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Identification and Characterization of the Cytochrome P450 Enzymes Involved in *N*-Dealkylation of Propafenone: Molecular Base for Interaction Potential and Variable Disposition of Active Metabolites

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SUMMARY

The activity of metabolizing enzymes determines plasma concentrations and hence effects of drugs. Identification of these enzymes may allow the prediction of both the interaction potential of drugs and the variability deriving from certain pathways. The antiarrhythmic propafenone is extensively biotransformed to the active metabolites 5-hydroxypropafenone and *N*-desalkylpropafenone. Whereas 5-hydroxylation is catalyzed by CYP2D6, the enzyme involved in *N*-dealkylation has not been identified. We, therefore, characterized the enzyme involved in the formation of *N*-desalkylpropafenone by using both *in vitro* [human liver microsomes, specific antibodies or inhibitors, and stably expressed cytochrome P450 (P450) enzymes] and *in vivo* (formation of *N*-desalkylpropafenone in patients under conditions of chronic therapy) approaches. Formation of *N*-desalkylpropafenone can be described by Michaelis-Menten kinetics. A strong correlation was observed between maximum rate of formation (V_{max}) of *N*-desalkylpropafenone and the amount of CYP1A2 ($r = 0.83$, $p < 0.001$) and CYP3A ($r = 0.54$, $p < 0.05$) in the microsomal fraction of 20 human livers. *In vitro* intrinsic clearances (derived from V_{max}/K_m) indicated a wide interindividual variability in seven human livers (from 0.01 to 0.1 ml/hr/mg of protein). Antibodies directed against CYP3A and CYP1A2 inhibited formation of *N*-desalkylpropafenone by 54 ± 10% and 24 ± 16%, respectively. The CYP2D6-mediated formation of 5-hydroxypropafenone was unaffected by these antibodies. Verapamil (substrate of CYP3A4 and CYP1A2) and midazolam (substrate of CYP3A4) were competitive inhibitors of *N*-desalkylpropafenone formation ($K_i = 70$ μM and 25 μM for verapamil and midazolam, respectively). Coding sequences for CYP1A2 and CYP3A4 were inserted in a yeast expression vector and introduced into *Saccharomyces cerevisiae* strain W(R). Both CYP1A2 and CYP3A4 catalyzed *N*-dealkylation of propafenone, with specific activities of 0.32 pmol/min/pmol of P450 and 0.16 pmol/min/pmol of P450, respectively. Our data indicate that *N*-dealkylation of propafenone is mediated via CYP3A4 and CYP1A2. From experiments on the molecular level interactions of propafenone with other drugs that are metabolized by CYP3A4 and CYP1A2 can be predicted. Such interactions have been reported for cyclosporin, rifampicin, warfarin, and theophylline. Moreover, *in vitro* intrinsic clearances showed a wide interindividual variability. Therefore, variable plasma concentrations of the active metabolite *N*-desalkylpropafenone are expected *in vivo*. We tested this hypothesis in 14 patients (dose of 150 mg of propafenone three times per day) during chronic oral therapy and observed steady state plasma concentrations of *N*-desalkylpropafenone ranging from 4 to 293 ng/ml. In summary, our work outlines the potential of different *in vitro* techniques in predicting interaction potential and interindividual variability of metabolite formation in humans.

The risk/benefit ratio of antiarrhythmic therapy has been the subject of intense discussions. Recently published data from the CAST (1) indicated a significantly higher mortality in patients treated with the antiarrhythmics flecainide or encainide, compared with placebo. The definite cause for the increased mortality has not been identified so far. It has become evident, however, that knowledge of factors determining individual antiarrhythmic response is crucial for the safe use of these drugs.

One important factor determining the intensity of response to and side effects of antiarrhythmics is the concentration of parent drug or its active metabolites in the heart. In the case of the widely used antiarrhythmic propafenone (2), which shows an electrophysiological profile similar to that of encain-

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ABBREVIATIONS: CAST, Cardiac Arrhythmia Suppresion Trial; P450, cytochrome P450; EOR, 7-ethoxyresorufin-*O*-deethylase; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; MOR, 7-ethoxyresorufin-*O*-demethylase; V_{max}, maximum rate of formation.

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ide (3) or flecainide (4), the antiarrhythmic action is mediated by both the parent compound and the active metabolites 5-hydroxypropafenone and *N*-desalkylpropafenone (5). In addition, the drug has β-blocking properties that reside in the parent compound (6). Pharmacokinetic parameters of 5-hydroxypropafenone and *N*-desalkylpropafenone differ from those of propafenone during long term therapy (7).

Some of the variability in response to propafenone has been explained by the variable extent of formation of 5-hydroxypropafenone, which leads to a wide interindividual variability in plasma concentrations of parent compound. 5-Hydroxypropafenone is formed by the P450 enzyme CYP2D6 (8, 9), which is polymorphically expressed in humans. Seven to 10% of a Caucasian population are devoid of CYP2D6 activity (10). Thus, propafenone could interact with drugs that are substrates for this enzyme. Such interactions have been described for metoprolol (increase of metoprolol concentrations) (11) and quinidine (increase of propafenone concentrations) (12). Therefore, coadministration of other drugs, which is a common procedure in a population treated for arrhythmias (e.g., patients enrolled in CAST were treated concomitantly with a variety of drugs) (1), may modulate the drug effects by means of drug interactions.

The other major metabolic pathway of propafenone besides 5-hydroxylation is formation of *N*-desalkylpropafenone. This metabolite has the same electrophysiological potency as propafenone (5, 13), and the plasma concentrations of *N*-desalkylpropafenone are similar to those of 5-hydroxypropafenone during chronic administration in humans (7). Therefore, *N*-desalkylpropafenone contributes to the antiarrhythmic effects of propafenone. The enzyme involved in *N*-dealkylation of propafenone has not been identified so far. Identification would enable prediction of drug interactions with other compounds that are metabolized by the same enzyme. In fact, there have been reports of metabolic interactions of propafenone with drugs that are not substrates for CYP2D6 [e.g., warfarin (14), cyclosporin (15), and theophylline (16)], indicating affinity of propafenone for other enzymes. Moreover, if the interindividual variability in expression of the enzyme involved in formation of *N*-desalkylpropafenone is known for a given population, then the resulting variability in formation and hence concentration of this active metabolite can be estimated. Based on these findings, one can predict whether formation of *N*-desalkylpropafenone is affected by genetic and/or environmental factors.

We, therefore, characterized the enzyme catalyzing *N*-dealkylation of propafenone both *in vitro* and *in vivo*, by adopting the following approach. (a) Formation of *N*-desalkylpropafenone was estimated in the microsomal fraction of human livers and the maximum rate of formation (V_{max}) was determined. (b) V_{max} was correlated with the individual content of various P450 enzymes as determined from immunoblotting in the same liver preparations. (c) Formation of *N*-desalkylpropafenone was inhibited with antibodies directed specifically against certain P450 enzymes and with drugs that are substrates for these enzymes. (d) Formation of *N*-desalkylpropafenone was investigated in microsomes obtained from yeast cells that were genetically engineered for stable expression of certain P450 enzymes.

This approach allows for both identification of the enzyme and a prediction of variability of *N*-dealkylation in humans. To assess the validity of such predictions, we compared results of *in vitro* experiments with *in vivo* data by investigating the variability of steady state plasma concentrations of *N*-desalkylpropafenone in a patient population treated with propafenone.

Patients and Methods

Microsomal preparations. The microsomal fraction was prepared from human livers as described previously (17). Liver samples were obtained from patients undergoing partial hepatectomy. The study was approved by the local ethics committee, and written informed consent was obtained from each patient. Individual protein and P450 contents were estimated according to the methods of Lowry *et al.* (18) and Omura and Sato (19), respectively.

Immunoblots and antibodies. Immunoblots were performed according to the method of Towbin *et al.* (20), as modified by Guengerich *et al.* (21). The amounts of the different P450s (CYP1A2, CYP2C, CYP2D6, CYP2E1, and CYP3A) were determined by densitometry of the spots.

Anti-rat CYP1A was prepared in our laboratory and checked against a preparation from Guengerich (21). It recognized rat and human CYP1A1 and CYP1A2. In human liver CYP1A1 protein concentration is negligible (22). Anti-CYP3A recognized the 3A subfamily. Human liver microsomes exhibited a single band co-migrating with pure CYP3A4 (23, 24). The different members of the CYP3A subfamily were probably estimated as a whole. Anti-CYP2C recognized the whole CYP2C subfamily (25) and human liver microsomes exhibited a single band in the P450 area. Antibodies against CYP2D6 and CYP2E1 were generous gifts from U. A. Meyer (Basel, Switzerland) and F. P. Guengerich (Nashville, TN), respectively.

Incubation conditions for *N*-dealkylation of propafenone. Incubations were carried out at 37° (pH 7.4) for 35 min in the presence of 50 μg of microsomal protein and 1 mg of NADPH. The final volume was 100 μl. Protein and time dependency have been described elsewhere (9). For kinetic experiments racemic propafenone was used in concentrations of 20, 40, 80, 160, 320, and 640 μM. In addition, microsomes from one liver were incubated with the enantiomers of propafenone, using the same concentrations. The reaction was stopped by addition of 10 μl of 30% (v/v) perchloric acid. After centrifugation at 10,000 × *g* for 5 min, 50 μl of the supernatant were injected in the HPLC system. Experiments were carried out in the microsomal fraction of 20 human livers. Complete Michaelis-Menten kinetics were obtained from seven preparations. Maximum rate of formation was estimated in 13 liver preparations.

HPLC assay for quantification of *N*-desalkylpropafenone. *N*-Desalkylpropafenone was quantified with an HPLC system with UV detection (λ = 220 nm). A 5-μm C_{18} reverse phase column (15 × 0.46 cm) was used, with a flow rate of 0.8 ml/min. The mobile phase consisted of aqueous tetrabutylammonium sulfate (0.01 M) and methanol (56:44, v/v). This HPLC system allows monitoring of the formation of both *N*-desalkylpropafenone and 5-hydroxypropafenone. The retention times were 6.4 and 8.4 min for 5-hydroxypropafenone and *N*-desalkylpropafenone, respectively. Calibration curves were linear from 50 to 1000 pmol. The interassay variability, as estimated from the slope of the calibration curves, averaged 8.7%.

Inhibition experiments using antibodies against CYP1A2 and CYP3A. Fifty micrograms of microsomal protein from three different liver preparations (no. 3, 5, and 7, with P450 contents of 0.069, 0.049, and 0.193 nmol/mg of protein, respectively; see Table 1) were preincubated with 0, 3.5, 7, 14, 35, 70, 105, 140, or 175 μg of either anti-CYP1A2 (21) or anti-CYP3A antiserum (24) at room temperature for 20 min. Subsequently, propafenone was added at a concentration of 320 μM. The incubation conditions and the HPLC system for quantification were identical to those described above. Control experiments were performed with the same concentrations of preimmune IgG.

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Inhibition experiments using substrates for CYP1A2 and for CYP3A. The drugs used as inhibitors were incubated simultaneously with propafenone. The experiments were carried out under the same incubation conditions as described above and at three different (80, 160, and 320 μM) concentrations of propafenone. Verapamil (substrate for CYP1A2 and CYP3A) (26, 27) at 5.1, 8.5, 17, 51, 153, 306, and 612 μM, 5, 10, 20, 40, 80, 110, and 220 μM midazolam (substrate for CYP3A) (28), and 5, 10, 20, 40, 80, 160, and 330 μM phenacetin (substrate for CYP1A2) (29) were added to the incubation mixtures.

Incubation conditions using microsomes from yeast cells expressing CYP1A1, CYP1A2, and CYP3A4. CYP1A1 and CYP1A2 coding sequences were cloned by polymerase chain reaction from human liver cDNA template and were inserted in the yeast expression vector YeDP60 (30).[1] Human CYP3A4 coding sequence was inserted in pYeDP60 by gap repair (31, 32). Each expression vector was introduced into the engineered *Saccharomyces cerevisiae* strain W(R), which overexpresses yeast NADPH-P450 reductase when grown with galactose as a carbon source.[2]

Culture medium S5 (containing galactose) without adenine, spheroplast preparation by enzymatic digestion of yeast cell walls, and subcellular fractionations were as described previously (33, 34). EOR and MOR enzymatic activities were measured in yeast microsomes using an adaptation of the methods of Lubet *et al.* (35) and Namkung *et al.* (36).

The testosterone 6-β-hydroxylase activity was determined by incubating 80 μM testosterone in 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 0.1 mg/ml NADPH, with 100 μg of microsomal protein in a final volume of 250 μl. After 15 min of incubation at 37°, the reaction was stopped with 5 μl of trifluoroacetic acid and the organic residue was extracted with dichloromethane. Metabolite separation was accomplished by HPLC using a 5-μm C_{18} column (3 × 0.21 cm) at a flow rate of 1 ml/min (10–40% acetonitrile gradient in water) and was monitored at 254 nm. 6β-Hydroxytestosterone standard used for this HPLC method was purchased from Steraloids (Wilton, NH). The peak of 6β-hydroxytestosterone was identified by retention time.

GC/MS assay for identification of *N*-desalkylpropafenone formed by stably expressed enzymes. For GC/MS analysis, *N*-desalkylpropafenone was derivatized as follows. An aliquot of the organic extract was evaporated to dryness with a gentle stream of nitrogen. Trifluoroacetylation was achieved by addition of 20 μl of *N*,*N*-bis-trifluoroacetylmethylamine acetonitrile (1:1). The mixture was allowed to react at room temperature for 30 min.

A HP 5985A GC/MS instrument was used for characterizing *N*-desalkylpropafenone. A chemically bounded SE52 fused silica capillary column (25-m length, 0.25-μm film thickness, and 0.32-mm internal diameter) was installed and directly inserted into the ion source. For GC, splitless injection at 280° was used; the temperature was started at 135°, maintained for 1 min, and then increased at a rate of 30°/min up to 320°. The retention time of *N*-desalkylpropafenone was 5.8 min. Spectra were obtained using negative ion chemical ionization mode with methane as reactant gas; the temperature of the transfer line was 300° and the temperature of the ion source was 200°. Spectra were provided by scanning a range from *m/z* 340 to 510.

Patients. Fourteen patients who were treated chronically for ventricular arrhythmias with propafenone (150 mg, three times per day) were included in this study (demographics of the patients, 9 men and 5 women; age, 60 ± 9 years; weight, 73 ± 11 kg). The study was approved by the local ethics committee and written informed consent was obtained from each patient. Blood samples were taken before the morning dose and after 1, 2, 3, 4, 6, and 8 hr. Plasma concentrations of *N*-desalkylpropafenone were determined by HPLC. Mean average steady state plasma concentrations (C_{ss}) were derived from the ratio of the plasma concentration versus time curve divided by the dosage interval.

Quantification of *N*-desalkylpropafenone in plasma. One milliliter of plasma was spiked with 250 ng of internal standard [2′-(2-hydroxy-3-ethylamino-propoxy)-3-phenylpropiophenone hydrochloride; Knoll Pharmaceuticals], adjusted to pH 9 with 500 μl of ammonium hydroxide/ammonium chloride buffer (1 mM), and extracted with 5 ml of dichloromethane. After centrifugation the aqueous phase was removed. The organic solvent was evaporated to dryness with a gentle stream of nitrogen. The residue was dissolved in 50 μl of mobile phase and injected into the HPLC system, which was identical to that described above for the microsomal incubation. The recovery of *N*-desalkylpropafenone in this assay was 83.5%; the within-day precision was 10.4% at a concentration of 50 ng/ml (10 experiments) and 3.5% at a concentration of 400 ng/ml (10 experiments).

Statistical calculation. V_{max} and K_m were calculated by an iterative curve-fitting computer program (37). K_i was determined mathematically and graphically from Dixon plots. The *in vitro* intrinsic clearance CL_{int} was determined from V_{max}/K_m.

Results

***In vitro* experiments.** Formation of *N*-desalkylpropafenone follows monophasic Michaelis-Menten kinetics. There was a minor difference in K_m [125, 157, and 104 μM for (*RS*), (*S*)-, and (*R*)-propafenone, respectively] and V_{max} [4.5, 6.5, and 5.8 pmol/μg/hr for (*RS*)-, (*S*)-, and (*R*)-propafenone, respectively] for the formation of *N*-desalkylpropafenone after incubation of the enantiomers of propafenone, contrasting with the results for formation of 5-hydroxypropafenone (38). Therefore, racemic propafenone was used in all experiments. CL_{int} obtained in microsomal fractions of seven livers showed a 10-fold difference, ranging from 0.01 to 0.1 ml/hr/mg of protein (Table 1).

Maximum rate of formation was correlated with expression of CYP1A2 and CYP3A, as determined by immunoblotting, thereby indicating a contribution of these enzymes to formation of *N*-desalkylpropafenone (Table 1). Antibodies directed against CYP1A2 and CYP3A inhibited formation of *N*-desalkylpropafenone by 24 ± 16% and 54 ± 10%, respectively. Formation of 5-hydroxypropafenone was unaffected (Fig. 1).

Verapamil inhibited formation of *N*-desalkylpropafenone in a competitive manner (K_i = 70 μM; the respective Dixon plot is shown in Fig. 2). Identical experiments were carried out using midazolam and phenacetin and resulted in competitive inhibition of *N*-dealkylation with a K_i of 25 μM and 200 μM, respectively. Maximum inhibition of formation of *N*-desalkylpropafenone was 100% for verapamil, 72% for midazolam, and 22% for phenacetin.

Incubation of propafenone with the microsomal fraction of yeast cells that were genetically engineered for stable expression of CYP1A1, CYP1A2, and CYP3A4 resulted in formation of *N*-desalkylpropafenone, as shown in Table 2. YeDP60 was used as a control. Control yeast had low activity, less than one third that of CYP3A4, which was subtracted from the results given for CYP1A1, CYP1A2, and CYP3A4. The microsomes from the stably expressing cells were tested for their EOR activities [<1, 476, 37, and <1 pmol/min/mg for yeast strains control-W(R), 1A1-W(R), 1A2-W(R), and 3A4-W(R), respectively], MOR activities [<1, 250, 59, and <1 pmol/min/mg for yeast strains control-W(R), 1A1-W(R), 1A2-W(R), and 3A4-W(R), respectively], and testosterone 6-β-hydroxylase activities [<1, 54, 1, and 67 pmol/min/mg for yeast strains control-

[1] Gautier, P. J.-C., Urban, P. Beaune, and D. Pompon. Yeast expression of human cytochrome P450 1A1 and microsomal epoxide hydrolase as a tool for the analysis of the first steps of benzo[*a*]pyrene activation. *Eur. J. Biochem.*, in press (1993).

[2] G. Truan, C. Cullin, P. Reisdorf, P. Urban, and D. Pompon. Associated protein environment strongly modulates the *in situ* activities of mammalian P450s 1A1 and 3A4 expressed in yeast. Submitted for publication.

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TABLE 1

Correlation of content of various P450 enzymes (reported as arbitrary units/mg of protein, from densitometric evaluation of immunoblots) versus maximum rate of formation (V_{max}) of *N*-desalkylpropafenone from propafenone in the microsomal fraction of 20 human livers

The apparent affinity (K_m) and the *in vitro* intrinsic clearance ($CL_{int} = V_{max}/K_m$) are reported for seven livers.

Liver number	P450 content					V_{max}	K_m	*In vitro* CL_{int}
	CYP1A2	CYP2C	CYP2D6	CYP2E1	CYP3A			
	units/mg of protein					pmol/mg of protein/min	μM	ml/hr/mg of protein
1	0.2	0.8	1.3	0.3	0.3	96.6	104	0.05
2	0.1	1.4	0.5	0.2	0.3	63.3	154	0.025
3	0.0	0.5	0.1	0.0	0.3	30.0	91	0.02
4	0.1	0.9	0.5	0.4	0.9	160.0	81	0.10
5	0.7	1.2	0.8	0.3	0.7	165.0	105	0.09
6	0.0	0.5	1.2	0.3	0.5	19.5	115	0.01
7	2.8	0.1	1.1	0.2	1.2	201.7	128	0.09
8	0.0	0.3	1.7	0.2	0.4	0.016		
9	1.6	1.1	1.9	0.8	1.2	173.3		
10	0.3	1.1	1.1	1.7	1.7	74.2		
11	0.2	0.7	1.4	1.5	1.8	76.2		
12	2.5	1.4	2.1	2.1	1.4	194.2		
13	2.3	1.1	2.2	1.7	1.2	251.7		
14	0.7	1.7	0.2	3.6	1.4	90.0		
15	2.7	1.8	1.3	1.6	1.6	215.7		
16	0.9	1.3	1.6	0.9	0.4	41.7		
17	2.0	1.1	0.4	1.1	1.2	158.3		
18	0.06	1.3	0.0	1.5	0.8	70.8		
19	0.2	1.7	0.2	0.6	0.4	0.16		
20	0.7	1.1	1.1	1.1	0.5	130.8		
Correlation[a]	$p < 0.01$	NS	NS	NS	$p < 0.05$			

[a] A significant correlation was observed for CYP1A2 ($p = 0.01$, $r = 0.83$) and CYP3A ($p < 0.05$, $r = 0.54$). NS, not significant.

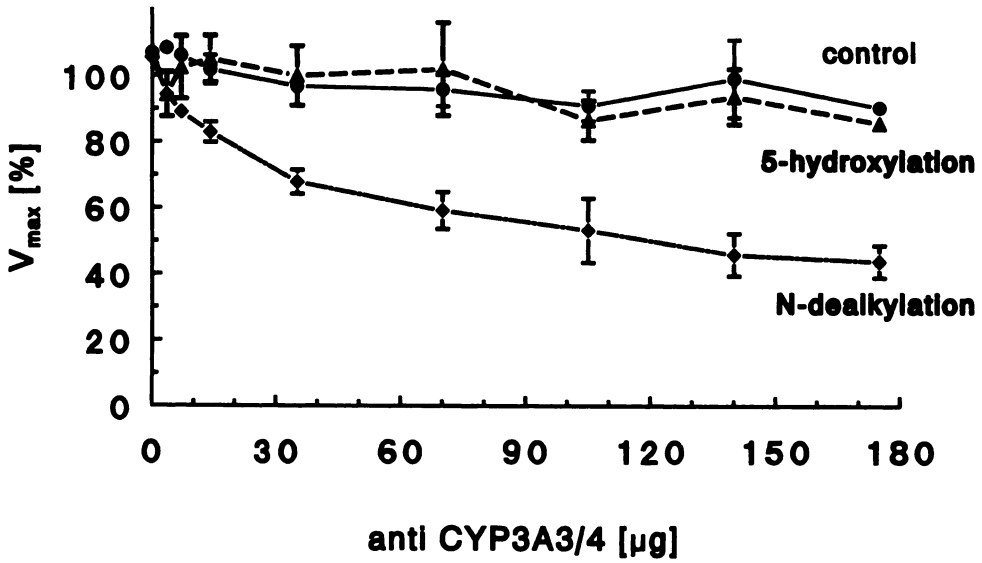

Fig. 1. Inhibition of *N*-dealkylation (◆) and 5-hydroxylation (▲) of propafenone in human liver microsomes (livers 3, 5, and 7; see Table 1) (50 μg of microsomal protein incubated for 35 min at 37°) after preincubation (20 min) with anti-CYP3A. Control experiments (●) were performed with preimmune IgG.

W(R), 1A1-W(R), 1A2-W(R), and 3A4-W(R), respectively; see Table 2].

The identity of *N*-desalkylpropafenone formed after incubation with stably expressed enzymes was confirmed by GC/MS. Spectra obtained from incubation mixtures were identical to those of the reference compound. The spectra of the derivatized compound showed the molecular ion at *m/z* 491 (10% relative abundance) and some characteristic fragments at *m/z* 471 (M−20, 12% relative abundance), *m/z* 451 (M−40, 6% relative abundance), *m/z* 375 (45% relative abundance), and *m/z* 355 (100% relative abundance).

***In vivo* experiments.** C_{ss} of *N*-desalkylpropafenone showed a wide interindividual variability in the 14 patients. The geometric mean of C_{ss} was 41 ng/ml, with a range of 4 to 293 ng/ml.

Discussion

The present investigation indicates that formation of *N*-desalkylpropafenone is mediated by both CYP3A4 and CYP1A2. Based on these findings, a variety of metabolic interactions of propafenone with other drugs can be explained readily. Coadministration of propafenone decreases clearance of warfarin and cyclosporin, both substrates for CYP3A4 (14, 15). Moreover, coadministration of rifampicin, an inducer of CYP3A4, decreases plasma concentrations of propafenone (39).

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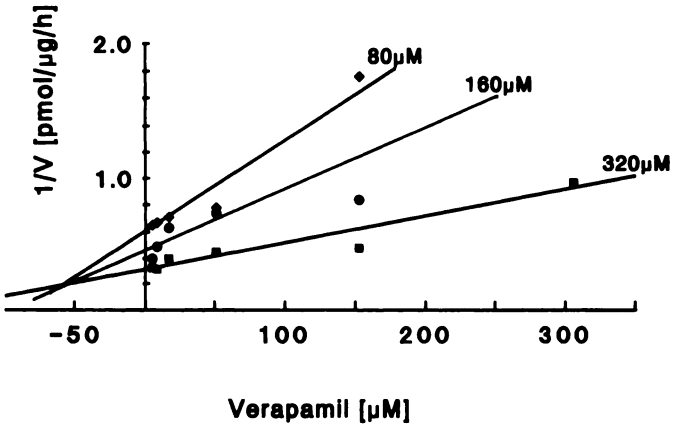

Fig. 2. Competitive inhibition of *N*-dealkylation of propafenone (substrate concentrations, 80, 160, and 320 µM) in human liver microsomes (50 µg of microsomal protein incubated for 35 min at 37°) with increasing concentrations of verapamil.

TABLE 2

Monooxygenase activities of microsomes from yeast expressing human P450s

Specific activities are expressed as pmol/min/mg of microsomal protein. Values in parentheses are turnover numbers (min^{-1}). Values are means of duplicate determinations.

Yeast strains	Monooxygenase activities			
	EOR	MOR	Testosterone 6β-hydroxylation	Propafenone *N*-dealkylation
	pmol/min/mg			
Control-W(R)	<1	<1	<1	0[a]
1A1-W(R)	476 (15.5)	250 (8.1)	54 (1.8)	69 (2.3)
1A2-W(R)	37 (1.0)	59 (1.5)	1 (0.02)	32 (0.83)
3A4-W(R)	<1	<1	67 (0.9)	12 (0.16)

[a] Control yeast had low activity, less than one third that of CYP3A4, which was subtracted from the results for CYP1A1, CYP1A2, and CYP3A4. [W(R) is a *S. cerevisiae* strain that overexpresses yeast NADPH-P450 reductase when grown with galactose as a carbon source].

It can be predicted that other drugs that are metabolized by CYP3A4 are likely candidates for interactions with propafenone [e.g., verapamil (40) and nifedipine (41); for a complete list of CYP3A4 substrates, see the work of Guengerich (42)]. With respect to the contribution of CYP1A2 to formation of *N*-desalkylpropafenone, a recent report indicated an interaction of propafenone and theophylline, leading to a substantial rise in theophylline plasma concentrations (16). Theophylline is known to be metabolized by CYP1A2. Thus, propafenone also has the potential to interact with drugs that are substrates for CYP1A2 (for a complete list, see Ref. 42).

The therapeutic consequences of drug interactions involving enzyme-mediated processes depend on the relative affinity of the interacting drugs for the catalyzing enzyme. Moreover, a pronounced decrease in clearance due to an interaction is expected only if a major fraction of a dose is metabolized via this pathway. *N*-Dealkylation of propafenone has been shown to contribute to the total clearance of propafenone to only a minor extent (12). Propafenone, however, may inhibit metabolism of other drugs via CYP3A4 and CYP1A2 and thus represents an example of a drug for which a minor pathway has considerable consequences for the interaction potential. A similar situation is observed for the antiarrhythmic quinidine, which is an effective blocker of CYP2D6 (12) although only a minor fraction of the dose is biotransformed via this enzyme (43).

Because CYP3A4 and CYP1A2 are inducible by concomitant administration of other drugs (CYP3A4) or smoking (CYP1A2) (42), the activity of these enzymes is subject to environmental modulation. In contrast, CYP2D6-mediated formation of 5-hydroxypropafenone is not inducible (44) and is subject to a genetic polymorphism. Therefore, metabolism of propafenone is under both genetic (5-hydroxylation) and environmental (*N*-dealkylation) control.

Expression of CYP3A4 exhibits a wide interindividual variability in humans (23, 45), which is monomodally distributed. From the fact that *N*-desalkypropafenone is formed via CYP3A4 one can predict considerable interindividual differences in formation and hence concentration of this active metabolite in a patient population. Both *in vitro* and *in vivo* lines of evidence support this assumption; intrinsic clearance (as derived from V_{max}/K_m) in seven human livers showed a 10-fold variation, ranging from 0.01 to 0.1 ml/hr/mg of protein. *In vivo* concentrations of *N*-desalkylpropafenone in patients being chronically treated with propafenone also indicated a pronounced variability; steady state plasma concentrations ranged from 4 to 293 ng/ml. Because *in vitro* antiarrhythmic activity of *N*-desalkylpropafenone is similar to that of 5-hydroxypropafenone and propafenone and plasma concentrations of *N*-desalkylpropafenone are in the same range as those of 5-hydroxypropafenone, it can be assumed that CYP3A4-based variability in *N*-desalkylpropafenone formation contributes to variable antiarrhythmic effects after propafenone administration. This is even more likely taking into account the higher free (non-protein-bound) fraction of *N*-desalkylpropafenone, compared with 5-hydroxypropafenone (29 ± 8% for *N*-desalkylpropafenone versus 17 ± 2% for 5-hydroxypropafenone).[3]

Our experiments using inhibitory antibodies indicated that the major fraction of *N*-desalkylpropafenone in human liver microsomes is formed via CYP3A (anti-CYP3A reduced formation of *N*-desalkylpropafenone by 54 ± 10% and anti-CYP1A2 reduced formation by 24 ± 16%). Data derived from microsomes from yeast cells that express CYP3A4 or CYP1A2, however, suggested a higher rate of formation of *N*-desalkylpropafenone by CYP1A2 (32 pmol/min/mg versus 12 pmol/min/mg). This apparent paradox is solved once the relative abundance of CYP3A4 and CYP1A2 in human liver is taken into account. Data obtained by Guengerich and Turvy (46) indicated the content of CYP1A2 and CYP3A4 to be 0.026 nmol/mg of protein and 0.248 nmol/mg of protein, respectively. CYP1A1 is present in human liver at very low levels (about one tenth that of CYP1A2) (22). Combining these data with the turnover rates shown in Table 2 allows calculation of the relative contributions of CYP1A2 and CYP3A4 to overall formation of *N*-desalkylpropafenone as 22% and 75%, respectively. These data correspond to results from experiments using immunoinhibition (54 ± 10% for anti-CYP3A and 24 ± 16% for anti-CYP1A2) and inhibiting substrates (100% for verapamil, 72% for midazolam, and 22% for phenacetin). Thus, the higher rate of turnover of propafenone to *N*-desalkylpropafenone observed in the CYP1A2-expressing yeast cells, compared with those expressing CYP3A4, is compensated for in an *in*

[3] H. K. Kroemer, unpublished observations.

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vivo situation by the greater abundance of CYP3A4 in human liver. Using liver microsomes has the advantage of reflecting the *in vivo* contribution of different P450 enzymes. Stable expression of single enzymes, however, yields unequivocal qualitative evidence that a certain enzyme is capable of catalyzing a defined metabolic step. Our investigation indicates that the different techniques, using human liver microsomes, antibodies, and stably expressed enzymes, are complementary for identification and quantification of P450 enzymes involved in the biotransformation of drugs.

In summary, this work identifies the enzymes involved in formation of the active metabolite *N*-desalkylpropafenone from propafenone. Both the variability in a given population and the interaction potential arising from metabolism of propafenone via CYP3A4 and CYP1A2 may be predicted from the *in vitro* experiments. These estimations were confirmed by an *in vivo* study. There is the possibility that a minor fraction of *N*-dealkylation of propafenone is catalyzed by other P450 enzymes, which were not tested in our experiments. However, no relevant *in vivo* interactions of propafenone have been reported that could be indicative of the involvement of additional enzymes in the *N*-dealkylation of propafenone.

As a consequence, precautions should be undertaken when propafenone is coadministered with other drugs that are known substrates for CYP3A4 and CYP1A2. As shown in CAST, coadministration of a variety of drugs with antiarrhythmics is common practice. *In vitro* identification and quantification of the metabolizing enzymes may contribute to prediction of the potential risk arising from these interactions and hence improve the safety of pharmacological treatment with arrhythmias.

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