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IDENTIFICATION OF CYTOCHROME P450 ENZYMES INVOLVED IN THE METABOLISM OF THE NEW DESIGNER DRUG 4'-METHYL-α-PYRROLIDINO-BUTYROPHENONE (MPBP)

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Identification of CYPs Responsible for MPBP Metabolism

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Abbreviations used are: CYP, cytochrome P450; MPBP, 4'-methyl-α-pyrrolidinobutyrophenone; PPP, 4'-methyl-α-pyrrolidinobutyrophenone; MPPP, 4'-methyl-α-pyrrolidinopropiophenone; MPHP, 4'-methyl-α-pyrrolidinohexanophenone; MOPPP, 4'-methoxy-α-pyrrolidinopropiophenone; MDPPP, 3',4'-methylenedioxy-α-pyrrolidinopropiophenone; PVP, α-pyrrolidinovalerophenone; HO-MPBP, 4'-hydroxymethyl-α-pyrrolidinobutyrophenone; ICM, insect cell microsomes; pHLM, pooled human liver microsomes; PMD6 HLM, single donor human liver microsomes from donors with poor metabolizer genotype for CYP2D6; PMC19 HLM, single donor human liver microsomes from donors with poor metabolizer genotype for CYP2C19; IS, internal standard; RAF, relative activity factor; TR, turnover rates; PS, probe substrate; LC-MS, liquid chromatography-mass spectrometry; AT, Agilent Technologies; APCI, atmospheric pressure chemical ionization; SIM, selected-ion monitoring.

ABSTRACT:

The involvement of human hepatic cytochrome P450 (CYP) isoenzymes in the metabolism of the new designer drug 4'-methyl-α-pyrrolidinobutyrophenone (MPBP) to 4'-(hydroxymethyl)-α-pyrrolidinobutyrophenone (HO-MPBP) was studied using insect cell microsomes with cDNA-expressed human CYPs and human liver microsomes (HLM). Incubation samples were analyzed by liquid chromatography-mass spectrometry. Only CYP2D6, CYP2C19, and CYP1A2 were capable of catalyzing MPBP 4'-hydroxylation. According to the relative activity factor approach, these enzymes accounted for 54%, 30%, and 16% of net clearance. At 1 µM MPBP, the chemical inhibitors quinidine (CYP2D6), fluconazole (CYP2C19), and α-naphthoflavone (CYP1A2) reduced metabolite formation in pooled HLM by 83%, 53%, and 47%, respectively, and at 50 µM MPBP by 41%, 47%, and 45%, respectively. In experiments with HLM from CYP2D6 and CYP2C19 poor metabolizers, HO-MPBP formation was found to be 78% and 79% lower in comparison to pooled HLM, respectively. From these data, it can be concluded that polymorphically expressed CYP2D6 is mainly responsible for MPBP hydroxylation.

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The cytochrome P450 (CYP) family accounts for more than 90% of oxidative metabolic reactions of xenobiotics (Guengerich, 2005). The involvement of particular CYP enzymes in the biotransformation of a new chemical entity is usually thoroughly investigated before it can be marketed in order to assess the risk of increased side effects in poor metabolizer subjects and of drug-drug or drug food interactions. However, such data are typically acquired for substances intended for therapeutic use, but not for drugs of the illicit market.

1-(4-Methylphenyl)-2-pyrrolidin-1-ylbutan-1-one (4'-methyl-α-pyrrolidinobutyrophenone, MPBP) is a new designer drug of the pyrrolidinophenone type. Together with α-pyrrolidino-propiophenone (PPP) (Roesner et al., 1999; Springer et al., 2003c), 4'-methyl-α-pyrrolidino-propiophenone (MPPP) (Roesner et al., 1999; Springer et al., 2002; Springer et al., 2003c), 4'-methyl-α-pyrrolidinohexanophenone (MPHP) (Springer et al., 2003f), 4'-methoxy-α-pyrrolidino-propiophenone (MOPPP) (Springer et al., 2003b), 3',4'-methylenedioxy-α-pyrrolidinopropio-phenone (MDPPP) (Roesner et al., 1999; Springer et al., 2003a), and α-pyrrolidinovalerophenone (PVP), MPBP belongs to the new class of pyrrolidinophenone-type designer drugs. The pyrrolidinophenones have entered the illicit drug market in Germany and are distributed among drug abusers as tablets, capsules, or powders (Roesner et al., 1999). Very little is known about the frequency of their occurrence, because these drugs cannot be detected with usual routine analysis procedures (Springer et al., 2002; Springer et al., 2003a; Springer et al., 2003b; Springer et al., 2003c; Springer et al., 2003f; Peters et al., 2005) and might, therefore, have been overlooked. So far, little information about the dosage of as well as the pharmacological and toxicological effects of the pyrrolidinophenones is available. However, they may be expected to be very similar to those of pyrovalerone (4'-methyl-α-pyrrolidinovalerophenone) due to their close structural relation to this drug. Pyrovalerone is a psychostimulant which acts by releasing dopamine and norepinephrine from the respective nerve terminals (Fauquet et al., 1976; Servin et al., 1978) and

inhibits the reuptake of these neurotransmitters (Meltzer et al., 2006). The latter was also shown for PVP. In comparison to amphetamine, pyrovalerone reportedly has similar psychostimulant effects but less influence on motor function in animals and humans (Stille et al., 1963; Holliday et al., 1964; Heimann and Lukacs, 1965). Pyrovalerone had been studied as a therapeutic drug (Heinmann and Vetter, 1965; Goldberg et al., 1973; Soderholm et al., 1976), but was withdrawn from the market and scheduled as a controlled substance after reports of its intravenous abuse (Deniker et al., 1975). A similar pharmacological profile of the pyrrolidinophenones would clearly be in line with their abuse as stimulant designer drugs.

The qualitative metabolism of PPP, MPPP, MPBP, MPHP, MOPPP, MDPPP, PVP and pyrovalerone has been studied in animals (Michaelis et al., 1970; Lho et al., 1996; Shin et al., 1996; Springer et al., 2002; Springer et al., 2003a; Springer et al., 2003b; Springer et al., 2003c; Springer et al., 2003f; Peters et al., 2005) and, in case of pyrovalerone, also in humans (Michaelis et al., 1970). The main metabolic steps of all 4'-methyl pyrrolidinophenones, i.e. MPPP, MPBP, MPHP, and pyrovalerone, were 4'-methyl hydroxylation followed by oxidation to the respective carboxylic acids, oxidation of the pyrrolidine moiety to the respective lactam, and reduction of the keto group. In all cases, the 4'-carboxy compounds were the main metabolites. The involvement of human CYP isoenzymes in the first step in their formation, namely hydroxylation of the 4'-methyl moieties, was studied for MPPP and MPHP (Springer et al., 2003d; Springer et al., 2003e). In both cases, CYP2D6 and CYP2C19 were found to be mainly involved in this metabolic reaction. In the case of MPHP, CYP1A2, CYP2B6, and CYP2C9 were also involved, but only to a minor extent.

So far no data are available on the CYP mediated metabolism of MPBP. Therefore, the aim of the presented study was to study the involvement of human CYP isoenzymes in formation of 4'-hydroxymethyl-α-pyrrolidinobutyrophenone (HO-MPBP).

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Materials and Methods

Materials. MPBP-HNO_3 and the hydrochloride of MDPPP were provided by the Hessian State Criminal Office (Wiesbaden, Germany) for research purposes. HO-MPBP was biotechnologically synthesized as described previously (Peters et al., 2007). $NADP^+$ was obtained from Biomol (Hamburg, Germany), isocitrate and isocitrate dehydrogenase from Sigma (Taufkirchen, Germany), all other chemicals and reagents from Merck (Darmstadt, Germany). The following microsomes were from Gentest and delivered by NatuTec (Frankfurt/Main, Germany): baculovirus-infected insect cell microsomes (ICM, Supersomes®) containing 1 nmol/ml human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, or 2 nmol/ml CYP2E1, CYP3A5, wild-type baculovirus-infected ICM (control Supersomes®), pooled human liver microsomes (pHLM 20 mg microsomal protein/ml, 400 pmol total CYP/mg protein) and single donor human liver microsomes (20 mg microsomal protein/ml) from donors with poor metabolizer genotype for CYP2D6 (PMD6 HLM) or CYP2C19 (PMC19 HLM). After delivery, the microsomes were thawed at 37°C, aliquoted, shock-frozen in liquid nitrogen and stored at –80°C until use.

Microsomal Incubations. Incubation mixtures (final volume: 50 µl) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM isocitrate, 1.2 mM $NADP^+$, 0.5 U/ml isocitrate dehydrogenase, 200 U/ml superoxide dismutase and substrate at 37°C. The substrate was added after dilution of a 250 mM methanolic stock solution in the above-mentioned phosphate buffer. The methanol concentration did not exceed 0.4%. Reactions were started by addition of ice-cold microsomes and terminated with 5 µl of 60% (w/w) perchloric acid. After addition of 3 µl of internal standard (IS) solution (0.05 mM MDPPP in methanol), the samples were centrifuged and the supernatants were transferred to autosampler vials.

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Initial Screening Studies. Incubations were performed with 50 µM MPBP and 50 pmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM or 90 mM Tris buffer, respectively, according to the Gentest manual.

Kinetic Studies. Kinetic constants of HO-MPBP formation were derived from incubations with the following MPBP concentrations, incubation times and protein concentrations: 2, 5, 10, 15, 20, 30, 40, 75, 120, 200, 400, 700 and 1000 µM MPBP with 30 pmol CYP2C19/ml for 15 min; 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 5, 7.5, 10, 15, 20, 30, 40, and 75 µM MPBP with 30 pmol CYP2D6/ml for 15 min; 75, 120, 200, 400, 700, 1000, 1500, 2000, 2500, 3000, and 5000 µM MPBP with 30 pmol CYP1A2/ml for 15 min; 2, 5, 10, 15, 20, 30, 40, 75, 120, 200, 400, 700 and 1000 µM MPBP with 0.5 mg HLM protein/ml for 30 min. Enzyme kinetic constants were estimated by non-linear regression using GraphPad Prism 3.02 software (San Diego, CA). The Michaelis-Menton equation (equation 1) was used to calculate apparent K_m and V_{max} values for single-enzyme systems.

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

Eadie-Hofstee plots were used to check for biphasic kinetics (Clarke, 1998). If the Eadie-Hofstee plot indicated biphasic kinetics, equation 1 and the alternative equation 2 for a two site binding model (Clarke, 1998) were applied to the respective data. If equation 2 was found to fit the data significantly better (F-test, $P < 0.05$), biphasic kinetics were assumed.

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$$V = \frac{V_{max,1} \times [S]}{K_{m,1} + [S]} + \frac{V_{max,2} \times [S]}{K_{m,2} + [S]} \quad (2)$$

Calculation of Relative Activity Factors, Contributions, and Percentages of Net Clearance. The relative activity factor (RAF) approach (Crespi and Miller, 1999; Venkatakrishnan et al., 2000a) was used to account for differences in functional levels of redox partners between the two enzyme sources. The turnover rates (TR) of CYP2C19 [probe substrate (PS) *S*-mephenytoin], CYP2D6 (PS bufuralol), and CYP1A2 (PS phenacetin) in ICM and HLM were taken from the supplier's data sheets. The RAFs were calculated according to equation 3.

$$RAF_{enzyme} = \frac{TR_{PS} \text{ in HLM [pmol/min/mg protein]}}{TR_{PS} \text{ in ICM [pmol/min/mg protein]}} \quad (3)$$

V_{max} values for MPBP 4'-methyl hydroxylation obtained from incubations with cDNA-expressed CYPs were then multiplied with the corresponding RAF leading to a value, which is defined as 'contribution':

$$contribution_{enyzme} = RAF \times V_{max} \text{ of HO - MPBP formation in ICM} \quad (4)$$

From these corrected activities (contributions) the percentages of net clearance by a particular CYP can be calculated according to equation 5, where clearance equals contribution/K_m:

$$clearance_{enzyme}\,[\%] = \frac{clearance_{enzyme}}{\sum clearances_{enzymes}} \times 100 \quad (5)$$

Chemical Inhibition Studies. The effect of 3 µM quinidine, 20 µM fluconazole, or 30 µM α-naphthoflavone on HO-MPBP formation was assessed in incubations containing 0.5 mg HLM protein/ml and 1 µM or 50 µM MPBP (n=6 each). Control incubations contained none of these chemical inhibitors but the same amount of methanol («0.1%) to control for any solvent effects (n=6 each). Significance of inhibition was tested by a one-tailed unpaired t-test using GraphPad Prism 3.02 software.

Studies for comparison of pHLM with PMD6 HLM and PMC19 HLM. Incubations were carried out with pHLM, PMD6 HLM, and PMC19 HLM (0.5 mg protein/ml, n=6 each) for 30 min. The MPBP concentrations were 1.3 µM (pHLM and PMD6 HLM), 9.0 and 280 µM (pHLM and PMC19 HLM). Significance of differences in metabolite formation was tested by one-tailed unpaired t-test using GraphPad Prism 3.02 software.

Liquid Chromatography-Mass Spectrometry (LC-MS) Conditions and Quantification of Metabolite. MPBP, HO-MPBP and MDPPP were analyzed using an Agilent Technologies (AT, Waldbronn, Germany) AT 1100 series LC-MSD, SL version, with atmospheric pressure chemical ionization (APCI) electrospray interface, and an LC-MSD ChemStation using the A.08.03 software.

LC conditions. Gradient elution was achieved on a Merck LiChroCART® column (125 x 2 mm I.D.) with Superspher®60 RP Select B as stationary phase and a LiChroCART®10-2 Superspher®60 RP Select B guard column. The mobile phase consisted of a 60:40 (v/v) mixture of 5 mM ammonium formate buffer (adjusted to pH 3 with formic acid) and acetonitrile containing 1 ml/l formic acid according to (Maurer et al., 2002). The flow rate was 0.4ml/min. The injection volume was 5 µl.

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APCI-MS conditions. The following APCI inlet conditions were applied: drying gas, nitrogen (7000 ml/min, 300°C); nebulizer gas, nitrogen (25 psi, 172.3 kPa); capillary voltage, 4000 V; drying gas temperature set at 300°C, vaporizer temperature set at 400°C; corona current was 5.0 µA; positive selected-ion monitoring (SIM) mode, *m/z* 232 for MPBP and *m/z* 248 for HO-MPBP and MDPPP; fragmentor voltage 50 V.

Metabolite quantification. Calibration curves were constructed plotting peak area ratios (HO-MPBP vs. IS) of spiked calibrators vs. their concentrations (0.1, 2.5, 5.0, 7.5, 10.0 µM). Quantification was carried out using a weighted ($1/x^2$) linear regression model.

Results

LC-MS Procedure. The mass fragmentograms in Fig. 1 show that the applied LC-MS conditions provided baseline separation of HO-MPBP, MPBP and the standard MDPPP. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and standard) and zero samples (control microsomes without substrate, but with standard). The method showed good linearity in a range of 0.1-10.0 µM HO-MPBP ($R^2 = 0.996$). Matrix effect studies comparing the peak areas of HO-MPBP in neat standard solutions with those in spiked incubation mixtures containing the same concentrations of HO-MPBP gave no indication of ion suppression.

Initial Screening Studies. Among the nine CYPs tested, only CYP2C19, CYP2D6 and CYP1A2 were markedly capable of catalyzing the hydroxylation of the 4'-methyl moiety of MPBP. In the incubations of the other CYPs only very little (CYP2B6) or no HO-MPBP was detectable.

Kinetic Studies. CYP2D6 (Fig. 2, upper left), CYP1A2 (Fig. 2, lower left), and pHLM (Fig. 2, lower right) showed typical hyperbolic metabolite formation profiles allowing use of equation 1

for estimation of the kinetic constants. The resulting K_m and V_{max} values are listed in Table 1. Visual inspection of the CYP2C19 Michaelis-Menten plot (Fig. 2, upper right) gave evidence of biphasic kinetics. The corresponding Eadie-Hofstee plot clearly confirmed this (data not shown). Hence the CYP2C19 kinetic parameters were estimated by fitting the data into equation 2 for a two-site binding model. The resulting $K_{m,1}$, $K_{m,2}$, $V_{max,1}$, and $V_{max,2}$ data are also reported in Table 1.

Calculation of Relative Activity Factors, Contributions, and Percentages of Net Clearance. The supplier provided turnover rates of the specific probe substrates in the used batches of ICM and pHLM, respectively, were as follows: 5000 pmol/min/mg protein and 48 pmol/min/mg protein for 4'-hydroxymephenytoin formation (CYP2C19), 5165 pmol/min/mg protein and 71 pmol/min/mg protein for 1'-hydroxybufuralol formation (CYP2D6), 6271 pmol/min/mg and 980 pmol/min/mg for paracetamol formation (CYP1A2). The resulting RAFs derived from these data. The RAFs, contribution and intrinsic clearance data, and percentages of net clearance calculated from these and the above-mentioned kinetic data are reported in Table 1.

Chemical Inhibition Studies and Comparative Studies of pHLM with PMD6 HLM and PMC19 HLM. The results of the experiments with the chemical inhibitors quinidine (CYP2D6), fluconazole (CYP2C19), and α-naphthoflavone are presented in Fig. 3. The results of the studies comparing HO-MPBP formation in pHLM with those in PMD6 HLM and PMC19 HLM are presented in Fig. 4.

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Discussion

The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in MPBP hydroxylation. According to the supplier's advice, the chosen incubation conditions are applicable for checking the general involvement of particular CYP enzymes. Because of the very low activity of CYP2B6 with respect to MPBP 4'-methyl hydroxylation, only CYP2C19, CYP2D6 and CYP1A2 were characterized by their kinetic profiles. For this reason, only the kinetic profiles of CYP2C19, CYP2D6 and CYP1A2 as single CYP enzyme sources and that of HLM were further investigated. Duration and protein content of all incubations in these studies were in the linear range of metabolite formation (data not shown). Less than 20% of substrate was metabolized in all incubations with exception of the lowest substrate concentrations. The kinetic data for CYP2D6 and CYP1A2 followed the expected classical hyperbolic Michaelis-Menten plots (Fig. 2). In contrast, CYP2C19 revealed a biphasic kinetic profile. This is in accordance with previous findings of our group (Springer et al., 2003d) where a biphasic kinetic profile of CYP2C19 had also been observed when studying 4'-hydroxylation of the MPBP analogue MPPP. CYP2D6 and CYP2C19 turned out to have the highest affinity towards MPBP with apparent K_m values markedly lower than the K_m of CYP1A2 (Table 1), whereas the capacity of CYP1A2 was considerably higher than those of the other two isoenzymes. However, with respect to expected plasma concentrations of MPBP, CYP1A2 should only play a minor role in MPBP metabolism because of its extremely high K_m value. The RAF approach (Crespi, 1995; Crespi and Penman, 1997; Venkatakrishnan et al., 2000a) is an accepted strategy to correct recombinant CYP formation rates for native human liver enzyme activity. According to the results of the RAF approach, CYP2D6 (54%) and CYP2C19 (30%) accounted for more than 80% of the net clearance. Because both of these CYPs are expressed polymorphically in humans further studies were performed to confirm their role in MPBP

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hydroxylation. One approach for confirming the roles of CYP2D6 and CYP2C19 were the studies comparing HO-MPBP formation in pHLM and liver microsomes from donors with poor metabolizer genotype with respect to CYP2D6 or CYP2C19. The concentrations in these experiments were chosen near the K_m values of the respective isoenyzmes, because the effect of these isoenzymes should be most prominent at these concentrations: At the K_m of CYP2D6 (higher affinity), metabolite formation by this isoenzyme should already be comparatively high, while that by CYP2C19 (lower affinity) should still be comparatively low. At the $K_{m,1}$ of CYP2C19, CYP2D6 should already be saturated and the contribution of CYP2C19 to metabolite formation should become more important. Finally, at the $K_{m,2}$ of CYP2C19 (lowest affinity but highest capacity), this isoenzyme should be mainly responsible for metabolite formation. The metabolite formation in both poor metabolizer microsomes was almost 80% lower than in pHLM, indicating that the *in vivo* hepatic clearance can be expected to be considerably lower in CYP2D6 and CYP2C19 poor metabolizer subjects who account for about 7% and 3% of the Caucasian population, respectively (Bertilsson, 1995; Smith et al., 1998). Hence MPBP users with poor metabolizer genotypes with respect to one or both of these enzymes might be at a higher risk of developing unwanted or even toxic effects. However, it cannot be unequivocally concluded whether these genetic polymorphisms and or the above-mentioned drug interactions are of clinical relevance for MPBP toxicokinetics, because alternative metabolic pathways such as lactam formation (Peters et al., 2005) might compensate for the reduced clearance via 4'-methyl hydroxylation. The second approach for confirming the role of CYP2D6 and CYP2C19 and also of CYP1A2 in MPBP 4'-hydroxylation was inhibition of these isoenzymes in pHLM using the chemical inhibitors quinidine, fluconazole, and α-naphthoflavone, respectively. In contrast to quinidine and α-naphthoflavone, fluconazole is not a specific inhibitor for a single isoenzyme but inhibits CYP2C19 and CYP3A4 (Venkatakrishnan et al., 2000b). Nevertheless, it could be used

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in the described experiments, because CYP3A4 was not capable of catalyzing the studied metabolic reaction. The substrate concentrations examined were 1 µM and 50 µM. The first concentration was chosen, because this was below the apparent K_m for the high affinity enzyme CYP2D6 and plasma levels of MPBP may be expected to be in this range. The second substrate concentration was selected to describe the effect of the inhibitor at an MPBP concentration, at which the role of CYP2C19 and the low affinity enzymes should become increasingly evident. The concentration of the inhibitors were based on average literature data (Newton et al., 1995; Bourrie et al., 1996; Wienkers et al., 1996; Clarke, 1998; Venkatakrishnan et al., 2000b) and were approximately ten times higher than the respective inhibition constants (K_i) of the inhibitors. The results of CYP2D6 inhibition study showed that at the high substrate concentration the overall turnover was inhibited significantly less ($p<0.05$) than in experiments with 1 µM MPBP (83% vs. 59%, Fig. 3A). This notable inhibition was consistent with the observation (although not exactly), that CYP2D6 accounted for about 54% of the net intrinsic clearance of MPBP. Moreover, they indicated that simultaneous intake of potent CYP2D6 inhibitory drugs, such as fluoxetine or paroxetine (Crewe et al., 1992), might lead to a decreased clearance of MPBP and, consequently, to elevated plasma concentrations. The effects of the inhibitors fluconazole and α-naphthoflavone were essentially the same with inhibition of HO-MPBP formation of about 50%. Moreover, they seemed to be independent of the substrate concentration. At the lower concentration, these inhibition effects are much higher than one would have expected considering the results of the percentage of net clearance and the extensive inhibition of HO-MPBP formation by quinidine. This discrepancy between the results of the RAF approach and those of the inhibition studies stands somewhat in contrast to the findings of (Venkatakrishnan et al., 2001) who had reported a generally good agreement between those approaches when studying the *N*-demethylation of amitriptyline. However, these authors had also

observed rather poor correlations for some of the tested isoenzymes, among them CYP1A2 and CYP2C19. One possible explanation for poor agreement of results obtained with the RAF approach and studies using chemical inhibitors would be complex interactions of multiple CYP isoenzymes in pHLM, which can alter the catalytic properties of individual CYP isoenzymes (Cawley et al., 2001; Hazai and Kupfer, 2005). In addition, unspecific binding in pHLM incubations might at least in part be responsible for the discrepancies observed in our study considering that protein concentrations in pHLM (0.5 mg/ml) were two to three times higher than in ICM (0.14-0.27 mg/ml). Such unspecific binding would also be an explanation for the high K_m value observed in pHLM.

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Legends to the Figures

FIG. 1. Typical mass fragmentograms of ions *m/z* 232 (dotted line) and *m/z* 248 (solid line) of a supernatant of an incubation mixture of 40 µM MPBP with pHLM. The HO-MPBP concentration in the incubation mixture was determined to be 2.7 µM.

FIG. 2. Michaelis-Menten plots for MPBP hydroxylation catalyzed by CYP2D6 (upper left), CYP2C19 (upper right), CYP1A2 (lower left), and pHLM (lower right). Data points represent means (solid squares) and ranges (error bars) of duplicate measurements. The solid curves were calculated by nonlinear regression according to equation 1 (one site binding model). The dotted curve in the upper right panel was calculated according to equation 2 (two site binding model).

FIG. 3. Effect (grey bars) of 3 µM quinidine (top), 20 µM fluconazole (middle), and 30 µM α-naphthoflavone on HO-MPBP formation in incubation mixtures containing 1 µM (left) and 50 µM MPBP (right). Controls (open bars) were set to 100%. Each bar represents the mean of six incubations ± standard error of the mean.

FIG. 4. Metabolite formation in PMD6 HLM (grey bars, top) and PMC19 HLM (grey bars, middle and bottom) incubation mixtures containing 1.3 µM (top), 9 µM (middle), or 280 µM MPBP (bottom). Controls with pHLM (open bars) were set to 100%. Each bar represents the mean of six incubations ± standard error of the mean.

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TABLE 1

Kinetic data of MPBP hydroxylation by CYP2C19, CYP2D6, CYP1A2 and HLM. Units are: K_m in µM, V_{max} and contribution in pmol/min/pmol CYP (CYP2D6, CYP2C19 and CYP1A2) or pmol/min/mg protein (pHLM).

	CYP2D6	CYP2C19 (1)	CYP2C19 (2)	CYP1A2	pHLM
K_m (best fit value ± standard error)	2.4±0.6[a]	$K_{m,1}$ 9.2 ± 5.3[b]	$K_{m,2}$ 283 ± 163[b]	1674±369[a]	174 ± 21[a]
V_{max} (best fit value ± standard error)	0.070±0.005[a]	$V_{max,1}$ 0.14±0.04[b]	$V_{max,2}$ 0.27±0.03[b]	0.89±0.08[a]	10.4±0.4[a]
RAF	0.01	0.01	0.01	0.16	
Contribution (V_{max}*RAF)	0.0007	0.0014	0.0027	0.143	
Clearance (contribution/K_m)	0.00029	0.00015	0.0000095	0.000085	
Percentage of net clearance	54	28	2	16	

[a] Kinetic data estimated according to equation 1

[b] Kinetic data estimated according to equation 2

FIG. 1

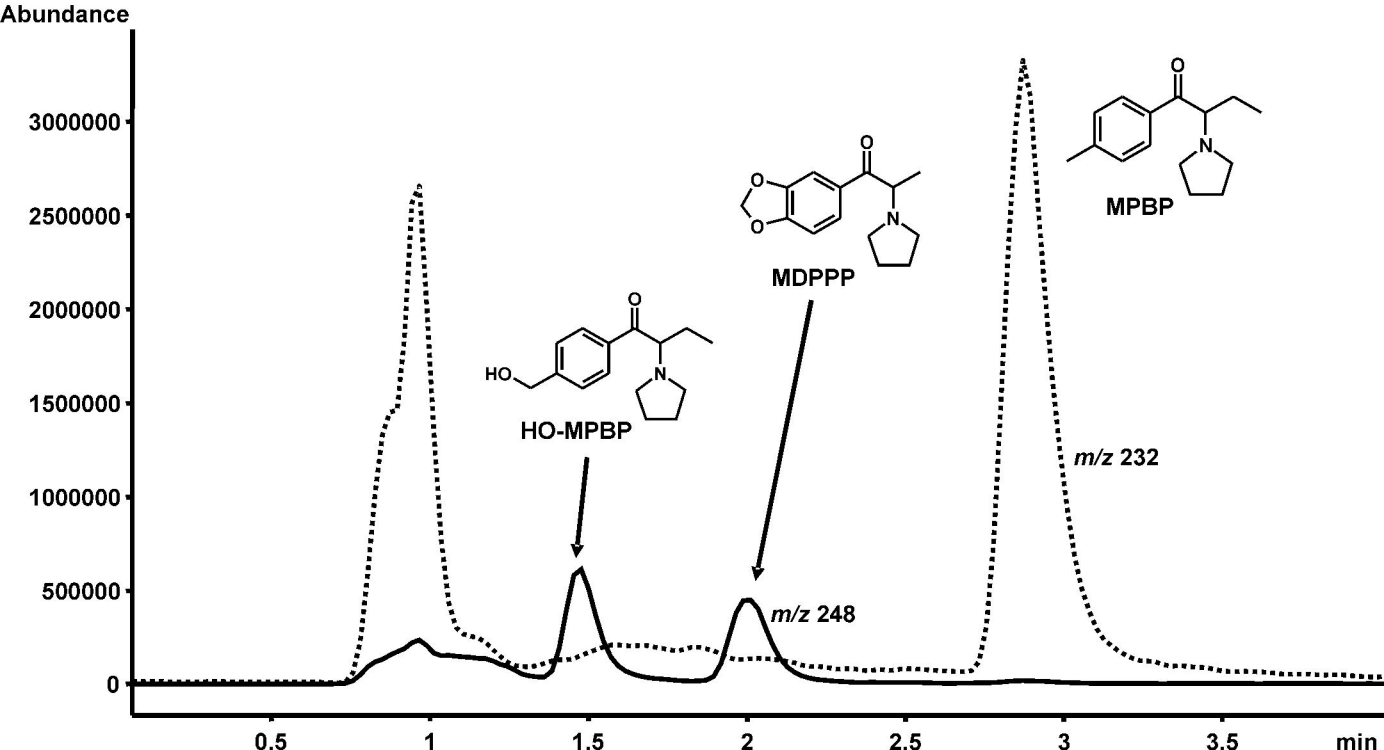
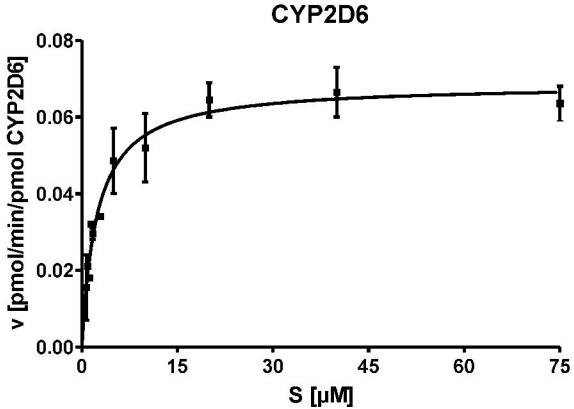
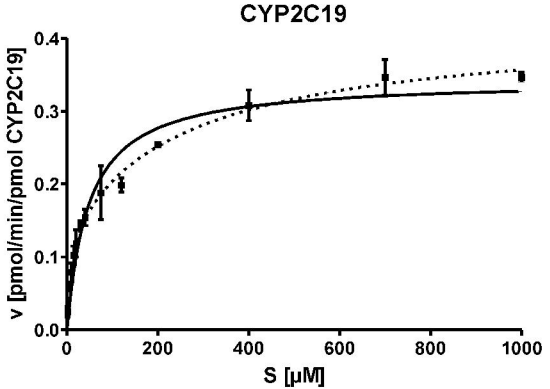
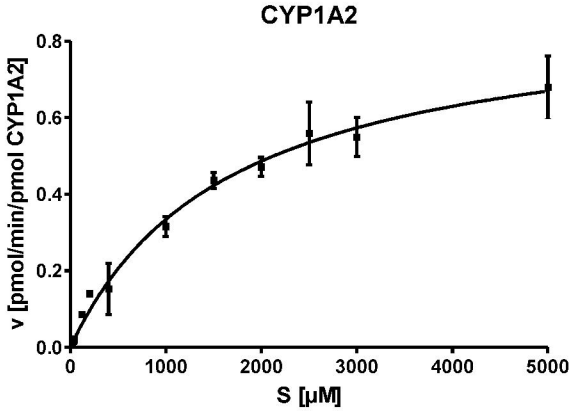

Abundance
3000000
2500000
2000000
1500000
1000000
500000
0
0.5
1
1.5
2
2.5
3
3.5
min
HO-MPBP
MDPPP
MPBP
m/z 232
m/z 248

FIG. 2


CYP2D6
v [pmol/min/pmol CYP2D6]
0.08
0.06
0.04
0.02
0.00
0
15
30
45
60
75
S [µM]


CYP2C19
v [pmol/min/pmol CYP2C19]
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
S [µM]


CYP1A2
v [pmol/min/pmol CYP1A2]
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
S [µM]

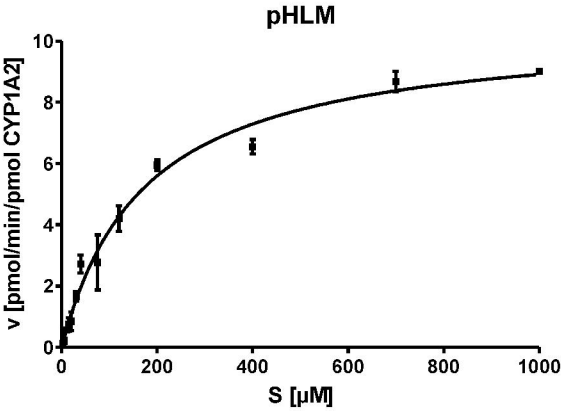

pHLM
v [pmol/min/pmol CYP1A2]
10
8
6
4
2
0
0
200
400
600
800
1000
S [µM]

FIG. 3

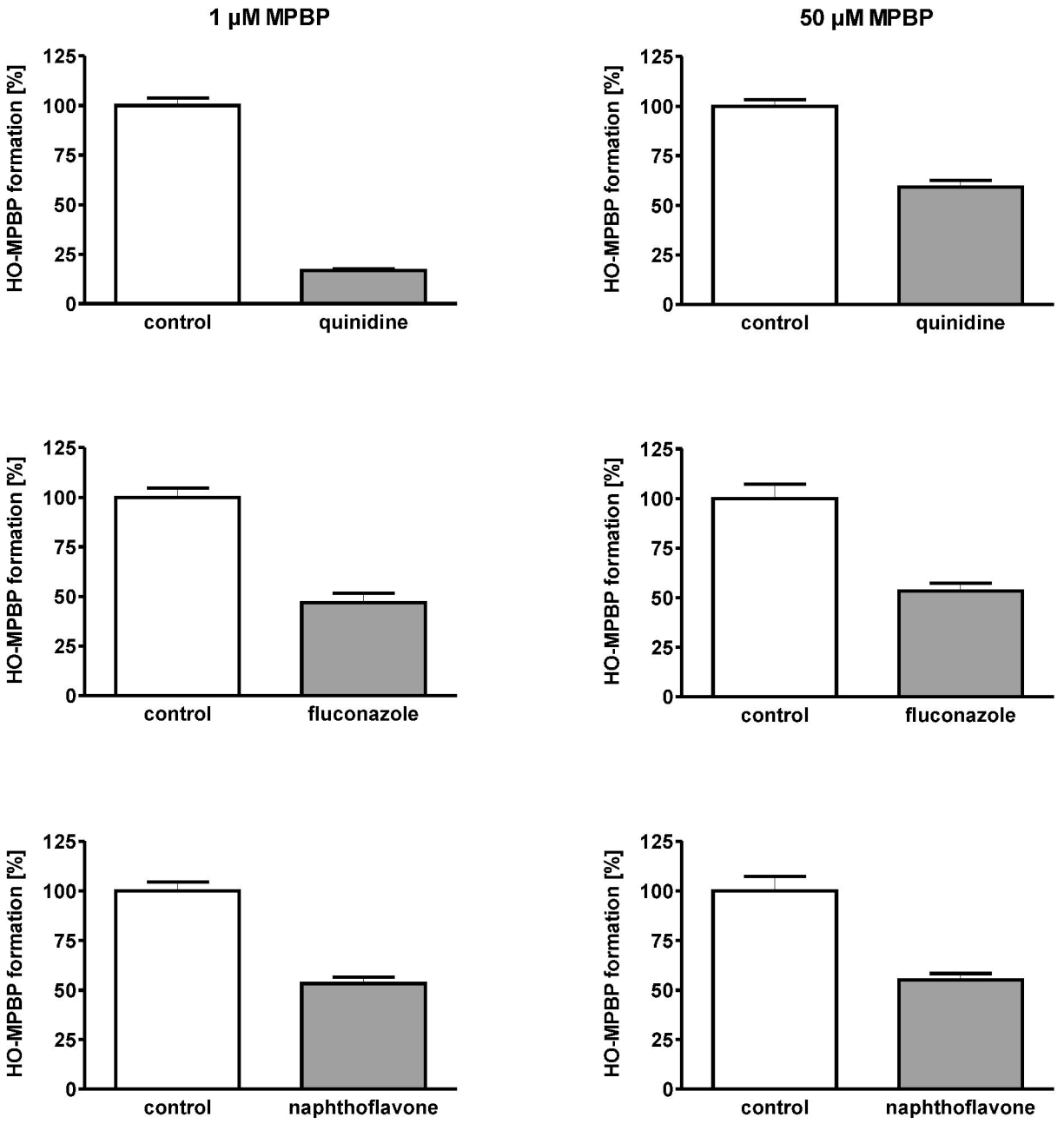
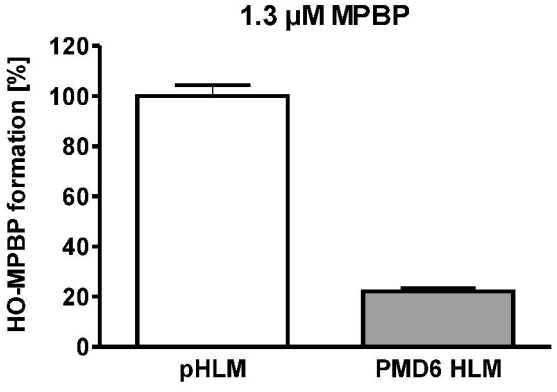
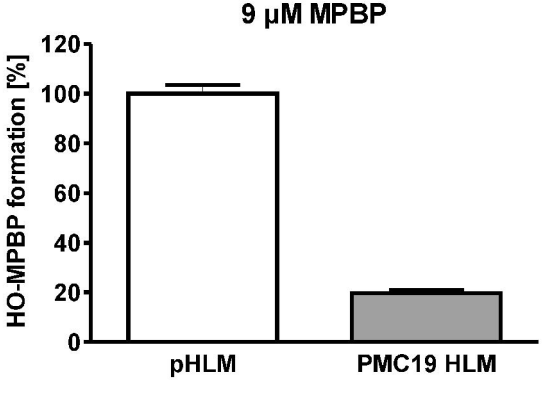
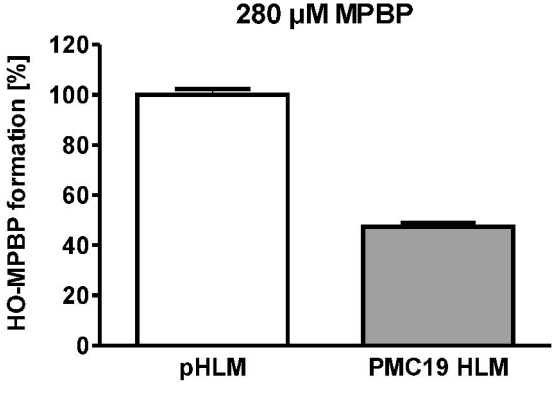

1 µM MPBP
50 µM MPBP
HO-MPBP formation [%]
125
100
75
50
25
0
control
quinidine
fluconazole
naphthoflavone

FIG. 4


1.3 µM MPBP
HO-MPBP formation [%]
120
100
80
60
40
20
0
pHLM
PMD6 HLM


9 µM MPBP
HO-MPBP formation [%]
120
100
80
60
40
20
0
pHLM
PMC19 HLM


280 µM MPBP
HO-MPBP formation [%]
120
100
80
60
40
20
0
pHLM
PMC19 HLM