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Metabolite Complex Formation of Orphenadrine with Cytochrome P450

INVOLVEMENT OF CYP2C11 AND CYP3A ISOZYMES

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ABSTRACT. Expression and inhibition of cytochrome P450 (CYP) isozymes capable of forming an orphenadrine metabolite complex were studied in microsomes of untreated and inducer-treated male and female rats. High levels of complex-forming isozymes were found in microsomes of untreated male as compared to female rats. Treatment of male rats with several P450 inducers did not considerably increase the extent of *in vitro* complex formation. In female rats, however, phenobarbital or dexamethasone treatments led to pronounced induction. The isozyme specifity of complex formation was investigated by several approaches including: 1. inhibition by orphenadrine of isozyme-specific P450 activities, such as hydroxylation of testosterone, O-dealkylation of pentoxy- and ethoxyresorufin and complex formation with triacetyloleandomycin (TAO), 2. inhibition of orphenadrine complex formation by metyrapone, TAO, and cimetidine, and 3. correlation of complex levels with immunochemically, enzymatically, or spectroscopically determined amounts of P450 isozymes. Our data suggest that CYP2C11, a CYP3A isozyme and an unidentified P450 species are involved in complex formation with orphenadrine, but exclude the involvement of CYP1A1/2 and CYP2B1/2. The capability of CYP2C11 to form a metabolite complex with orphenadrine is strongly suggested for the following reasons: 1. Efficient inhibition of testosterone 2α- and 16α-hydroxylation by complex formation with orphenadrine in microsomes of untreated male rats, 2. high expression of orphenadrine-complexing isozymes in untreated male compared to female rats, 3. specific inhibition of *in vitro* complex formation by cimetidine, 4. suppression of complex-forming isozymes by 3-methylcholanthrene and β-naphthoflavone, and 5. concomitant induction of complex-forming isozymes, immunodetectable CYP2C11, and testosterone 2α-hydroxylase by stanozolol. That at least one, but not all, CYP3A isozymes is involved in complex formation is concluded from inhibition experiments with TAO that show that orphenadrine complexation can be significantly inhibited in microsomes of dexamethasone-treated, but not in microsomes of untreated rats. Furthermore, complex formation with TAO is not inhibited by orphenadrine in microsomes of phenobarbital (PB)-treated rats. In PB-treated female rats, a further unidentified complex-forming isozyme can be detected that is not inhibited by complex formation with TAO. BIOCHEM PHARMACOL 52;1: 73–84, 1996., 1996.

KEY WORDS. P450; CYP2C11; CYP3A; orphenadrine; metabolite complex; inhibition

The xenobiotic-metabolizing potential of the liver microsomal CYP† system can be altered by different mechanisms acting on the transcriptional, translational, and posttranslational levels. Modulatory effects on the protein level are exerted by ligand binding [1, 2], protein phosphorylation [3], and inhibition of enzymatic activities [4, 5]. Among the latter, suicide inhibition [5–7] or enzyme inactivation by formation of P450 metabolic intermediate complexes [8–11] are of special significance either because of the irreversibility of the reaction or the relatively high stability of the formed complexes [9], which may persist within the organism after the free compound has been cleared [5]. Formation of such complexes has been shown for a number of compounds, including macrolide antibiotics [10], methylene dioxyphenyl derivatives [8], and amphetamines [11]. The isozyme specificity of complex formation is often insufficiently known. Knowledge, however, is a prerequisite for predictability of drug interactions. From induction, inhibition, and reconstitution experiments, it has been shown that, for example, nortriptyline, SKF 525-A, and N-hydroxyamphetamine form complexes with CYP2C11 [12–14] and also TAO interacts with CYP3A isozymes [1, 15]. Deduc-

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† *Abbreviations:* CYP, cytochrome P450; DEX, dexamethasone; EROD, ethoxyresorufin O-dealkylase; HCB, hexachlorobenzene; 3MC, 3-methylcholanthrene; βNF, β-naphthoflavone; PB, phenobarbital; PROD, pentoxyresorufin O-dealkylase; STZ, stanozolol; TAO, triacetyloleandomycin; UT, untreated.

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tion of isozyme specificity is not easy, because *in vivo* or in microsomal samples manifold interactions between P450 isozymes and xenobiotics can be expected (i.e., involvement of several isozymes in sequential metabolic steps, in one of these steps, or in final complex formation). Furthermore, differential inhibitory or stimulatory effects of formed metabolites can be exerted on the isozymes involved [16].

Complex-forming substrates with defined isozyme specificity can be used for spectroscopic *in vitro* quantitation of the respective isozymes. This has been shown for the complex formed between CYP3A isozymes of the rat and a metabolite of TAO [15]. In this respect, we also studied complex formation with the myotonolytic drug orphenadrine, which is used in the treatment of Parkinson's disease. Orphenadrine is metabolized by liver microsomal cytochrome P450. Prospective sites for enzymatic attack are the phenyl residues, the alkylether function, and the dimethylamino group of the molecule. A number of phase I and phase II metabolites have been identified in humans [17]. Discrepancies in single and multiple-dose kinetics of orphenadrine were explained by product inhibition of its metabolism [18]. This hypothesis was verified by the finding that P450-dependent N-demethylation of orphenadrine and the subsequent formation and binding of a reactive metabolite lead to enzymatic inactivation of the respective P450 molecule [19, 20]. This type of mechanism-based inhibition has also been shown for compounds such as SKF 525-A [13], benzphetamine [9], and TAO [10]. Complex formation with orphenadrine has been studied in rats and, based on induction and inhibition experiments, the involvement of CYP2B1 and CYP2C6 in complex formation has been proposed [19, 21]. To confirm the isozyme specificity of complex formation with orphenadrine and to examine its suitability for spectroscopic isozyme quantitation, we studied kinetics, extent, and inhibition of complex formation and the effects of orphenadrine on several P450 activities in microsomes of untreated and inducer-treated male and female rats. Our findings strongly suggest that CYP2C11 and a CYP3A isozyme(s), rather than CYP2B1, are involved in complex formation with orphenadrine in Sprague-Dawley rats.

MATERIALS AND METHODS

Animals and Animal Treatment

Male and female Sprague-Dawley rats (200–250 g) were purchased from Lippische Versuchstierzucht (Extertal, Germany). Induction regimes were as follows: PB: 0.1% in drinking water *ad lib.* for 4 days. Lipophilic inducers in corn oil were applied by intraperitoneal injections on 3 subsequent days in the following amounts (mg/kg body weight): 3MC, 20; βNF, 40; HCB, 80; DEX, 30; STZ, 10. The next day, liver microsomes were prepared as described [22].

Enzyme and Protein Assays

In vitro formation of metabolite complexes with orphenadrine and TAO: 3 mL samples containing 3 nmol microsomal P450, NADP (350 μM), Tris-HCl (50 mM, pH 7.5), KCl (50 mM), $MgCl_2$ (10 mM), isocitrate (5 mM), isocitrate dehydrogenase (0.5 U/mL) and catalase (230 U/mL) were preincubated for 1 min at 37°C. For complexation with TAO, 300 μM NADH was also added. After recording and storing a baseline, the reaction was started by addition of 30 μM orphenadrine (in buffer) or 10 μM TAO (in DMSO). A corresponding volume of buffer or DMSO, respectively, was added to the reference. Complex formation was monitored by repetitive scanning in the wave length range of 510 nm to 400 nm up to 30 min with cycles of 3 min. The baseline was subtracted, and the amount of formed complex calculated from the absorbance difference at 455 nm (orphenadrine) or 456 nm (TAO) and 500 nm using extinction coefficients for the metabolite complexes of 78 $mM^{-1}* cm^{-1}$ (this paper) and 69 $mM^{-1} cm^{-1}$ [15], respectively. Consecutive complex formation with TAO and orphenadrine was essentially as described above. Formation of the primary complex was allowed to proceed until completion (30 min). Then, the contents of the sample and the reference cuvettes were used as starting materials for subsequent complex formation with the second substrate (TAO or orphenadrine). For this, the contents of one cuvette were equally distributed on two further cuvettes, a new baseline was recorded, and the reaction was started with the second substrate and monitored as described above.

Testosterone hydroxylation assays were performed as described [15]. For quantitation of testosterone metabolism after complex formation with orphenadrine, formation of the complex was monitored over a period of 30 min, as described above. Then, 250 μM testosterone was added to both the sample and reference cuvettes and incubated for a further 10 min at 30°C. Concomitantly, spectra of the formed metabolite complex were monitored to show its stability. The reaction was terminated by adding 3 mL ice-cold diethylether to extract the testosterone metabolites. The organic phases were removed and the extraction was repeated with an equal amount of ether. Subsequently, the organic phases were combined and evaporated under nitrogen. Methanol was used to dissolve the residues for further analysis by reversed-phase HPLC. Testosterone metabolites were separated on a Supelcosil LC-18 column (5 μm, 150 × 4.6 mm) with the following eluents: (A) methanol-H_2O (25:75) and (B) methanol-acetonitrile-H_2O (60.5:7.7:31.8) (gradient: from 40% B to 100% B within 60 min, flow rate: 0.9 mL/min). Peak areas and retention times were determined by a computer program (PC Integration Pack, Kontron, Gräfelfing, Germany).

Pentoxyresorufin and ethoxyresorufin O-dealkylation (PROD and EROD) were quantified spectrofluorometrically by continuous monitoring of the formation of resorufin with excitation and emission wavelengths of 530 nm and 585 nm, respectively [23]. The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 2.7 mM sodium isocitrate, 0.3 U/mL isocitrate dehydrogenase, 2 mM magnesium sulfate,

50 μg/mL bovine serum albumine, 76 U/mL catalase, 170 μM NADP, and 2.5 μM pentoxyresorufin or ethoxyresorufin. The reaction was started with the microsomal preparation, giving a final concentration between 1 and 100 nM P450, depending on the CYP2B (PROD) or CYP1A content (EROD) of the microsomes.

For inhibition of PROD activity and complex formation with orphenadrine, the inhibitors were either dissolved in DMSO (TAO, stanozolol) or in 100 mM Tris-HCl, pH 7.4 (orphenadrine, cimetidine, metyrapone, tofenacine). CYP2C11-specific inhibition by cimetidine was essentially done as previously described [24].

Protein and cytochrome P450 concentrations were determined by standard methods [25, 26]. Polyacrylamide gel electrophoresis in the presence of SDS and subsequent transfer of the proteins to nitrocellulose by transblotting for immunostaining was performed as described [27, 28]. Stained protein bands were quantified by a CCD camera and 2D-analyzing software (Cybertech, Berlin, Germany). Antibodies to P450 2B1, purified by the method of Ryan *et al.* [29], were obtained by immunization of rabbits as described [15].

Chemicals

Androstenedione, DEX, testosterone, orphenadrine, and cimetidine were purchased from Sigma (Deisenhofen, Germany), hydroxylated testosterone derivatives as standards for HPLC from Steraloids (Wilton, U.S.A.) and goat anti-rabbit IgG peroxidase conjugate from Nordic (Bochum, Germany). Orphenadrine citrate, TAO, STZ, and tofenacine were kind gifts of 3M Medica (Borken, Germany), Pfizer GmbH (Karlsruhe, Germany), Sanofi Winthrop GmbH (Munich, Germany) and Prof. Dr. H. Timmerman (Amsterdam, The Netherlands), respectively.

RESULTS

Metabolite Complex Formation with Orphenadrine in Vitro

Kinetic studies showed that *in vitro* formation of the orphenadrine metabolite complex was more than 95% complete after incubation of microsomes for 30 min at 37°C, irrespective of whether the animals were untreated or inducer-treated (Fig. 1). The orphenadrine concentration for half-maximal metabolite complex formation was 2.9 ± 0.5 μM and 1.9 ± 0.7 μM for microsomes of untreated and phenobarbital-treated rats, respectively. For quantitative complex formation, orphenadrine concentrations of at least 25 to 100 μM were required (not shown). Therefore, in all further experiments, complex formation was allowed to proceed for at least 30 min in the presence of 30 μM orphenadrine. Under these conditions, comparable complex formation was found in liver microsomes of untreated as well as inducer-treated male rats (Table 1). Low, but significant, inductions by a factor of about 1.4 were obtained with PB and the synthetic anabolic steroid STZ. On the other hand, significant suppression of complex-forming isozymes was observed with the typical CYP1A inducers 3-MC and β-NF. In liver microsomes of untreated female rats, only negligible amounts of orphenadrine-complex-forming P450 isozymes were present. Pronounced induction of the latter in females, however, was observed after PB or DEX treatment, but not with STZ (Table 1).

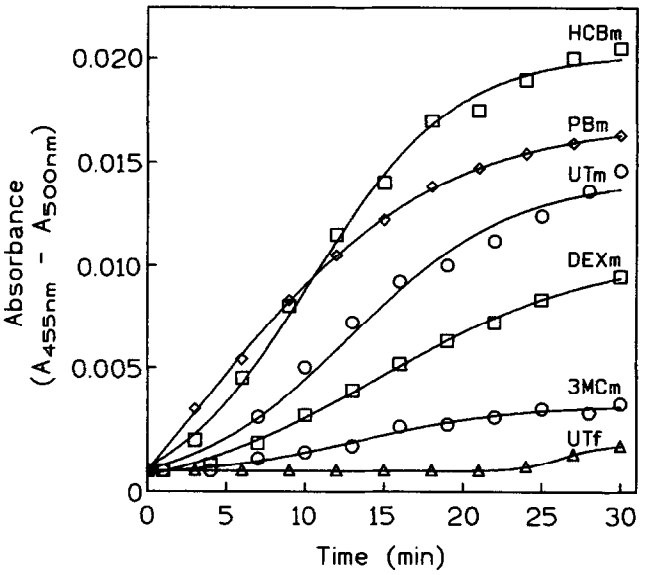

FIG. 1. Kinetics of orphenadrine complex formation in liver microsomes of untreated (UT) and inducer-treated rats (DEX, HCB, 3MC, PB). Final P450 concentrations: 1 μM. Orphenadrine citrate: 100 μM. For experimental details see Materials and Methods. m, male; f, female.

Spectral Properties of the Metabolite Complexes

The spectral characteristics of the orphenadrine metabolite complexes formed with microsomes of either untreated or

TABLE 1. Extent of *in vitro* complex formation with orphenadrine in liver microsomes of untreated and inducer-treated rats

Microsomes (treatment/sex)	pmol Complex/ nmol P450	n	pmol Complex/ mg protein	n
UTm	168 ± 44	16	159 ± 45	16
PBm	118 ± 27	7	220 ± 43	7
DEXm	110 ± 19	4	154 ± 10	3
βNFm	72 ± 12	4	85 ± 35	4
3MCm	22 ± 14	4	39 ± 23	4
HCBm	221 ± 41	2	221 ± 41	2
STZm	251 ± 9	5	214 ± 7	5
UTf	17 ± 9	3	16 ± 11	3
PBf	85 ± 26	3	148 ± 44	3
DEXf	66 ± 10	3	115 ± 17	3
STZf	35 ± 9	2	29 ± 7	2

UT, untreated; PB, phenobarbital; DEX, dexamethasone; βNF, β-naphthoflavone; 3MC, 3-methylcholanthrene; HCB, hexachlorobenzene; STZ, stanozolol; m, male; f, female; *n*, number of determinations. Figures give means ± standard deviations. An extinction coefficient of 78 mM^{-1*} cm^{-1} was used for the P450 orphenadrine metabolite complex.

inducer-treated rats were very similar, with an absorption maximum at 455 nm and an isosbestic point at 436 nm (not shown). However, the spectrum obtained with microsomes of PB-treated rats and orphenadrine in the presence of NADPH shows an additional shoulder at about 434 nm (Fig. 2). This signal was probably due to the binding of the orphenadrine metabolite tofenacine to a PB-inducible P450 isozyme. Accordingly, a respective signal was obtained by addition of tofenacine to microsomes of PB-treated, but not of untreated, rats (not shown). Formation of the 434 nm complex during orphenadrine metabolism was kinetically distinct from that of the metabolic intermediate complex described thus far. Within 4 min, 50% of the 434 nm complex were formed, whereas half-maximal formation of the 455 nm metabolic intermediate complex took about 15 min.

Formation of the orphenadrine metabolite complex was accompanied by a loss in carbon monoxide binding capacity of dithionite-reduced P450. Based on this effect, and on the extinction coefficient of the P450-CO-complex of 91 mM^{-1}* cm^{-1} [26], an extinction coefficient for the orphenadrine metabolite complex of 78 ± 8 mM^{-1} cm^{-1} was determined with microsomes of untreated and inducer-treated male rats.

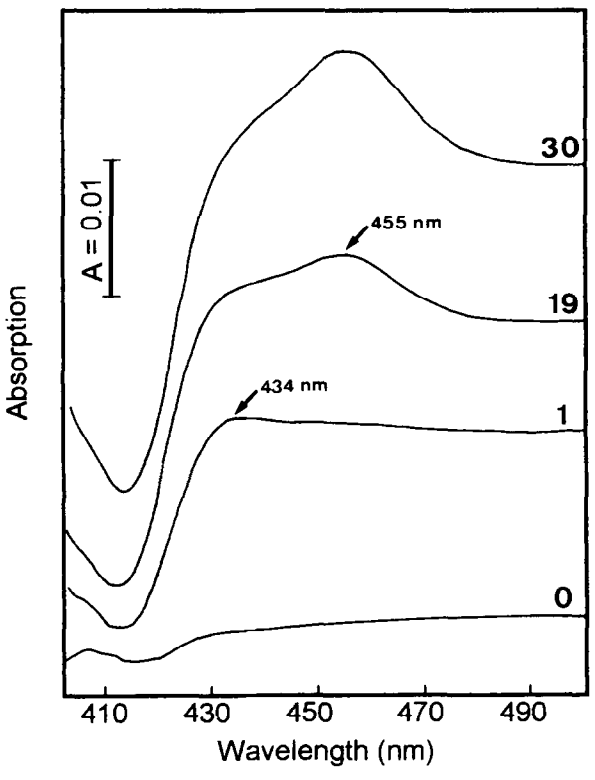

FIG. 2. Time-dependent development of the orphenadrine metabolite complex (455 nm) in microsomes of PB-treated rats showing the formation of an additional complex with an absorption maximum at λ = 434 nm. P450 concentration: 1 μM. Numbers specify the incubation times in min.

P450 Isozyme Involvement

On the basis of the induction experiments and data from the literature [16, 19, 21, 30], possible P450 candidates for complex formation with orphenadrine are CYP2C11, CYP2B and CYP3A isozymes, and CYP2C6 (see Discussion).

P450 ISOZYME CYP2C11. CYP2C11 is a sex-specific isozyme expressed constitutively in liver microsomes of male rats only. Thus, this isozyme may account for the high level of orphenadrine metabolite complex formed in microsomes of untreated male rats compared to female rats, amounting to 159 and 16 pmol/mg protein, respectively (Table 1). Expression of CYP2C11 cannot be increased by treatment with typical P450 inducers. On the contrary, inducers of CYP1A isozymes such as polycyclic aromatic hydrocarbons or β-NF lead to a significant suppression of CYP2C11 [31, 32]. Accordingly, orphenadrine-complex-forming isozymes were suppressed to levels of 52% and 24% by treatment with β-NF and 3-MC, respectively (Table 1). An induction of CYP2C11 by a factor of about 1.5 was obtained by treatment of rats with the anabolic steroid STZ as estimated by immunoblotting using a CYP2C11-specific antibody and by determination of CYP2C11-specific testosterone 2α-hydroxylase activity (not shown). Accordingly, the extent of metabolite complex formation with orphenadrine also increased by a factor of about 1.4 in these microsomes (Table 1). As will be shown in a forthcoming paper, STZ does not induce CYP2B or CYP3A isozymes, which, therefore, cannot be responsible for the increase in complex formation [33]. In liver microsomes of untreated or STZ treated female rats, which exhibit only low complex-forming activities with orphenadrine (Table 1), neither testosterone 2α-hydroxylase activity nor immunoreactive CYP2C11 protein was detected.

CYP2C11-dependent testosterone metabolism by microsomes of untreated male rats was efficiently inhibited by prior complex formation with orphenadrine. In the experiment shown in Table 2, complex formation was allowed to proceed for 30 min so that complete conversion of respective P450 isozymes into the complex could be assumed (Fig. 1). Furthermore, it was shown that the metabolite complex, which comprised about 23% of total P450, remained stable after addition of 250 μM testosterone to the sample. Quantitation of formed testosterone metabolites revealed that CYP2C11-dependent 2α- and 16α-hydroxylations were both inhibited by about 83%, whereas CYP3A- and CYP2A1-dependent activities such as 2β-, 6β-, 18- and 7 α-hydroxylation of testosterone were not inhibited or even stimulated by prior complex formation with orphenadrine.

As shown by Chang *et al.* [24], cimetidine is a specific inhibitor of CYP2C11 activities under certain conditions. Following their protocol, orphenadrine complex formation can be effectively inhibited by cimetidine in microsomes of untreated male rats (Fig. 3). Under the same conditions, and even with cimetidine concentrations up to 500 μM,

TABLE 2. Inhibition of P450-dependent testosterone metabolism by complex formation with orphenadrine

Activity	Microsomes of untreated rats		
	-Orphenadrine	+Orphenadrine	
Metabolite complex formation: pmol complex/mg protein			% Complex of total P450
Incubation for 30 min	–	205 ± 31	23.5
+250 μM Testosterone for 10 min	–	194 ± 29	22.3
Testosterone metabolism: pmol metabolite/mg protein · min			% Inhibition (*stimulation*) by orphenadrine
6β-Hydroxytestosterone	239 ± 40	269 ± 17	*(12.6)*
7α-Hydroxytestosterone	199 ± 56	266 ± 53	*(33.4)*
16α-Hydroxytestosterone	1315 ± 114	209 ± 29	84.1
16β-Hydroxytestosterone	261 ± 82	44 ± 2	83.0
18-Hydroxytestosterone	20 ± 1	25 ± 11	*(23.3)*
2α-Hydroxytestosterone	1477 ± 226	227 ± 36	84.6
2β-Hydroxytestosterone	60 ± 9	51 ± 3	15.6
Androstenedione	1281 ± 154	1101 ± 139	14.0

Sample: microsomes of untreated male rats. P450, 1 μM. Orphenadrine, 25 μM. *In vitro* formation of the metabolite complex was done as described in Materials and Methods. After complex formation had proceeded for 30 min, 250 μM testosterone was added to the sample and reference cuvette, and the stability of the complex monitored for an additional 10 min. The reaction was stopped by immediate extraction of the substrates and metabolites with 3 mL ice-cold diethylether. Testosterone metabolites formed in the samples with and without orphenadrine were analyzed by HPLC. Values give means ± standard deviation based on 3 independent determinations.

CYP2B-dependent PROD activity was not significantly affected in microsomes of PB-treated rats (Table 3). In microsomes of either untreated or inducer-treated rats, complex-forming isozymes were inhibited by cimetidine without preincubation. However, a pronounced increase in inhibition by preincubation with NADPH was only observed with microsomes of untreated male rats and not with PB- or DEX-treated rats.

CYP3A ISOZYMES. To a limited extent, CYP3A isozymes appeared to be involved in metabolite complex formation with orphenadrine. As shown in Table 1, DEX and PB treatment of female rats led to pronounced induction of orphenadrine-complex-forming liver microsomal P450 isozymes. Because both compounds are known inducers of CYP3A isozymes [1, 34] (Table 4), involvement of the latter in complex formation with orphenadrine is probable. Inhibition studies with TAO, which forms a stable metabolic intermediate complex with CYP3A [10], show that CYP3A isozymes are, in part, responsible for complex formation. After quantitative complex formation with TAO, the extent of orphenadrine complex formation was decreased by ≥50% in microsomes of DEX-treated male and female rats, but not in microsomes of untreated or PB-treated rats of either sex (Fig. 4b). In another set of experiments, the effect of primary complex formation with orphenadrine on the extent of subsequent TAO complex formation was studied. Figure 4a shows that the extent of formed TAO complex could be diminished by about 60% in microsomes of DEX-treated rats by primary complex formation with orphenadrine but was only slightly affected in PB-treated and untreated animals. Because microsomes of

TABLE 3. Effect of cimetidine on CYP2B-dependent PROD activity

	μM Cimetidine	Pre-incubation	nMol resorufin/mg protein · min
Experiment no. 1	–	–	3.20
	100	+	3.48
	750	–	3.03
Experiment no. 2	–	–	2.97
	500	+	2.83

Cimetidine was used with (+) and without (–) preincubation as described in Materials and Methods. The results of 2 independent experiments are given.

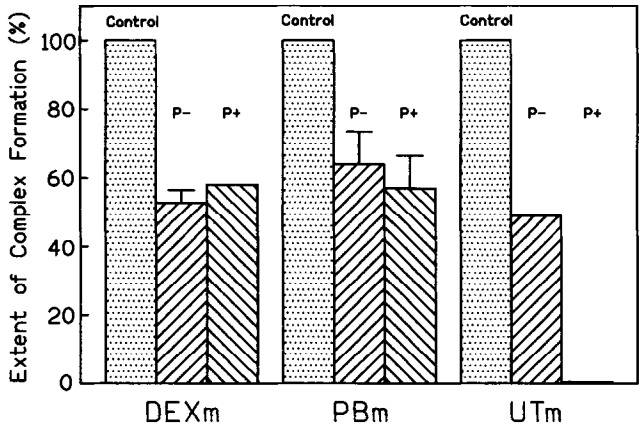

FIG. 3. Inhibition of *in vitro* orphenadrine complex formation by cimetidine in microsomes of untreated, PB-treated, and DEX-treated rats. Cimetidine was added to the samples containing microsomes, NADP, and an NADPH-generating system either 15 min prior to (P+) or concomitantly with addition of orphenadrine (P-). Control: uninhibited sample. Extent of formed complex corresponding to 100% ($A_{455nm-500nm}$/μM P450); DEXm, 0.0076; PBm, 0.0109; UTm, 0.0099.

TABLE 4. CYP3A levels compared to the level of orphenadrine-complex-forming isozymes

Microsomes (treatment/sex)	CYP3A level (nmol/mg protein)	Orphenadrine complex (nmol/mg protein)
UTm	0.081 ± 0.049	0.159 ± 0.045
PBm	0.296 ± 0.060	0.220 ± 0.043
DEXm	0.822 ± 0.252	0.154 ± 0.010
UTf	0.010	0.016 ± 0.011
PBf	0.130 ± 0.045	0.148 ± 0.044
DEXf	0.730 ± 0.070	0.115 ± 0.017

Samples: liver microsomes of untreated and inducer-treated rats. CYP3A was quantified spectroscopically by complex formation with TAO (extinction coefficient 69.0 $mM^{-1} \cdot cm^{-1}$, [15]). m, male; f, female. Figures give means ± standard deviations (at least 3 experiments) or single values when only one experiment was performed.

female rats do not contain CYP2C11 and because orphenadrine complex formation with microsomes of PB-treated female rats is not inhibited by TAO, at least one further P450 species is involved in complex formation with orphenadrine, in addition to CYP2C11 and CYP3A isozymes.

Metyrapone is a potent inhibitor of liver microsomal CYP3A-dependent enzymatic activities [15]. In dexamethasone-treated rats that express orphenadrine-complexing TAO-sensitive CYP3A isozymes, metyrapone efficiently inhibits orphenadrine complex formation with an I_{50} of about 5 μM (Fig. 5a,c). Less effective inhibition by metyrapone was also observed with microsomes of untreated and PB-treated rats with I_{50}-values of about 50 μM (Fig. 5b,c). The shapes of inhibition curves obtained with microsomes of untreated and PB-treated rats suggest the involvement of at least 2 different isozyme components in complex formation. Clarification of this question deserves further examination.

CYP2B ISOZYMES. Inducible CYP2B isozymes are apparently not involved in complex formation with orphenadrine. In liver microsomes of untreated and inducer-treated rats, there was no correlation between the number of orphenadrine-complex-forming isozymes and the level of CYP2B-dependent PROD activities (Table 5, $r^2 = 0.259$) or the isozyme levels determined by immunoblotting. Microsomes of untreated male rats showed low PROD activity and expressed only minute amounts of isozyme CYP2B (Fig. 6). However, these microsomes can form high amounts of orphenadrine complex. Although PB treatment of rats resulted in high microsomal levels of both CYP2B1 and CYP2B2 (Fig. 6) and a concomitant 170-fold increase in CYP2B-dependent PROD activity (Table 5), the extent of orphenadrine complex formation was only slightly higher in these microsomes compared to microsomes of untreated rats.

In addition to CYP3A isozymes, metyrapone efficiently inhibited enzymatic activities of CYP2B isozymes. For example, CYP2B-dependent PROD activity was inhibited by metyrapone with K_I = 0.3 μM [35]. On the contrary, metyrapone was only a poor inhibitor of orphenadrine complex formation in microsomes of PB-treated rats. Inhibition of complex formation by metyrapone was determined by the extent of complex formed after 30 min of incubation in the presence of varying metyrapone concentrations. By this method, an I_{50}-value of about 50 μM was determined, which is equal to that obtained with nearly CYP2B-deficient microsomes of untreated rats. For comparison, the I_{50}-value of 3A-dependent complex-forming activity in microsomes of DEX-treated rats was one order of magnitude lower (5 μM, see above).

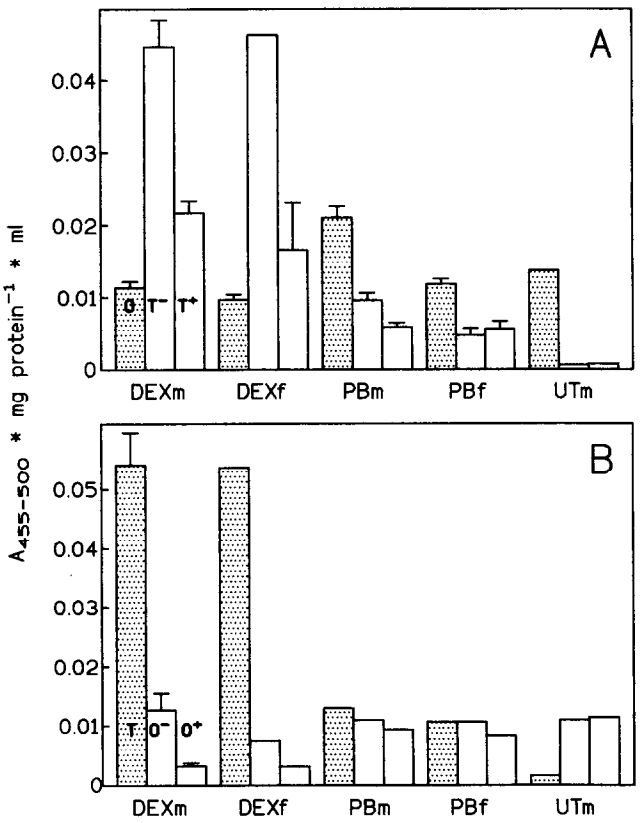

FIG. 4. A. Effect of primary complex formation with orphenadrine (O) on subsequent formation of a TAO metabolite complex. B. Effect of primary complex formation with TAO (T) on subsequent formation of an orphenadrine complex. Left bars (dotted): extent of complex with first substrate (O, T). Centre bars: extent of complex with second substrate (O^-, T^-). Right bars: extent of complex with second substrate after complex formation with the first substrate (O^+, T^+). Formation of the complexes was allowed to proceed for 30 min at 37°C. The absorption differences were normalized on a protein basis. Samples: liver microsomes of untreated (UT) and inducer-treated rats as indicated. DEX, dexamethasone; PB, phenobarbital; m, male; f, female.

If CYP2B isozymes were involved in complex formation with orphenadrine, 2B-dependent activities would be blocked after formation of the metabolite complex. As shown in Fig. 7, dealkylation of pentoxyresorufin by CYP2B could, indeed, be competitively inhibited by addition of orphenadrine (K_I = 7.2 μM). However, during concomitant formation of the orphenadrine complex, the inhibition remained constant up to at least 30 min (Fig. 8). Incubation

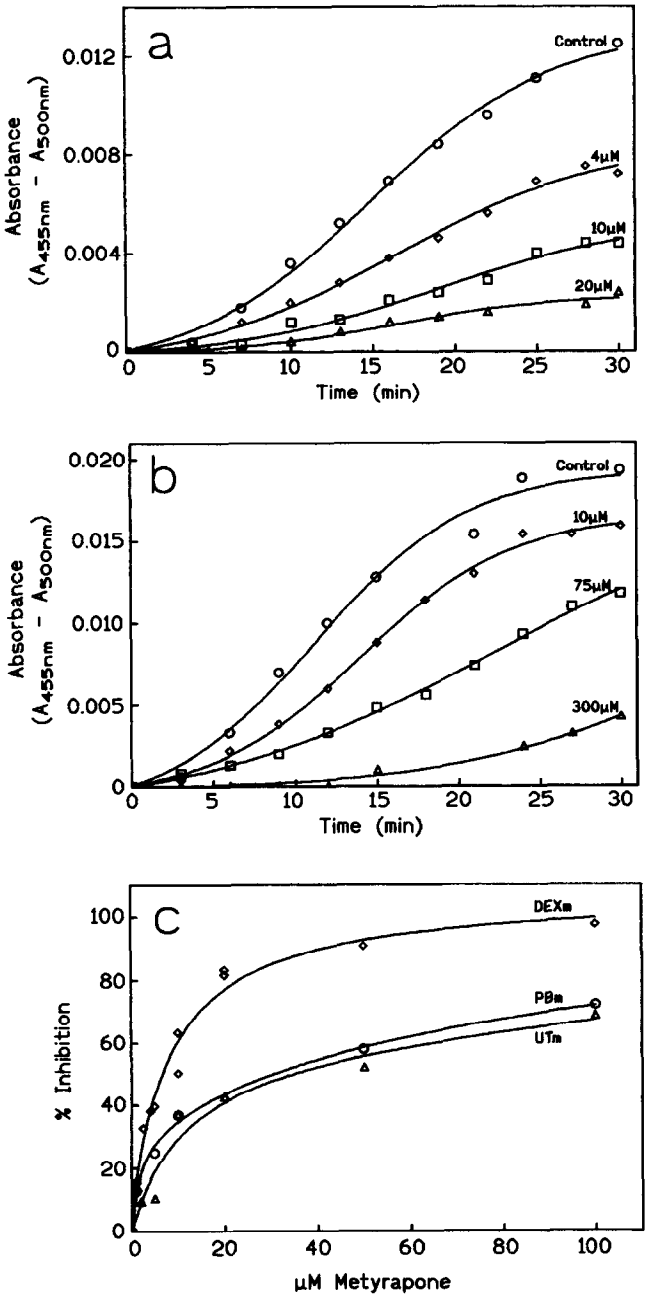

FIG. 5. Inhibition of orphenadrine complex formation by metyrapone. (a) Kinetics of complex formation with microsomes of DEX-treated male rats in the presence of various metyrapone concentrations. P450 concentration: 1.5 μM. (b) Kinetics of complex formation with microsomes of untreated male rats in the presence of various metyrapone concentrations. P450 concentration: 1 μM. (c) Inhibition of complex formation in microsomes of untreated, PB-treated, and DEX-treated male rats as a function of metyrapone concentration. Inhibition is given by the extent of formed complex after 30 min of incubation in relation to maximally formed complex.

for 20 min with orphenadrine in the presence of NADPH prior to PROD measurement did not show a significant difference in reaction velocity compared to a control

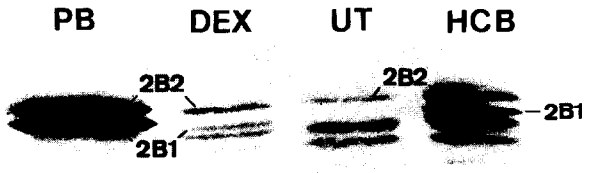

FIG. 6. Immunoblot of microsomal P450 separated by SDS-PAGE using an antibody against CYP2B1/2B2. Samples: liver microsomes of male rats either untreated (UT) or treated with DEX, PB, or HCB. Each sample contained 5 μg microsomal protein.

sample without orphenadrine. Thus, complex formation, as such, did not lead to inhibition of CYP2B-specific PROD activity. However, efficient inhibition of PROD activity was obtained with tofenacine, the monodemethylated metabolite of orphenadrine. Analysis by a Dixon-plot revealed an inhibitor constant of 0.15 μM (not shown), which was about 50-fold lower than with orphenadrine.

CYP1A ISOZYMES. CYP1A-specific EROD activity was only slightly, if at all, affected by orphenadrine up to a concentration of 66 μM. As for PROD activity, there was no correlation between EROD activities and the levels of orphenadrine metabolite complex-forming cytochromes P450 (Table 5). Treatment of rats with the CYP1A inducers 3-MC and β-NF led to high induction of CYP1A-dependent EROD activities and concomitant suppression of orphenadrine-complex-forming isozymes (Table 1).

DISCUSSION

Microsomal metabolism of orphenadrine results in formation of an enzymatically inactive P450 metabolite complex [16, 19–21] with an absorption maximum at 455 nm. We have investigated the potential of liver microsomes of untreated and inducer-treated rats to form this metabolite

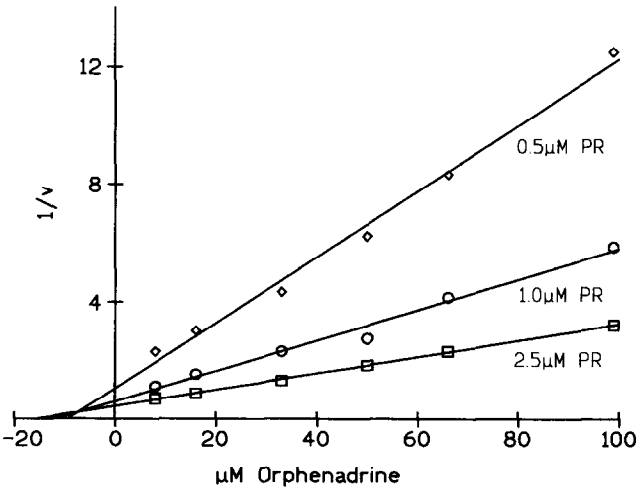

FIG. 7. Inhibition of CYP2B-dependent pentoxyresorufin O-dealkylase activity by orphenadrine analyzed by a Dixon plot. Substrate and inhibitor were added concomitantly to the microsomal sample. PR, pentoxyresorufin.

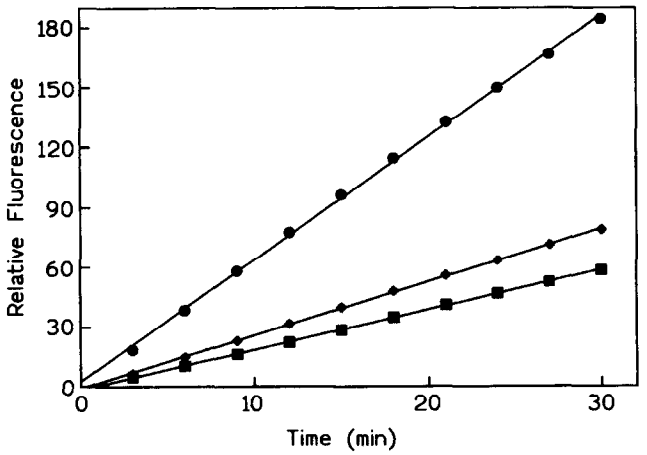

FIG. 8. Effect of orphenadrine on the kinetics of pentoxyresorufin O-dealkylation performed by microsomes of PB-treated male rats. Activity was monitored spectrofluorometrically in the absence (●) and presence of 30 μM orphenadrine (◆,■). Orphenadrine was added either concomitantly (◆) with pentoxyresorufin or 20 min prior to addition of the substrate (■). The preincubation was performed in the presence of NADP and an NADPH-generating system at 37°C. P450 concentration: 1.9 nM.

complex. From these investigations and enzymatic studies, it appears that at least 2 and possibly 3 different P450 isozymes are involved in complex formation with orphenadrine, and that one of these isozymes is a male-specific form.

We have found that, to a variable degree, liver microsomes of Sprague-Dawley rats are capable of metabolite complex formation with orphenadrine, irrespective of gender or of treatment with common P450 inducers (Table 1). Compared to females, untreated male rats express high levels of complex-forming isozymes (160 pmol/mg protein vs 16 pmol/mg protein). Similarly, Bast and Nordhoek [16] also found high amounts of metabolite complex formed *in vitro* in liver microsomes of untreated male Wistar rats with the primary orphenadrine metabolite tofenacine as substrate. From their data, a level of about 33% complex-forming P450 isozymes can be calculated using an extinction coefficient of $\epsilon_{complex}$ = 78 mM^{-1} cm^{-1}. These data suggest the involvement of the male-specific constitutively expressed isozyme CYP2C11 in complex formation with orphenadrine. This conclusion is confirmed by nearly complete inhibition of CYP2C11-dependent 2α- and 16α-hydroxylation of testosterone after metabolite complex formation with orphenadrine (Table 2). Further support comes from inhibition studies with cimetidine. As shown by Chang *et al.* [24], cimetidine is a specific inhibitor of CYP2C11-dependent enzymatic activities when preincubated, at relatively low concentrations (50 μM), with microsomes in the presence of NADPH. Using this method, orphenadrine complex formation is differentially inhibited in microsomes of untreated male rats when done with or without preincubation in the presence of inhibitor (Fig. 3). Significant differences in inhibition of complex formation by cimetidine with and without preincubation are obtained with microsomes of untreated male rats. These differences in inhibition potential are lower or absent in microsomes of PB- or DEX-treated rats, indicating that complex formation with orphenadrine is predominantly performed by P450 isozymes other than CYP2C11 in these microsomes. As was shown by Bast *et al.* [36], cimetidine inhibits the formation of a metabolite complex in microsomes of PB-treated rats with tofenacine as substrate (an intermediate in the process of complex formation with orphenadrine) [16, 20]. They used inhibitor concentrations of up to 400 μM which, in addition to CYP2C11, considerably affect other P450-dependent enzymatic activities such as ethoxyresorufin O-deethylase [37], ethoxycoumarin O-deethylase [38], and testosterone hydroxylation in the 2β-, 6β-, and 16β-position [24]. Under the conditions described by Chang *et al.* [24], however, cimetidine does not affect or only marginally affects enzymatic activities of the P450 isozymes 2A1, 2B1/2, and 3A1/2.

Treatment of rats with the anabolic steroid STZ does not alter the liver microsomal levels of CYP2B and CYP3A isozymes, but results in a significant induction of CYP2C11, as shown by immunoquantitation and determination of testosterone 2α-hydroxylase activity. Concomitantly, a respective increase in the level of orphenadrine-complexing isozymes is observed. On the other hand, treatments of rats with classical P450 inducers, such as PB, β-NF, pregnenolone 16α-carbonitrile, isosafrole, 3-MC, and 3,4,5,3′,4′,5′-hexachlorobiphenyl, results in significant suppression of CYP2C11 [32, 39, 40]. βNF and 3MC suppress the CYP2C11 level to 53% and 29%, respectively [31, 32]. This agrees well with the lower levels of orphenadrine-complexing isozymes (54% and 25%) after treatment with these CYP1A inducers (Table 1). However, PB treatment of rats was also shown to considerably decrease the level of CYP2C11 to about 30% compared to untreated animals [31, 32], and orphenadrine-complexing isozymes were increased by 40%. This suggests that, in addition to suppression of CYP2C11, PB concomitantly induces the expression of other P450 species capable of complex formation with orphenadrine to a moderate degree (see below for further discussion).

It is obvious that complex formation with orphenadrine in microsomes of untreated rats cannot be attributed to CYP1A1 or CYP2B1, because both isozymes are not expressed constitutively or only in small amounts in rat liver microsomes [41–43], documented here by the negligible levels of isozyme-specific EROD and PROD activities and by immunoblotting responses (Table 5, Fig. 6). Treatment of rats with typical CYP1A inducers such as β-NF and 3-MC led to a significant suppression of complex-forming isozymes by 47% and 75%, respectively, and EROD activities were concomitantly induced by factors of 125 and 160, respectively. In fact, induced EROD activities are negatively correlated with the extent of orphenadrine-complex-forming P450 isozymes. From these findings, and from the

TABLE 5. PROD activities and EROD activities compared to the level of orphenadrine-complex-forming isozymes

Microsomes (treatment)	PROD activity (nmol Res/ mg protein · min)	EROD activity (nmol Res/ mg protein · min)	Orphenadrine complex (nmol/mg protein)
UT	0.017 ± 0.008	0.16 ± 0.05	0.159 ± 0.045
PB	2.899 ± 0.718	0.55 ± 0.04	0.220 ± 0.043
DEX	0.268 ± 0.119	0.24 ± 0.01	0.154 ± 0.010
3MC	0.176 ± 0.047	25.10 ± 4.18	0.039 ± 0.023
βNF	0.063 ± 0.004	19.95	0.085 ± 0.035
STZ	0.016 ± 0.003	0.03 ± 0.01	0.214 ± 0.007

Samples: liver microsomes of untreated and inducer-treated male rats. Res, resorufin; m, male; f, female; n, number of determinations. Figures give means ± standard deviations.

fact that orphenadrine up to a concentration of 66 μM does not show any inhibitory effect on EROD activity after preincubation in the presence of NADPH, the conclusion can be drawn that CYP1A isozymes are not capable of complex formation with orphenadrine.

By PB-treatment of male rats, complex-forming isozymes are induced 1.4-fold, and the level of CYP2B isozymes is increased by 2 orders of magnitude (Table 5, Fig. 6) [15, 44]. Thus, in PB-treated rats, CYP2B isozymes do not appear to play a significant role in microsomal complex formation with orphenadrine. However, CYP2B-dependent pentoxyresorufin O-depentylation is efficiently inhibited by orphenadrine (K_I = 7.2 ± 0.3 μM). As shown by a kinetic experiment, this inhibition is not due to formation of a stable metabolic intermediate complex (Fig. 8), but is explained by competitive inhibition of CYP2B isozymes by orphenadrine or orphenadrine metabolites (Fig. 7). The lowering of the inhibitor constant of orphenadrine (K_I = 3.4 μM) by preincubation of microsomes in the presence of inhibitor and NADPH for 4 min (K_I = 0.13 μM) as observed by Reidy *et al.* [19] is probably caused by interaction of CYP2B and the primary orphenadrine metabolite tofenacine (monodemethylated orphenadrine). As shown here, the inhibitor constant of this compound for PROD activity is 0.15 μM, differing by a factor of 50 compared to orphenadrine (7.2 μM). Interaction of orphenadrine metabolites with CYP2B isozymes is also apparent from formation of a complex with an absorption maximum of 434 nm (Fig. 2), which is characteristic for microsomes of PB-treated rats. Binding studies with orphenadrine and some of its metabolites suggest that tofenacine is responsible for formation of this complex (not shown).

Inhibition of P450-dependent *in vitro* androstenedione metabolism by orphenadrine treatment of rats has been studied by Reidy *et al.* [19]. Activity measurements before and after dissociation of *in vivo* formed complex by ferricyanide revealed significant effects on recovered hydroxylation activities in the 16α and 16β positions. These reactions are performed by CYP2C11 and CYP2B1, respectively [45, 46]. 6β- and 7a-hydroxylations were not affected in these experiments. Although the authors argue against the involvement of CYP2C11 in complex formation with orphenadrine, their enzymatic data suggest the contrary. In fact, recovery of CYP2C11-dependent 16α-hydroxylation (+50%) in microsomes of orphenadrine-treated animals was more pronounced than that of 16β-hydroxylation (+26%).

Differential inhibition of complex formation with orphenadrine in microsomes of untreated and inducer-treated rats by cimetidine, metyrapone, or TAO and induction of complex-forming isozymes in female rats by PB and DEX suggest that other P450 isozymes in addition to CYP2C11 are involved in this process. TAO has been shown to be a specific inhibitor for rat microsomal CYP3A isozymes by formation of a stable metabolic intermediate complex [10, 15]. In microsomes of dexamethasone-treated rats, enzymatic inactivation of CYP3A isozymes by quantitative *in vitro* complex formation with TAO results in a 75% decrease in the extent of subsequent complex formation with orphenadrine. This means that CYP3A isozymes are predominantly responsible for orphenadrine complex formation in microsomes of DEX-treated rats. In similar experiments, only negligible inhibitory effects of TAO were observed with microsomes of untreated or PB-treated rats. Involvement of CYP3A is also shown in experiments where complex formation with orphenadrine was performed prior to that with TAO. It is shown here that the extent of *in vitro* formed TAO complex can be decreased by prior complex formation with orphenadrine, to about 50% in microsomes of DEX-treated male and female rats.

There are several lines of evidence indicating that only some of the several CYP3A forms that can be expressed in rat liver microsomes of male and female rats [34] are capable of forming an orphenadrine metabolite complex: 1. dexamethasone, a strong inducer of CYP3A, does not increase the level of complex-forming isozymes in male rats (there is no correlation between spectroscopically determined CYP3A levels and the extent of complex formation, r^2 = 0.016), 2. orphenadrine-complex formation only partly prevents complex formation with TAO in dexamethasone-treated rats (Fig. 4a), 3. although microsomes of untreated and phenobarbital-treated rats contain about 10% and 20% CYP3A, respectively, based on total P450 [15], TAO does not affect complex formation with orphenadrine

(Fig. 4b), and 4. CYP3A dependent 6β-, 2β-, and 18-hydroxylations of testosterone are not inhibited by complex formation with orphenadrine in microsomes of untreated male rats.

We have recently shown the existence of at least 4 different CYP3A isozymes in rat liver microsomes [34, 47]. The four isozymes known so far, 3A1, 3A2, cDEX, and 3A18 (cUT) [31, 47–49], are very similar, and there are no clear-cut enzymatic differences that would allow an easy distinction of the isozymes on the protein level. From our data, we cannot determine which isozyme from the CYP3A pool is involved in complex formation with orphenadrine. Evidence for the capability of individual CYP3A isozymes to form an orphenadrine metabolite complex may be obtained by enzymatic studies with heterologously expressed proteins. Orphenadrine may allow enzymatic discrimination between several CYP3A isozymes.

CYP2C6 has been proposed to form a metabolite complex with orphenadrine [21]. This isozyme is expressed constitutively in the livers of male, as well as female, rats in comparable amounts, making up about 0.35 nmol/mg microsomal protein [32] or 23.7% to 29% of total P450 [31, 32, 42]. Accordingly, similar levels of CYP2C6-mRNA have been determined in liver cells of male and female rats [50]. Because of the low levels of orphenadrine-complex-forming isozymes in untreated female compared to male rats (<2% vs 16%), CYP2C6 is probably not involved in complex formation with orphenadrine. However, constitutive testosterone 16β-hydroxylase activity in microsomes of untreated male rats, which is exerted by several P450 isozymes including CYP2C6 [51], is efficiently inhibited by prior complex formation with orphenadrine (Table 2).

Our studies suggest that CYP2C11, a CYP3A isozyme(s) and possibly a PB-inducible isozyme, are involved in complex formation with orphenadrine. Overlap in substrate specificity concerning members of the CYP2C and CYP3A subfamilies has also been shown for nifedipine [52], mephenytoin [53], and diazepam [54] in the rat. This substrate sharing by CYP2C11 and CYP3A has been considered characteristic for the rat and dog isozymes, in contrast to the corresponding human P450 species [55]. Overlapping specificity is also known for formation of metabolic intermediate complexes. In this respect, nortriptyline has been shown to result in complex formation with CYP2C11 and, partially, with CYP3A2 [12], thus exhibiting great similarity to isozyme specificity in orphenadrine complex formation. In a recent publication, the biotransformation of dapsone was investigated using the same approach and the same inducers and inhibitors as in the present paper [56]. Interestingly, inducibility and sensitivity to inhibitors of dapsone metabolism are strikingly similar to that of orphenadrine complex formation as described here. Accordingly, the authors also concluded that CYP2C11 and CYP3A isozyme are involved.

Among the different approaches to assess the multiple interactions of a drug with the liver microsomal P450 system, the analysis of intact microsomes of untreated and inducer-treated animals in combination with specific substrates and specific inhibitors is most suitable. This method allows recognition of multiple isozymes responsible for a certain reaction, evaluation of their relative contributions to this reaction and, possibly, a biochemical dissection of several consecutive enzymatic steps that may be catalyzed by different isozymes. In these respects, this strategy is superior to the use of heterologously expressed isozymes, as discussed by Halpert *et al.* [57]. To further enhance the suitability and reliability of this method, additional inhibitors with well-defined isozyme specificities will be necessary. Such isozyme-specific inhibitors are often used as diagnostic tools. For example, orphenadrine, itself, has been used to assess the isozyme specificity of ethosuximide metabolism [58]. In general, knowledge of the specificities of the involved enzymes and the underlying mechanisms of their induction and inhibition is important for evaluation of drug interactions and possible sex-dependent differences in drug susceptibility due to differential sex-linked expression of certain P450 isozymes, such as CYP2C11, CYP2C12, or several CYP3A forms [57].

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