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Cytochrome P450IIIA Enzymes in Rat Liver Microsomes: Involvement in C_3-Hydroxylation of Diazepam and Nordazepam but Not *N*-Dealkylation of Diazepam and Temazepam

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SUMMARY

Microsomes prepared from livers of male and female rats of nine inbred and outbred strains and of male Sprague-Dawley rats pretreated with monooxygenase-inducing agents were used to study *N*-dealkylation of diazepam and temazepam and C_3-hydroxylation of diazepam and nordazepam. Both C_3-hydroxylation reactions were more rapid in male than in female liver preparations, but this gender-dependent pattern was not seen with the *N*-dealkylation reactions. These results indicate the lack of identity of the monooxygenases responsible for the two kinds of reaction and suggest that male-specific enzyme(s) are responsible for the C_3-hydroxylations. Induction studies were undertaken to further define these enzymes. To do this, liver microsomes prepared from male Sprague-Dawley rats pretreated with a variety of agents known to have different monooxygenase induction effects were used. With triacetyloleandomycin, dexamethasone, and phenobarbital pretreatment, the specific activities of the C_3-hydroxylation reactions were selectively elevated over corresponding control values. These particular xenobiotics are known to enhance the abundance of cytochrome P450IIIA family enzymes, and our results strongly suggest the involvement of these enzymes in the benzodiazepine B ring monooxygenations. Formation of temazepam was also shown to be inhibited by triacetyloleandomycin. This effect was demonstrated to be equal in both saline-treated and dexamethasone-treated male Sprague-Dawley rat liver microsomes, with the antibiotic present either with diazepam throughout the entire incubation period or initially with NADPH in a preincubation mix for 15 min, following which C_3-hydroxylation was initiated by the addition of diazepam. These results confirm the uniformity of the involvement of cytochrome P450IIIA family enzymes in diazepam C_3-hydroxylation in untreated and inducer-treated rat liver microsomes. Recent studies with human and rabbit liver microsomal preparations have shown that orthologues of these enzymes also catalyze an equivalent hydroxylation in the B ring of midazolam. These findings, considered with the present results showing that the adjacent methyl *N*-substituent (absent in nordazepam but present in diazepam) did not affect the selectivity of these enzymes for the C_3-hydroxylation reaction, suggest that neither steric nor electronic factors markedly influence catalysis of this monooxygenation by these enzymes.

Diazepam is a prototypic member of the 1,4-benzodiazepine class of drugs, with hypnotic, anxiolytic, and muscle relaxant properties (1). It is highly lipophilic, is extensively protein bound in plasma, and has a distribution volume of approximately 1 liter/kg of body weight (2). In normal human medical usage, it is present in extracellular fluid at low micromolar concentrations and is cleared predominantly in urine as conjugates of polar metabolites, which are formed principally in liver by P450-mediated monooxygenations (3). Its clearance varies widely between individuals, showing dependence on gender, age, and exposure to a variety of other drugs and xenobiotics that alter the activity of P450 isoenzymes (4–8). In addition, diazepam clearance is reduced in people who metabolize (*S*)-mephenytoin poorly by the phenyl *p*-hydroxylation route (9), and studies with human liver microsomes have shown that diazepam potently inhibits this (*S*)-mephenytoin oxidation reaction (10, 11). The most important P450-mediated oxidative biotransformations leading to diazepam elimination in humans, dogs, and rats, which are the best studied species, are carbon hydroxylations in the 5-phenyl and heterocyclic ring moieties and *N*-dealkylation (12). The products resulting from *N*-dealkylation (nordazepam) and C_3-hydroxylation (temazepam) are themselves pharmacologically active, and each may undergo further metabolism (by C_3-hydroxylation and *N*-dealkylation, respectively) to yield oxazepam (see Fig. 1). Oxazepam is the predominant ultimate oxidized metabolite of diazepam in humans and, like nordazepam and temazepam, is pharmacologi-

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ABBREVIATIONS: P450, cytochrome P450; βNF, β-naphthoflavone; CIM, cimetidine; DEX, dexamethasone; G6PDH, glucose-6-phosphate dehydrogenase; HPLC, high performance liquid chromatography; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin; SD, Sprague-Dawley; TAO, triacetyloleandomycin; 3MC, 3-methylcholanthrene; CABH, Central Animal Breeding House.

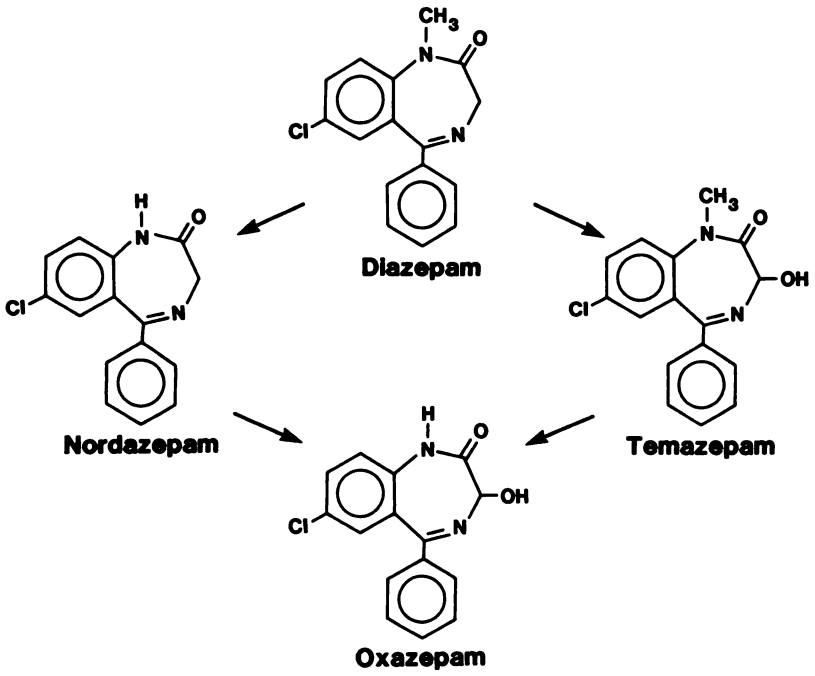

Fig. 1. Illustration of the monooxygenase-dependent interrelations between the benzodiazepines studied here. Diazepam undergoes either *N*-dealkylation, producing nordazepam, or C_3-hydroxylation, yielding temazepam. Each of these products is convertible to oxazepam by C_3-hydroxylation or *N*-dealkylation, respectively.

cally active. Because each member of this group of biologically active drugs is generated in the course of diazepam clearance, we considered it important to understand which P450 isoenzymes may contribute to their production. The identification of these enzymes would also be of value in the study of the mechanisms underlying interactions between the benzodiazepines and coadministered drugs and other xenobiotics. The complementary nature of the two oxidative routes leading from diazepam to oxazepam also suggested the value of separately studying all four of the monooxygenations shown in Fig. 1 to obtain insights into the structural features of the substrates that may influence regioselectivity.

Interspecies comparative studies of P450 have revealed families of isozymic forms that exhibit related structural, catalytic, and regulatory properties (13, 14). These classifications have helped to improve understanding of the marked gender differentiation of P450 monooxygenases exhibited by adult rat liver. Male animals have higher liver P450 specific contents and higher liver microsomal steroid 16α- and 6β-hydroxylase and erythromycin, *d*-benzphetamine, and ethylmorphine demethylase activities than females (14–16), and these differences are recognized as being attributable to differential expression of particular liver P450 isoenzymes in families II and III (16). Constitutively expressed male-specific enzymes in rat liver may be distinguished by differential regulation during development, by regio- and stereoselectivity of endobiotic steroid hydroxylations, by induction responses to administration of xenobiotics, and by specificity for particular xenobiotic monooxygenations (17). The P450IIIA family contains enzymes that are induced by PB and DEX in rabbits, rats, and humans and by TAO in rats and rabbits (15, 18). PCN induces these enzymes in rats but not in rabbits, whereas RIF is an inducer in rabbits, mice, and humans but not in rats (15, 18). These enzymes are principally responsible for the hydroxylation of a number of natural steroid substrates such as testosterone and cortisol (19, 20), for the metabolism of macrolide antibiotics such as erythromycin and TAO (21), and for the oxidation of nifedipine and cyclosporin A (22, 23). They were first characterized by their high activity for *N*-demethylation of ethylmorphine (24) and one of them (P450IIIA2) is male specific in untreated rats (19). Very recently, human and rabbit orthologues in this family of enzymes have been implicated in catalyzing monooxygenations that mediate the clearance of midazolam, in particular hydroxylation at C_4 of the B ring of this benzodiazepine (25, 26), a site that is structurally equivalent to the C_3 position of the B ring of diazepam. These observations suggested to us that early findings (27) showing C_3-hydroxylation, but not *N*-dealkylation, of diazepam to be higher in male than in female rat liver homogenate supernatants could indicate C_3-hydroxylation being attributable to members of this family of enzymes. Because these isoenzymes become substantially inactivated upon purification (28), we have sought to clarify their involvement in benzodiazepine metabolism by studying a number of microsomal monooxygenations. This was done by determining the inhibitory effects of TAO on diazepam C_3-hydroxylation in liver microsomes prepared from saline-treated and DEX-treated male SD rats and by determining correlations between all of the reactions shown in Fig. 1 and other characteristic monooxygenase activities, using washed liver microsomes prepared from untreated male and female rats of nine inbred and outbred strains and from male SD rats treated with selected monooxygenase-inducing agents.

Experimental Procedures

Materials. D-Glucose-6-phosphate (disodium salt) and G6PDH (yeast enzyme, grade 1) were obtained from Boehringer Mannheim Pty. Ltd. (Sydney, Australia). DEX, βNF, RIF, and 3MC were obtained from Sigma Chemical Co. (St Louis, MO). Ethylmorphine hydrochloride was a product of Macfarlan Smith Ltd. (Edinburgh, Scotland) and *p*-nitroanisole was obtained from the Aldrich Chemical Co. (Milwaukee, WI). PB was obtained from Queensland Ethicals (Brisbane, Australia). CIM, *d*-benzphetamine, erythromycin, TAO, and camazepam were gifts from Smith Kline and French Laboratories Ltd. (Sydney, Australia), Upjohn Co. (Kalamazoo, MI), Fauldings Pty. Ltd. (Adelaide, Australia), Pfizer (Brussels, Belgium), and Boehringer-Ingelheim Pty. Ltd. (Syd-

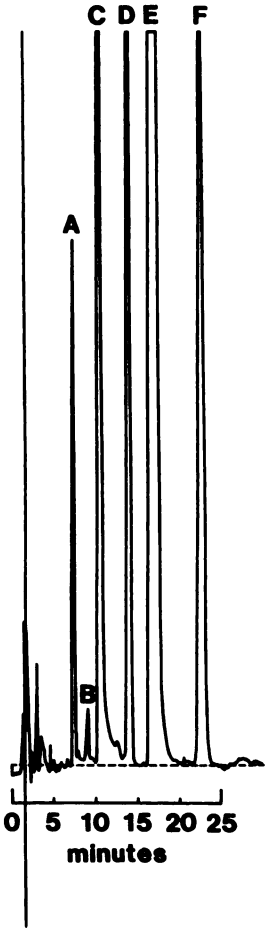

Fig. 2. Reverse phase HPLC analysis, with detection at 236 nm, of benzodiazepines in a mixture resulting from incubation of liver microsomes of PB-treated SD rats with 200 μM diazepam, 1 μM NADPH, and 1 μM P450 in 100 mM Tris·HCl, pH 7.9, buffer for 10 min at 37°. *Peak A* is a nonquantified unidentified polar product present in incubation mixtures with all microsomal preparations used here, *peak B* is oxazepam (a minor product with diazepam as substrate), and *peaks C, D, E,* and *F* indicate the elution pattern for temazepam, nordazepam, diazepam, and camazepam, respectively.

ney, Australia), respectively. Oxazepam, temazepam, nordazepam, and diazepam were all gifts from Roche Products Pty. Ltd. (Sydney, Australia). HPLC grade methanol was purchased from Mallinckrodt Pty. Ltd. (Clayton, Australia), and HPLC grade ethyl acetate was obtained from Waters Associates Inc. (Milford, MA). Other chemicals and solvents were of reagent grade; water that was purified using an Elga Spectrum system and that had a conductance of 0.07 μS/cm was used throughout.

Animals. Random outbred Wistar and SD rats of both genders were obtained from the University of Queensland CABH and from the University of Queensland Medical School animal house, respectively. Male and female rats of the inbred Buffalo, Dark Agouti, and Hypertensive strains were obtained from CABH, and male and female rats of the inbred Lewis and F344 strains were obtained from the Commonwealth Scientific and Industrial Research Organization Laboratories (Long Pocket, Brisbane, Australia) and from the John Curtin School of Medical Research (Canberra, Australia), respectively. CABH also supplied males and females of semi-inbred Noble (originating as a gift in 1984 from the Cancer Control Agency, Vancouver, British Columbia, Canada, and maintained through inbreeding) and Gunn (glucuronosyl transferase-deficient, supplied by the National Institutes of Health to the University of Queensland in 1978 and maintained by inbreeding) strains of rats. Monooxygenase inducer and vehicle treatments, given only to groups of six male SD rats, were as follows: isotonic sterile saline or sterile pure cold pressed corn oil, 0.5 ml/animal, given once per day by intraperitoneal injection for 5 days; PB or CIM, 1 mg/ml dissolved in drinking water, given for 7 and 14 days, respectively (drinking water consumption indicates average drug dosage of 120 mg/kg of body weight/day in each case); ethanol, 10 mg/ml in drinking water, given for 14 days; 3MC, βNF, and DEX, administered suspended in corn oil, intraperitoneally, once daily for 3, 4, and 5 days, respectively, at dose rates of 25, 40, and 20 mg/kg of body weight/day, respectively; and RIF, dissolved in saline, administered intraperitoneally once daily for 5 days at a dose rate of 50 mg/kg of body weight/day. In addition nine male SD rats caged individually were given either corn oil or TAO suspended in corn oil, intraperitoneally, at a dose rate of 500 mg/kg of body weight/day for 5 days. All intraperitoneal treatments were administered in 0.5 ml with the exception of TAO, which was given as a 1-ml injection, and all treatments were withheld 24 hr before sacrifice; animals receiving inducers as drinking water supplements were given tap water for the corresponding period. All animals were 6–10 weeks of age, were maintained in a temperature-controlled room (20–25°) under a 12-hr light/dark cycle, with food and water available *ad libitum*, and were acclimatized to this environment for 1 week before use.

Methods. Pooled microsomal suspensions were prepared (29) from livers taken from groups of six male and six female rats of each of the strains and from the groups of male SD rats given inducing agents as detailed above. Microsomal suspensions were also prepared separately from livers taken from the individually caged corn oil- and TAO-treated male SD rats. Microsomal P450 and protein concentrations were determined (30, 31) and the Hantzsch reaction was used (32) to quantitate formaldehyde produced in assays for ethylmorphine and *d*-benzphetamine (33) and erythromycin (34). The demethylation of *p*-nitroanisole was determined by recording *p*-nitrophenol production spectrophotometrically (35). The P450/TAO metabolite complex in microsomes prepared from rats that had received TAO was dissociated with ferricyanide (36), the preparations were washed by recentrifugation and resuspension immediately before P450 was quantitated, and monooxygenase activity measurements were made on these preparations. The corresponding corn oil control preparations were also treated in this way immediately before use. Monooxygenase activities with diazepam (200 μM), nordazepam (100 μM), or temazepam (100 μM) as substrates were determined in 200-μl reaction mixtures containing 100 mM Tris·HCl, pH 7.9, buffer, 2.5 mM glucose-6-phosphate, 1 IU G6PDH, 1.0 mM NADPH, and 1 μM P450. Due to the lipophilic nature of the benzodiazepines, these substrates were dispensed into monooxygenase assay tubes from methanolic stock solutions (stored in glassware treated with 0.2 g/liter cetyltrimethylammonium bromide to minimize adsorption), and the samples were evaporated to dryness at room temperature using an air stream. The benzodiazepines were then dissolved by sonication at room temperature for 15 min with a Bransonic 2200 bath sonifier, using an aliquot of the monooxygenase reaction buffer. This procedure was adopted to allow exclusion of propylene glycol and other benzodiazepine-solubilizing agents that had been demonstrated in preliminary studies to inhibit these monooxygenations. Mixtures containing all components except microsomal suspension were preincubated at 37°C for 3 min, to allow temperature equilibration, and monooxygenase reactions were started by the addition of microsomal suspensions. All reactions were conducted over 10 min. Incubations prepared in the absence of G6PDH and NADPH did not produce benzodiazepine metabolites, and these mixtures were used as reaction blanks for each of the microsomal preparations. Preliminary kinetic studies with diazepam had demonstrated that these monooxygenations obeyed apparent first-order kinetics up to the highest substrate concentrations attainable with or without the addition of solubilizing agents, and, at the concentrations of benzodiazepines used here, analyses of these monooxygenations were not limited by substrate concentration; linear yields of all the products with respect to both assay duration and P450 concentration were obtained. Reactions were stopped by the addition of 50 μl of internal standard (camazepam, 10

TABLE 1

P450 specific contents and monoxygenase specific activities of liver microsomal preparations of male and female rats of various inbred and outbred strains

Details of the methods are given in Experimental Procedures. The results are means ± standard deviations of three to six determinations on microsomal samples obtained from pooled livers of six rats. Significance levels of gender differences in P450 specific content and ethylmorphine, *d*-benzphetamine, and erythromycin *N*-demethylase activities are indicated.

Rat gender and strain	P450 specific content	Specific activity			
		Ethylmorphine *N*-demethylase	Benzphetamine *N*-demethylase	Erythromycin *N*-demethylase	*p*-Nitroanisole *O*-demethylase
	nmol/mg of protein	*μmol of HCHO/min/μmol of P450*			*μmol of p-nitrophenol/min/μmol of P450*
Male SD	0.79 ± 0.02[a]	4.82 ± 0.11[a]	2.89 ± 0.10[a]	0.85 ± 0.08[a]	1.67 ± 0.08[b]
Female SD	0.46 ± 0.02	2.37 ± 0.01	1.26 ± 0.07	0.55 ± 0.02	2.77 ± 0.38
Male Lewis	0.83 ± 0.02[b]	7.8 ± 0.10[a]	4.67 ± 0.16[a]	1.06 ± 0.01[a]	1.63 ± 0.04[b]
Female Lewis	0.70 ± 0.04	2.45 ± 0.10	2.14 ± 0.04	0.29 ± 0.03	1.29 ± 0.08
Male Gunn	0.75 ± 0.04	9.51 ± 0.35[a]	4.18 ± 0.29[a]	1.40 ± 0.08[a]	2.22 ± 0.02
Female Gunn	0.69 ± 0.04	1.98 ± 0.10	2.69 ± 0.06	0.50 ± 0.05	2.30 ± 0.10
Male Buffalo	0.79 ± 0.06	7.63 ± 0.24[a]	3.62 ± 0.09[a]	1.63 ± 0.05[a]	1.69 ± 0.06[b]
Female Buffalo	0.73 ± 0.03	4.16 ± 0.21	1.85 ± 0.05	0.73 ± 0.01	1.42 ± 0.02
Male F344	0.79 ± 0.04	8.78 ± 0.41[a]	3.90 ± 0.20[a]	1.18 ± 0.01[a]	2.35 ± 0.06
Female F344	0.72 ± 0.02	3.30 ± 0.07	2.87 ± 0.05	0.68 ± 0.06	2.45 ± 0.04
Male Dark Agouti	0.95 ± 0.02[a]	8.40 ± 0.19[a]	3.26 ± 0.09[a]	1.34 ± 0.04[a]	1.79 ± 0.01[a]
Female Dark Agouti	0.71 ± 0.04	2.51 ± 0.08	2.16 ± 0.31	0.24 ± 0.06	1.30 ± 0.01
Male Noble	0.67 ± 0.04	8.45 ± 0.17[a]	4.43 ± 0.09[a]	1.28 ± 0.01[a]	2.25 ± 0.02[b]
Female Noble	0.70 ± 0.06	1.85 ± 0.02	2.01 ± 0.09	0.40 ± 0.01	1.93 ± 0.08
Male hypertensive	0.91 ± 0.06[b]	7.06 ± 0.28[a]	3.43 ± 0.11[a]	1.61 ± 0.10[a]	1.47 ± 0.07[b]
Female hypertensive	0.61 ± 0.03	2.16 ± 0.23	1.05 ± 0.02	0.80 ± 0.01	1.22 ± 0.06
Male Wistar	0.85 ± 0.04[c]	6.34 ± 0.12[a]	2.90 ± 0.03[a]	1.11 ± 0.13[a]	1.32 ± 0.02
Female Wistar	0.71 ± 0.06	2.70 ± 0.02	2.40 ± 0.08	0.22 ± 0.04	1.29 ± 0.03

[a] $p < 0.005$.
[b] $p < 0.01$.
[c] $p < 0.05$.

μg/ml in tetrahydrofuran), and mixtures were vortexed and placed on ice. Benzodiazepines were extracted using 0.2 ml of 1 M carbonate buffer, pH 10, and 0.8 ml ethyl acetate, by vortex mixing. Samples were centrifuged (10,000 × *g* for 4 min at room temperature), 0.5 ml of the ethyl acetate layer was transferred to a tapered plastic centrifuge tube, and solvent was removed under a gentle air stream at room temperature. Samples were reconstituted for HPLC by the addition of 100 μl of mobile phase (55% methanol/45% water containing 0.02% triethylamine, adjusted to pH 5.0 with 1 M H_3PO_4) and sonication as described above. HPLC analyses were carried out using mobile phase filtered through a 0.22-μM membrane (Durapore; Millipore Corp, Bedford, MA), with additional gas removal being achieved with an in-line ERMA model ERC3522 membrane degassing unit. A Shimadzu LC-6A liquid chromatography pump was used to deliver mobile phase at 1.8 ml/min, and 20-μl aliquots of samples were applied to a Waters 8-mm × 10-cm Nova-Pak reverse phase C-18 cartridge in a Waters 8 × 10 radial compression module, using a Shimadzu model SIL-9A automatic injector fitted with a 150-μl sample loop. Products were detected using a Shimadzu SPD-6AV variable wavelength monitor set at 236 nm, and absorbance data relating to oxazepam, temazepam, and nordazepam were collected and analyzed in terms of peak area ratios with reference to camazepam, using a Shimadzu CR4-A Chromatopac recording integrator.

Results and Discussion

Fig. 2 shows the HPLC elution profile of reaction products obtained with PB-treated rat liver microsomes resulting from the analysis procedure described above. The unidentified peak A was present at approximately the same magnitude in all incubation mixtures and was not quantified.

Table 1 shows that liver microsomes of males of all the strains of rat used here exhibited significantly greater erythromycin, ethylmorphine, and *d*-benzphetamine demethylase activities than did the corresponding female preparations. The demethylation of *p*-nitroanisole, by contrast, did not show any consistent gender dependence, in keeping with the broad enzyme specificity recorded for this substrate (15). The oxidation of *d*-benzphetamine in uninduced male rat liver microsomes is attributable to catalysis by multiple male-specific enzymes (37, 38), whereas the oxidation of erythromycin and ethylmorphine, as noted above, is principally attributable to P450IIIA2 activity in these animals. Fig. 3 shows that there is also a relationship between gender and temazepam production with these microsomal preparations. In all cases, temazepam production by male liver preparations markedly exceeds that for corresponding female preparations. It is notable that this pattern is not seen with nordazepam production, and this indicates lack of identity between the catalytic species responsible for production of the two diazepam metabolites. This conclusion was also reached in work with human liver microsomes (39). In a recent report that addressed this same question in clinical studies, it was concluded that the demethylation of diazepam and the hydroxylation of nordazepam were both catalyzed by the same enzyme, (*S*)-mephenytoin 4-hydroxylase (9). This is clearly incompatible with the *in vitro* observations noted above, which indicate that different enzymes are responsible for these two monooxygenations. We are unable to explain this apparent discrepancy between clinical and biochemical studies, except to note that (*S*)-mephenytoin 4-hydroxylation has been demonstrated to be catalyzed by more than one isoenzyme in human liver and co-purification of diazepam *N*-demethylation and (*S*)-mephenytoin 4-hydroxylation was shown with one of these isozymes (10). This observation also allows rationalization of the finding (10, 11) that diazepam is a potent inhibitor of microsomal (*S*)-mephenytoin 4-hydroxylation.

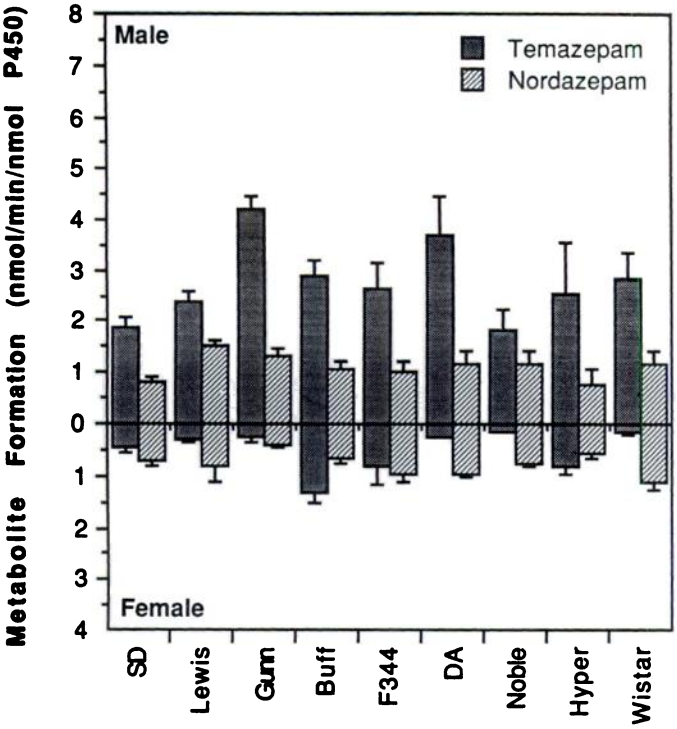

Fig. 3. Gender-dependent variation in temazepam and nordazepam production obtained with microsomes prepared from livers of groups of six male and female rats of nine inbred and outbred strains. Incubations contained 200 μM diazepam, 1 mM NADPH, and 1 μM P450 in 100 mM Tris·HCl, pH 7.9, buffer and were for 10 min at 37°. Diazepam metabolites were quantitated as described in the text. Standard deviations of results from four to six estimates on each pooled microsomal sample are shown; data for preparations of male liver microsomes are shown in the *upper section* and results for female liver preparations are given in the *lower section*. C_3-hydroxylase activity was significantly greater in male than in female liver microsomes in all strains ($p < 0.005$). *DA*, Dark Agouti; *Hyper*, hypertensive; *Buff*, Buffalo.

TABLE 2

Effects of TAO on diazepam C_3-hydroxylation

Microsomes containing 0.2 nmol of P450 prepared from saline- or DEX-treated male SD rats were assayed for C_3-hydroxylase activity with diazepam (200 μM) as substrate, in the presence or absence of 100 μM TAO; C_3-hydroxylase activity was initiated by the addition of microsomes according to the general procedure described in Experimental Procedures or after preincubation for 15 min at 37° in the presence or absence of 1 mM NADPH, in which case C_3-hydroxylase activity was initiated by the addition of diazepam dissolved in acetone (final concentration, 1%). In all cases, temazepam production was measured over 10 min at 37° in 100 mM Tris·HCl, pH 7.9, buffer according to the procedures detailed in Experimental Procedures. The results are the means of duplicate incubations and the numbers in parentheses are percentages of control values. Equivalent patterns of inhibition by TAO under both sets of conditions are seen in the two microsomal preparations.

Preincubation conditions	C_3-Hydroxylase	
	Saline-treated[a]	DEX-treated[a]
	nmol/min/nmol of P450	
None	1.90 (100)	3.18 (100)
None + TAO	1.19 (62)	2.12 (67)
NADPH	1.46 (100)	2.83 (100)
NADPH + TAO	0.49 (34)	0.82 (29)

[a] Enzyme source.

The present results using washed liver microsomes prepared from nine inbred and outbred strains of rat show gender dependence of diazepam C_3-hydroxylation and reinforce and extend earlier findings obtained using supernatants of rat liver homogenates (27). The involvement of male-specific enzyme(s) in diazepam C_3-hydroxylation may be inferred from these results.

TAO has recently been identified as a specific inhibitor of P450IIIA enzymes (40, 41), and use was made of this to further assist in the provisional identification of the enzymes responsible for C_3-hydroxylation of diazepam. Table 2 shows that, with saline control microsomes, TAO inhibited C_3-hydroxylase activity when either present with diazepam throughout the course of the assay or present with NADPH in a preincubation mix with microsomes initially in the absence of diazepam. These results provide firm evidence in support of the involvement of P450IIIA family enzymes in diazepam C_3-hydroxylation. Results similar to these have been used to assist in the assignment of P450IIIA family enzymes to other monooxygenations (40, 41). Potent inhibition of ethylmorphine *N*-demethylation by midazolam in microsomes prepared from human liver and PCN-treated SD rat liver (42) also suggests involvement of P450IIIA enzymes, rather than the other male-specific species, in diazepam C_3-hydroxylation.

Induction studies with xenobiotics known to cause changes in the abundance of different classes of P450 isozymes were undertaken to explore this finding more fully, and the results are shown in Fig. 4 and Table 3. Fig. 4 shows the concordant elevations in temazepam production from diazepam catalyzed by microsomal preparations of liver obtained from male SD rats pretreated with PB, DEX, and TAO, compared with the corresponding control preparations. The lack of effect of the other inducing agents on this oxidation is clear, and the characteristic pattern of change seen with C_3-hydroxylation is not seen for nordazepam production. Table 3 shows the P450 specific contents and specific activities of ethylmorphine, *d*-benzphetamine, erythromycin, and *p*-nitroanisole demethylases in these microsomal preparations. Marked elevations are seen only in ethylmorphine and erythromycin demethylase specific activities in microsomes from livers of animals treated with PB, DEX, and TAO, compared with the corresponding control preparations. This induction pattern is characteristic of increases in P450IIIA family isozymes. The specific activities of *d*-benzphetamine and *p*-nitroanisole demethylations did not alter in this characteristic way. The concordance of the elevations in the specific activities of erythromycin and ethylmorphine demethylations and diazepam C_3-hydroxylation resulting from administration of the P450IIIA family inducers confirms the involvement of these enzymes in this benzodiazepine oxidation. The differences in the relative magnitudes of the induction events seen with PB, DEX, and TAO with respect to each of the three xenobiotic oxidations may be attributable to the multiplicity of the inducible isoenzymes in this family (43, 44) and to the differences in induction mechanisms (45, 46). Of interest in this connection, however, are the further observations reported in Table 2, showing the congruence of the inhibitory effects of TAO on diazepam C_3-hydroxylation in liver microsomes isolated from DEX-treated and saline-treated rats.

These data are to be contrasted somewhat with results (41) showing that inhibition by TAO of the oxidation of a number of P450IIIA family substrates is less effective for control than for PCN-induced microsomes. The reason for this difference is not clear but may relate to differences in substrates or inducers used in this and the previous study.

Fig. 5 shows the effects of each of the vehicle and inducer

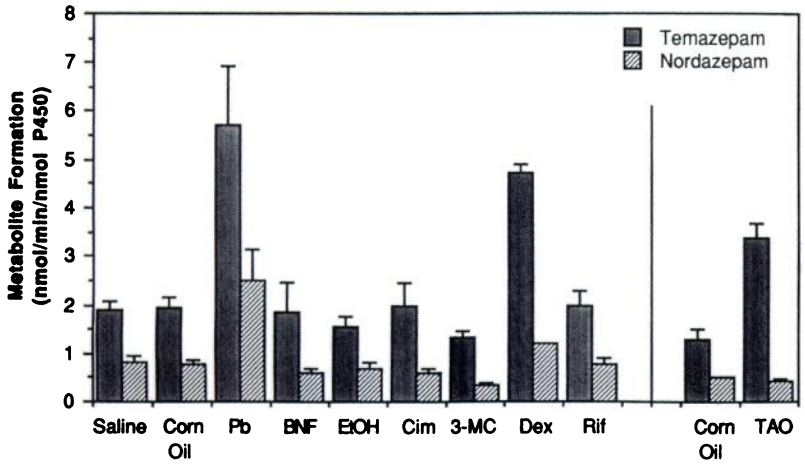

Fig. 4. Temazepam and nordazepam production from diazepam using microsomes isolated from livers of groups of male SD rats pretreated with inducers and vehicles as shown. Incubations contained 200 μM diazepam, 1 mM NADPH, and 1 μM P450 in 100 mM Tris·HCl, pH 7.9, buffer and were for 10 min at 37°. Diazepam metabolites were quantitated as described in the test. Data to the *right* of the *vertical dividing line* relate to microsomes prepared from the three individually housed corn oil-treated control and the six individually housed TAO-treated rats. *Error bars* associated with these data indicate standard deviations of results obtained for means of duplicate analyses of the individually prepared microsomal samples. Standard deviations shown for the other results are from four to six estimates on each pooled microsomal sample. C_3-hydroxylase activity was significantly induced by PB, DEX, and TAO ($p < 0.005$ when compared with the appropriate control in each case). *EtOH*, ethanol.

TABLE 3

P450 specific contents and monooxygenase specific activities of liver microsomal preparations of male SD rats given saline, corn oil, or xenobiotics

Analytical procedures are described in Experimental Procedures. Results are means ± standard deviations of three to six determinations on microsomal samples from pooled livers of six rats except for corn oil control and TAO-treated animals, shown in the bottom two rows, where numbers of individually analyzed livers are shown in parentheses. Significance levels of differences between results for inducers and corresponding controls are shown.

Rat treatment	P450 specific content	Specific activity			*p*-nitroanisole *O*-demethylase
		Ethylmorphine *N*-demethylase	Benzphetamine *N*-demethylase	Erythromycin *N*-demethylase	
	nmol/mg of protein	*μmol of HCHO/min/μmol P450*			*μmol of p-nitrophenol/min/μmol of P450*
Saline	0.79 ± 0.12	4.82 ± 0.11	2.89 ± 0.10	0.85 ± 0.08	1.67 ± 0.08
PB	1.95 ± 0.16[a]	7.65 ± 0.07[a]	5.42 ± 0.14[a]	1.23 ± 0.01[b]	2.06 ± 0.08[b]
RIF	0.77 ± 0.06	5.73 ± 0.35[c]	2.95 ± 0.19	0.82 ± 0.01	1.72 ± 0.07
CIM	1.08 ± 0.07[c]	2.52 ± 0.28[a]	1.75 ± 0.10[a]	0.73 ± 0.02	1.18 ± 0.02[a]
Ethanol	0.91 ± 0.07	5.33 ± 0.14[b]	2.78 ± 0.13	0.92 ± 0.04	2.10 ± 0.03[a]
Corn Oil	1.16 ± 0.04	5.19 ± 0.14	2.73 ± 0.13	0.60 ± 0.02	1.27 ± 0.02
DEX	0.85 ± 0.08[b]	15.09 ± 0.04[a]	4.08 ± 0.17[a]	3.75 ± 0.08[a]	2.87 ± 0.26[a]
3MC	1.57 ± 0.13[b]	3.66 ± 0.42[b]	1.23 ± 0.04[a]	0.42 ± 0.03[a]	1.90 ± 0.05[a]
βNF	1.14 ± 0.13	2.84 ± 0.11[a]	1.34 ± 0.04[a]	0.39 ± 0.01[a]	4.48 ± 0.09[a]
Corn Oil	1.26 ± 0.11 (3)	3.93 ± 0.46 (3)	2.90 ± 0.33 (3)	0.85 ± 0.13 (3)	0.89 ± 0.15 (3)
Triacetyloleandomycin	3.83 ± 0.22 (6)[c]	5.16 ± 0.36 (6)[c]	1.37 ± 0.15 (6)	5.07 ± 0.27 (6)[a]	0.81 ± 0.09 (6)

[a] $p < 0.005$.
[b] $p < 0.01$.
[c] $p < 0.05$.

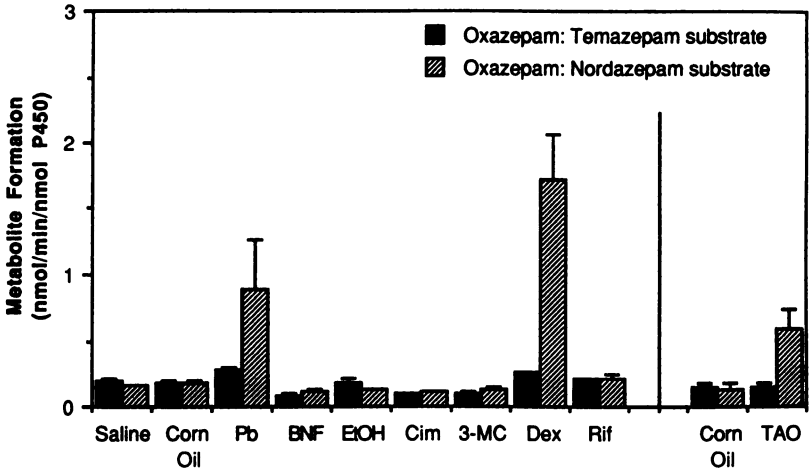

Fig. 5. Effects of pretreatment of male SD rats with vehicle and monooxygenase-inducing agents on liver microsomal oxazepam production from temazepam and nordazepam. Incubations contained 100 μM nordazepam or temazepam, 1 mM NADPH, and 1 μM P450 in 100 mM Tris·HCl, pH 7.9, buffer and were for 10 min at 37°. Oxazepam was quantitated as described in the text. Data to the *right* of the *vertical dividing line* relate to microsomes prepared from the three individually housed corn oil-treated control and the six individually housed TAO-treated rats. Error bars associated with these data indicate standard deviations of results obtained for means of duplicate analyses of each of the individually prepared microsomal samples. Standard deviations shown for the other results are from four to six estimates on each pooled microsomal sample. C_3-Hydroxylase activity was significantly induced by PB, DEX, and TAO ($p < 0.005$ when compared with the appropriate control in each case). *EtOH*, ethanol.

treatments on microsomal oxazepam production from temazepam and nordazepam. As with diazepam, increases are seen only in the specific activity of the C_3-hydroxylation reaction and are clearly identified with the P450IIIA family inducers. This strongly suggests identity between the isozymes responsible for C_3-hydroxylation of diazepam and of nordazepam and indicates that the methyl substituent on the B ring nitrogen of diazepam does not affect hydroxylation at the adjacent C_3 position. Carbon oxidation at the structurally equivalent 4-position in the B ring of midazolam has also recently been

reported to be a property of this group of enzymes (25, 26), and this suggests that the imidazole substituent of midazolam (incorporating the B ring nitrogen atom) also has negligible effects on these enzymes in determining their capacity to catalyze this monooxygenation.

The P450IIIA family of isozymes is clearly of great importance in the metabolism of a wide variety of endobiotics and xenobiotics, with C_3 of the 1,4-benzodiazepines now being recognized as an important monoxygenation site for these enzymes. In view of the multiple controls that regulate the stability and synthesis (gender-dependent expression in rodents, xenobiotic inducibility, and genetically determined polymorphisms in human populations) of these enzymes, their study offers many opportunities for further understanding of complex interrelations between multiple classes of monooxygenations.

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References

1. Sternbach, L. H. The discovery of CNS active 1,4-benzodiazepines in *The Benzodiazepines: From Molecular Biology to Clinical Practice* (E. Costa, ed.). Raven Press, New York, 1–6 (1983).
2. Klotz, U., G. R. Avant, A. Hoyumpa, S. Schenker, and G. R. Wilkinson. The effects of age and liver disease on the disposition and elimination of diazepam in adult man. *J. Clin Invest.* **55:** 347–359 (1975).
3. Kaplan, S. A., and M. L. Jack. Metabolism of the benzodiazepines: pharmacokinetic and pharmacodynamic considerations, in *The Benzodiazepines: From Molecular Biology to Clinical Practice* (E. Costa, ed.). Raven Press, New York, 173–199 (1983).
4. Greenblatt, D. J., J. S. Harmatz, and R. I. Shader. Factors influencing diazepam pharmacokinetics: age, sex, and liver disease. *Int. J. Clin. Pharmacol.* **16:** 177–179 (1978).
5. Macleod, S. M., H. G. Giles, B. Bengert, F. F. Liu, and E. M. Sellers. Age and gender related differences in diazepam pharmacokinetics. *J. Clin. Pharmacol.* **19:** 15–19 (1979).
6. Ohnhaus, E. E., B. K. Park, J. P. Colombo, and P. Heitzman. The effect of enzyme induction on diazepam metabolism in man. *Br. J. Clin. Pharmacol.* **8:** 557–563 (1979).
7. Marcucci, F., R. Fanelli, E. Mussini, and S. Garattini. Effect of phenobarbital on the *in vivo* metabolism of diazepam in several animal species. *Biochem. Pharmacol.* **19:** 1771–1776 (1970).
8. Klotz, U., and I. Reimann. Delayed clearance of diazepam due to cimetidine. *N. Engl. J. Med.* **302:** 1012–1014 (1980).
9. Bertilsson, L., T. K. Henthorn, E. Sanz, G. Tybring, J. Sawe, and T. Villen. Importance of genetic factors in the regulation of diazepam metabolism: relationship to *S*-mephenytoin, but not debrisoquin, hydroxylation phenotype. *Clin. Pharmacol. Ther.* **45:** 348–355 (1989).
10. Hall, S. D., F. P. Guengerich, R. A. Branch, and G. R. Wilkinson. Characterization and inhibition of mephenytoin 4-hydroxylase activity in human liver microsomes. *J. Pharmacol. Exp. Ther.* **240:** 216–222 (1987).
11. Inaba, T., M. Jurima, W. A. Mahon, and W. Kalow. *In vitro* inhibition studies of two isozymes of human liver cytochrome P450: mephenytoin *p*-hydroxylase and sparteine monooxygenase. *Drug Metab. Dispos.* **13:** 443–448 (1985).
12. Caldwell, J. Comparative aspects of detoxication in mammals, in *Enzymatic Basis of Detoxication* (W. B. Jacoby, ed.), Vol. 1. Academic Press Inc., New York 85–114 (1980).
13. Nebert, D. W., D. R. Nelson, M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8:** 1–13 (1989).
14. Gonzalez, F. J. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40:** 243–288 (1989).
15. Guengerich, F. P. Enzymology of rat liver cytochromes P450, in *Mammalian Cytochromes P450* (F. P. Guengerich, ed.), Vol. 1. CRC Press, Boca Raton, FL 1–54 (1987).
16. Waxman, D. J. Interactions of hepatic cytochromes P450 with steroid hormones. *Biochem. Pharmacol.* **37:** 71–84 (1988).
17. Dannan, G. A., F. P. Guengerich, and D. J. Waxman. Hormonal regulation of rat liver microsomal enzymes. *J. Biol. Chem.* **261:** 10728–10735 (1986).
18. Schwab, G. E., and E. F. Johnson. Enzymology of rabbit cytochromes P450, in *Mammalian Cytochromes P450* (F. P. Guengerich, ed.). Vol. 1. CRC Press, Boca Raton, FL 55–105 (1987).
19. Gonzalez, F. J., B.-J. Song, and J. P. Hardwick. Pregnenolone-16α-carbonitrile-inducible P450 gene family: gene conversion and differential regulation. *Mol. Cell. Biochem.* **6:** 2969–2976 (1986).
20. Ged, C., J. M. Rouillon, L. Pichard, J. Combalbert, N. Bressot, P. Bories, H. Michel, P. Beaune, and P. Maurel. The increase in urinary excretion of 6-β-hydroxycortisol as a marker of human hepatic cytochrome P450IIIA induction. *Br. J. Clin. Pharmacol.* **28:** 373–388 (1989).
21. Wrighton, S. A., E. G. Schuetz, P. B. Watkins, P. Maurel, J. Barwick, B. S. Bailey, H. T. Hartle, B. Young, and P. S. Guzelian. Demonstration in multiple species of inducible hepatic cytochromes P450 and their mRNAs related to the glucocorticoid-inducible cytochrome P450 of the rat. *Mol. Pharmacol.* **28:** 312–321 (1985).
22. Guengerich, F. P., M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, and D. J. Waxman. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261:** 5051–5060 (1986).
23. Combalbert, J., I. Fabre, G. Fabre, I. Dalet, J. Derancourt, J. P. Cano, and P. Maurel. Metabolism of cyclosporin A. IV. Purification and identification of the rifampicin-inducible human liver cytochrome P-450 (cyclosporin A oxidase) as a product of P450IIIA gene subfamily. *Drug Metab. Dispos.* **17:** 197–207 (1989).
24. Lu, A. Y. H., A. Somogyi, S. West, R. Kuntzman, and A. H. Conney. Pregnenolone-16α-carbonitrile: a new type of inducer of drug-metabolizing enzymes. *Arch. Biochem. Biophys.* **152:** 457–462 (1972).
25. Kronbach, T., D. Mathys, M. Umeno, F. J. Gonzalez, and U. A. Meyer. Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. *Mol. Pharmacol.* **36:** 89–96 (1989).
26. Fabre, G., P. Crevat-Pisano, S. Dragna, J. Covo, Y. Barra, and J. P. Cano. Involvement of the macrolide antibiotic inducible cytochrome P450 $LM3_e$ in the metabolism of midazolam by microsomal fractions prepared from rabbit liver. *Biochem. Pharmacol.* **37:** 1947–1953 (1988).
27. Nau, H., and C. Liddiard. Postnatal development of sex-dependent differences in the metabolism of diazepam by rat liver. *Biochem. Pharmacol.* **29:** 447–449 (1980).
28. Shimada, T., and F. P. Guengerich. Participation of a rat liver cytochrome P450 induced by pregnenolone-16α-carbonitrile and other compounds in the 4-hydroxylation of mephenytoin. *Mol. Pharmacol.* **28:** 215–219 (1985).
29. Guengerich, F. P. Microsomal enzymes involved in toxicology-analysis and separation, in *Principles and Methods in Toxicology* (A. W. Hayes, ed.). Raven Press, New York, 609–634 (1982).
30. Estabrook, W. R., J. Peterson, J. Baron, and A. Hildebrandt. The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. In *Methods in Pharmacology* (C. F. Chignell, ed.), Vol. 1. Appleton Century Crofts, Meredith Corp., NY, 303–350 (1972).
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275 (1951).
32. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55:** 416–421 (1953).
33. Reilly, P. E. B., and D. J. Winzor. Adverse drug interactions with cimetidine: competitive inhibition of monooxygenase-dependent *N*-demethylation of morphine. *Biochem. Pharmacol.* **33:** 1151–1153 (1984).
34. Wrighton, S. A. P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P450p. *Biochemistry* **24:** 2171–2178 (1985).
35. Reilly, P. E. B., D. J. O'Shannessy, and R. G. Duggleby. Non-Michaelian monooxygenase kinetics: studies using competitive inhibitors. *FEBS Lett.* **119:** 63–67 (1980).
36. Pessayre, D., V. Descatoire, M. Konstantinova-Mitcheva, J.-A. Wandscheer, B. Cobert, R. Level, J.-P. Benhamou, M. Jaouen, and D. Mansuy. Self-induction by triacetyloleandomycin of its own transformation into a metabolite forming a stable 456 nm-absorbing complex with cytochrome P450. *Biochem. Pharmacol.* **30:** 553–558 (1981).
37. Ryan, D. E., S. Iida, A. W. Wood, P. E. Thomas, C. S. Lieber, and W. Levin. Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *J. Biol. Chem.* **259:** 1239–1250 (1984).
38. Larrey, D., L. M. Distlerath, G. A. Dannan, G. R. Wilkinson, and F. P. Guengerich. Purification and characterization of the rat liver microsomal cytochrome P-450 involved in the 4-hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. *Biochemistry* **23:** 2787–2795 (1984).
39. Inaba, T., A. Tait, M. Nakano, W. A. Mahon, and W. Kalow. Metabolism of diazepam *in vitro* by human liver: independent variability of *N*-demethylation and C_3-hydroxylation. *Drug. Metab. Dispos.* **16:** 605–608 (1988).
40. Guengerich, F. P. Oxidation of 17α-ethynylestradiol by human liver cytochrome P-450. *Mol. Pharmacol.* **33:** 500–508 (1988).
41. Namkung, M. J., H. L. Yang, J. E. Hulla, and M. R. Juchau. On the substrate specificity of cytochrome P450IIIA1. *Mol. Pharmacol.* **34:** 628–637 (1988).
42. Ladona, M. G., D. J. M. Spalding, L. Ekman, B. Lindstrom, and A. Rane. Human fetal and adult liver metabolism of ethylmorphine. *Biochem. Pharmacol.* **38:** 3147–3155 (1989).
43. Graves, P. E., L. S. Kaminsky, and J. Halpert. Evidence for functional and structural multiplicity of pregnenolone-16α-carbonitrile-inducible cyto-

chrome P-450 isozymes in rat liver microsomes. *Biochemistry* **26:** 3887–3894 (1987).
44. Hostetler, K. A., S. A. Wrighton, P. Kremers, and P. S. Guzelian. Immunochemical evidence for multiple steroid-inducible hepatic cytochromes P-450 in the rat. *Biochem. J.* **245:** 27–33 (1987).
45. Larroque, C., R. Lange, P. Maurel, R. Langlois, and J. E. van Lier. Rat liver microsomal progesterone metabolism: evidence for differential troleandomycin and pregnenolone-16α-carbonitrile inductive effects in the cytochrome P-450 III family. *J. Steroid Biochem.* **33:** 277–286 (1989).
46. Nebert, D. W., D. R. Nelson, and R. Feyereisen. Evolution of the cytochrome P450 genes. *Xenobiotica* **19:** 1149–1160 (1989).

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