYP3A2, cytochrome b_5 (30 pmol) was also added and in place of DLPC, a 1:1:1 mixture of DLPC, DOPC and PS (10 μg) was used in analogous fashion to that described

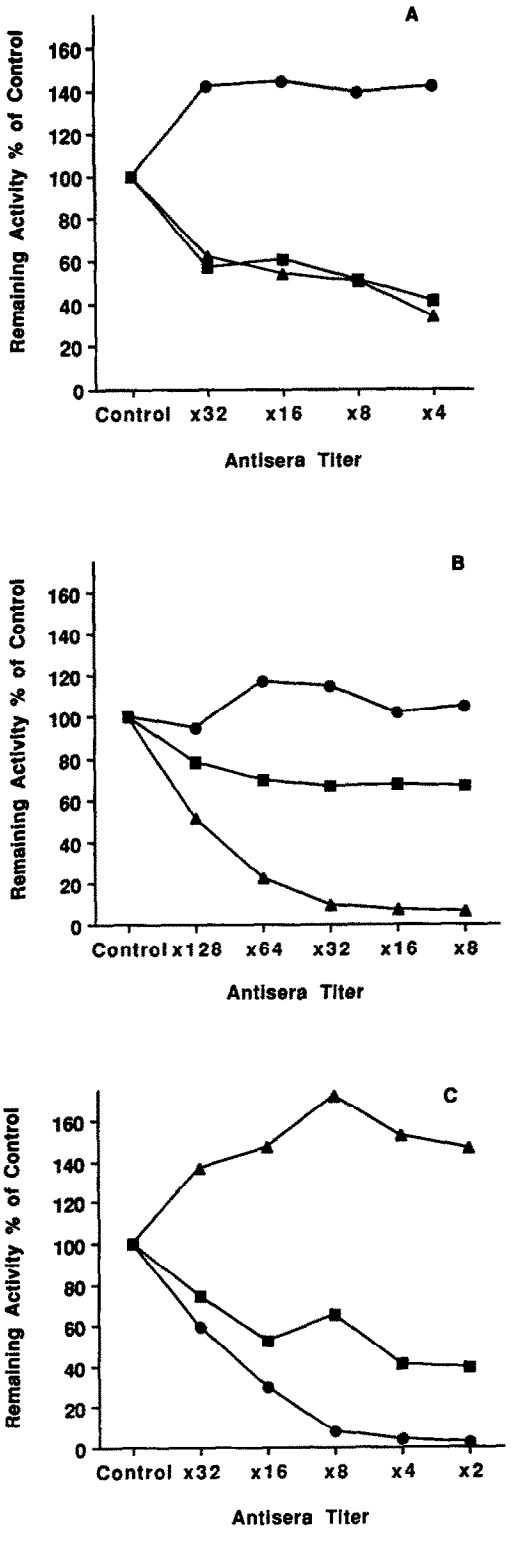

Fig. 2. Influence of antisera of (A) CYP2C11, (B) CYP3A2 and (C) CYP2D1 on DZ metabolism in microsomes of adult male rats. Results are presented as the remaining activity relative to control samples which were treated with non-immunized serum, and are the means of two incubations. Experiments were conducted as described under Materials and Methods. The symbols represent; (■) NDZ, (▲) 3HDZ and (●) 4'HDZ.

Table 1. Reconstruction experiments with DZ and the purified P450s: CYP2C11, CYP3A2 and CYP2D1

Reconstitution system	nmol metabolite formed/min/nmol P450		
	NDZ	3HDZ	4'HDZ
CYP2C11	0.42	0.10	—
+ non-immunized serum	0.37	0.10	—
+ anti-2C11 serum	—	—	—
CYP3A2	—	0.32	—
+ non-immunized serum	—	0.26	—
+ anti-3A2 serum	—	—	—
CYP2D1	0.44	—	2.57
+ non-immunized serum	0.48	—	2.68
+ anti-2D1 serum	0.22	—	0.66

The cytochrome P450s CYP2C11 and 2D1 (30 pmol) were assayed in the presence of 0.3 U of cytochrome P450 reductase and 5 μg of DLPC. In the case of CYP3A2 the mixtures also contained cytochrome b_5 (30 pmol) and in place of DLPC, 10 μg of a 1:1:1 aqueous suspension of the lipids DLPC, DOPC and PS. Incubations were carried out in 0.1 M Tris buffer, in buffer containing non-immunized rabbit or goat serum or in buffer containing the corresponding P450 antiserum.

Values are expressed as nanomoles of metabolite formed per minute per nanomole of cytochrome P450 and are the means of two assays.

Catalytic activities less than 0.1 nmol of metabolite/min/nmol cytochrome P450 are designated —.

previously [30]. They were then preincubated at 37° for 3 min. Buffer (0.1 M Tris–HCl; pH 7.4), buffer containing non-immunized serum or the corresponding P450 antiserum, and the substrate [^{14}C]DZ (5–8 μM in 10% ethanol–saline) were then added to give a final volume of 500 μL. Samples containing sera were preincubated at room temperature for 25 min. Reactions were initiated by the addition of NADPH (10 mM) and the mixtures incubated at 37° for a further 15 min. They were also stopped by addition of ice-cold methanol (0.5 mL) and analysed by TLC, again as described later.

Analysis of metabolites. Using a Drummond Microdispenser (Series 300), aliquots (10 μL) of the incubation mixtures were applied to TLC plates: precoated silica gel, type 60F_{254} (20 × 20 cm), layer thickness 0.25 mm, Merck art No. 5715. They were developed with the solvent system chloroform:hexane:acetic acid:ethanol (10:10:1:1). In such a system DZ and its metabolites had the following R_F values: DZ, 0.55; NDZ, 0.42; 3HDZ, 0.37; 3HNDZ, 0.20; 4'HDZ, 4'HNDZ, 0.12. In some instances resolution was not always possible for the more polar metabolites 4'HDZ and 4'HNDZ.

Developed plates were then exposed to Fuji imaging plates (Type BA), placed in corresponding cassettes and maintained in a lead-shielded box for 48 hr. These plates are coated with a photostimulatable phosphor layer which detects and accumulates energy of radiation from radiolabelled

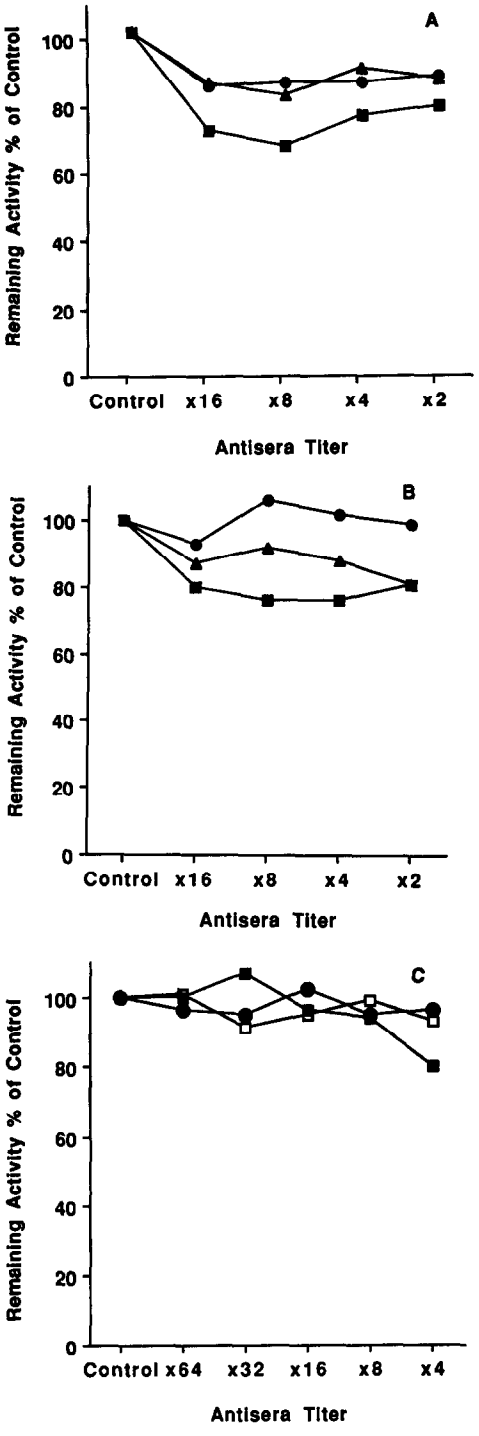

Fig. 3. Influence of antisera of (A) CYP2B1, (B) CYP2C6 and (C) CYP2E1 on DZ metabolism in microsomes of adult male rats. Symbols and data presented are the same as described in the legend of Fig. 2.

substances. They are then scanned with a Fuji bioimage analyser (BA100) which releases the trapped energy of radiation and produces an image showing separated spots of the unchanged labelled substrate or its metabolites. Relative percentages of unchanged DZ and its metabolites were then determined using the appropriate computer software. Data were calculated and presented as the amount of remaining activity relative to control samples which were treated with non-immunized sera. The characteristics of the bioimage analyser system have been described previously [31].

RESULTS

CYP2C11 was isolated from microsomes of untreated male rats, designated UT-2 and shown to have considerable activity towards the N-demethylation of benzphetamine [22]. When we pretreated microsomes of untreated adult male rats (aged 7 weeks old) with antisera against this enzyme both DZ N^1-demethylation and C_3 allylic hydroxylation were partially inhibited; in both cases, by approximately 60% at low titers of antisera. This concomitantly resulted in decreased formation of NDZ and 3HDZ, respectively, as shown in Fig. 2A, and suggested that this enzyme was involved in both these reactions in adult male rats. The results also showed an apparent increase in the rate of formation of aromatic hydroxylated DZ metabolites such as 4'HDZ. This affect may be attributable to the switching of the microsomal metabolism from pathways which involve inhibited P450s to those which are unaffected and probably reflects limited availability of substrate in the incubation system. Activity of purified CYP2C11 was subsequently studied in a reconstituted system with added phospholipids and cytochrome P450 reductase. Results showed (Table 1) that it catalysed formation of both NDZ and 3HDZ; however, the latter was obtained in only about 30% of the amounts of the former and provided strong evidence that this P450 enzyme was only primarily involved with the N^1-demethylation of DZ. As with all purified enzymes studied in reconstitution experiments, non-immunized serum did not greatly affect the activity of 2C11, as similar catalytic properties were observed in reconstituted assays with normal reaction buffer or buffer containing non-immunized sera (see also Table 1). In contrast, addition of antisera raised against CYP2C11 inhibited the activity of the enzyme and thus the formation of NDZ and 3HDZ was again reduced.

The male-specific enzyme CYP3A2 was first obtained [19] from microsomes of adult male rats which were treated with either pregnenolone-16α-carbonitrile (PCN) or phenobarbital (PB) and thus referred to as P450 PB/PCN. Imaoka and coworkers [22, 32, 33] have since obtained this P450 from PB-induced microsomes, as PB-1, and also in significant amounts from untreated rats. It was subsequently characterized by its ability in hydroxylation of testosterone at both the 2β and 6β positions; with the latter however being the most pronounced. In these studies addition of antisera against CYP3A2 to microsomal incubations inhibited C_3 hydroxylation of DZ and thus 3HDZ formation (Fig. 2B). The observed inhibition was strong at low titers of added antisera (*ca.* 94%), in contrast to N^1-demethylation, and NDZ formation, which showed only slight

inhibition (30%). This indicated that CYP3A2 is one of the principal P450 enzymes involved in 3HDZ formation. A reconstitution study with purified CYP3A2 under the same conditions as used with CYP2C11, and hence with DLPC as added lipid, did not show any catalytic activity. As in earlier reconstitution studies with CYP3A2 [28, 32, 33], use of a 1:1:1 lipid mixture of DLPC, DOPC and PS was however more successful. The purified 3A2 was then observed to catalyse the allylic hydroxylation of DZ to give 3HDZ. Formation of NDZ was not detected. Activity in the reconstituted system was inhibited by the addition of antisera of 3A2 (Table 1).

CYP4502D1 was also isolated from untreated male rats, as UT-7 [24]. It has since been associated with aminopyrine demethylation [34] and debrisoquine allylic hydroxylation [35]. In these studies, pretreatment of adult male microsomes with raised antisera of this P450 enzyme subsequently inhibited the C-4′ aromatic hydroxylation of DZ and thus formation of metabolites such as 4′HDZ (Fig. 2C). Since this inhibition was again strong (*ca.* 98% at low titers), it appears that 2D1 is one of the principal enzymes responsible for the formation of these metabolites in microsomes of adult male rats. Similarly, CYP2D1 antisera partially inhibited (60%) DZ *N*-demethylase activity and thus NDZ formation; which suggested that this P450 enzyme is also involved in the N^1-demethylation of DZ. This latter observation could perhaps account for the uninhibited portion of DZ *N*-demethylase activity we observed in earlier studies with antisera of CYP2C11 (cf. Fig. 2A) and that reported by Fujita *et al.* [10] with antisera raised against their male-specific form of 2C11. Furthermore, in our studies pretreatment of microsomes with CYP2D1 antisera also apparently caused an increased rate of 3HDZ formation. As previously described for data with antisera of 2C11, this may be due to transfer of metabolic activity to pathways that involve P450 enzymes which are uninhibited and maybe again demonstrate limited substrate availability. In a reconstitution study with purified 2D1 (Table 1), this P450 enzyme catalysed formation of both NDZ and 4′HDZ; relative rates of formation were 15 and 85%, respectively. These activities were again inhibited by addition of the corresponding antisera against CYP2D1 to the reconstituted system.

DISCUSSION

In previous unreported studies in our laboratories we have investigated the sex and ageing differences of DZ metabolism in rat liver microsomes, during ontogeny (development). Neonatal rats showed higher formation of 3HDZ than NDZ (data not shown). In contrast formation of C-4′hydroxylated metabolites of DZ, such as 4′HDZ, showed increased rates in young adult male animals, >5 weeks old in particular, when compared to neonatals, and thus suggested that this metabolic pathway was more active in young adult animals than in neonatals that are still in the early stages of development. These results again suggested that DZ is subject to metabolism by different P450 enzymes the relative amounts of which vary as a function of age and sex.

Our results have indicated that in adult male rat microsomes CYP2C11 plays a significant role in the N^1-demethylation of DZ to form NDZ. In a recent study [10], a P450 enzyme corresponding to CYP2C11 was also isolated and termed a "male-specific" cytochrome P450 (P450 mL). Antibodies were raised against it and were observed to inhibit partially the demethylation of imipramine and DZ in microsomes of adult male rats. In a subsequent reconstitution study with DZ, it also catalysed NDZ and 3HDZ formation; activity of the former being about three times that of the latter. Hence, the results we have obtained in this study with DZ correlate very well with those which Fujita *et al.* [10] reported. Jauregui *et al.* [26] also reported recently that the PB-inducible enzyme CYP2B1 was implicated in the N-demethylation of DZ in hepatic microsomes and cultured hepatocytes, both of which had been induced with PB. In these studies antisera of the enzymes CYP3A2, 2B1 and 2C6, (Figs 2B, 3A and B, respectively) also showed slight inhibition (*ca.* 25–30%) of DZ N-demethylation. As these enzymes are all inducible by PB their influence may explain the data observed by Jauregi *et al.* [26]. We concluded however that the inhibition values we observed were too low to warrant further investigation. Antisera of the ethanol-inducible P450 enzyme CYP2E1 (Fig. 3C) did not have any influence on the microsomal metabolism of DZ.

Results from these studies also strongly indicated that the PB-inducible enzyme CYP3A2 is the principal P450 enzyme involved in the C_3 allylic hydroxylation of DZ to give 3HDZ. Previous studies [27], using either monooxygenase (P450) inducers or selective enzyme inhibitors, demonstrated that P450 enzymes of the CYP3A family were involved in formation of both 3HDZ and the secondary metabolite 3HNDZ via the C_3 hydroxylation of DZ and its primary metabolite NDZ, respectively (cf. Fig. 1). To the best of our knowledge however, this is the first time when the specific enzyme involved in 3HDZ formation in *in vitro* systems has been identified. Under the incubation conditions used in these studies, formation of the secondary metabolite 3HNDZ was rarely observed.

Cytochrome P450 enzymes, in particular of the CYP2C11, 3A2 and 2D1 gene families, also show interspecies differences in drug metabolism [36]. In these studies the role of CYP2D1 in DZ metabolism in rat microsomes perhaps exemplifies such differences. Although enzymes of the CYP2D gene family, such as 2D1, are known for their *N*-demethylase activities, they have been more commonly characterized by their ability to form hydroxylated metabolites of drugs. In substrates these hydroxylations are usually directed towards the *para* position of their aromatic rings or to alkyl substituents situated therein [36]. In the case of the β-adrenoreceptor blocking drugs such as propranolol, bufurolol and metoprolol, and the antihypertensive agent debrisoquine, these type of reactions are catalysed by a comparable CYP2D enzyme present in both rats and humans [37]. Hence, antibodies raised against the rat form of this enzyme, at first referred to as $P450_{UT\text{-}H}$ and later as db1 [35], inhibited the hydroxylase activity of some of these substrates in microsomes or both humans and rats

[37]. We have shown that DZ aromatic hydroxylation at the C-4′ *para* position of the substrate, to give 4′HDZ, is catalysed by CYP2D1 in male rat microsomes. However, formation of a corresponding 4′-hydroxylated metabolite in humans is not observed. Furthermore, it is noteworthy that antibody raised against rat P450 CYP2D1 enzyme did not influence greatly DZ *N*-demethylase activity in human liver [37]; a metabolic pathway which is a major route for DZ metabolism in humans [2, 3, 6, 7] and which we have also observed to be catalysed by this enzyme in rat microsomes. These differences in DZ metabolism may be due to interspecies variation in the relative affinities of the CYP2D enzyme for the substrate. In any case it suggests that DZ shows substrate specificity, for different forms of P450, during its metabolism by different species.

In summary, our results from these studies indicated that during DZ metabolism in microsomes of adult male rats, the P450s CYP2C11, 3A2 and 2D1 are chiefly implicated in its N^1-demethylation, C_3 allylic hydroxylation and C-4′ aromatic hydroxylation, respectively. Reconstitution studies with DZ and the corresponding purified P450 enzymes of CYP2C11, CYP3A2 and CYP2D1 thus primarily catalysed formation of NDZ, 3HDZ and 4′HDZ.

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