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Cytochrome P450 dependent metabolism of the new designer drug 1-(3-trifluoromethylphenyl)piperazine (TFMPP) *In vivo* studies in Wistar and Dark Agouti rats as well as *in vitro* studies in human liver microsomes

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Abstract

1-(3-Trifluoromethylphenyl)piperazine (TFMPP) is a designer drug with serotonergic properties. Previous studies with male Wistar rats (WI) had shown, that TFMPP was metabolized mainly by aromatic hydroxylation. In the current study, it was examined whether this reaction may be catalyzed by cytochrome P450 (CYP)2D6 by comparing TFMPP vs. hydroxy TFMPP ratios in urine from female Dark Agouti rats, a model of the human CYP2D6 poor metabolizer phenotype (PM), male Dark Agouti rats, an intermediate model, and WI, a model of the human CYP2D6 extensive metabolizer phenotype. Furthermore, the human hepatic CYPs involved in TFMPP hydroxylation were identified using cDNA-expressed CYPs and human liver microsomes. Finally, TFMPP plasma levels in the above mentioned rats were compared. The urine studies suggested that TFMPP hydroxylation might be catalyzed by CYP2D6 in humans. Studies using human CYPs showed that CYP1A2, CYP2D6 and CYP3A4 catalyzed TFMPP hydroxylation, with CYP2D6 being the most important enzyme accounting for about 81% of the net intrinsic clearance, calculated using the relative activity factor approach. The hydroxylation was significantly inhibited by quinidine (77%) and metabolite formation in poor metabolizer genotype human liver microsomes was significantly lower (63%) compared to pooled human liver microsomes. Analysis of the plasma samples showed that female Dark Agouti rats exhibited significantly higher TFMPP plasma levels compared to those of male Dark Agouti rats and WI. Furthermore, pretreatment of WI with the CYP2D inhibitor quinine resulted in significantly higher TFMPP plasma levels. In conclusion, the presented data give hints for possible differences in pharmacokinetics in human PM and human CYP2D6 extensive metabolizer phenotype subjects relevant for risk assessment.

Keywords: 1-(3-Trifluoromethylphenyl)piperazine; TFMPP; Metabolism; Cytochrome P450; Wistar rats; Dark Agouti rats

1. Introduction

TFMPP is a new designer drug of the group of piperazine-derived compounds, which has appeared on the illicit drug market. It is mentioned as active hallucinogen in scene books [1] and on so-called drug information web sites (http://www.erowid.org, http://www.lycaeum.org). Generally, these piperazine drugs are sold and consumed as an alternative to amphetamine-derived designer drugs [2]. Seizures could be made throughout the world [3–11] and organizations which check the ''purity'' of illegally sold tablets have reported their occurrence more and more often (http://www.dancesafe.org). A fatality involving piperazine derived compounds has been reported [12].

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Abbreviations: TFMPP, 1-(3-trifluoromethylphenyl)piperazine; MDMA, methylenedioxymethamphetamine; MDEA, methylenedioxyethylamphetamine; CYP, cytochrome P450; WI, male Wistar rats; HO-TFMPP, hydroxy TFMPP; mCPP, 1-(3-chlorophenyl)piperazine; fDA, female Dark Agouti rats; PM, human CYP2D6 poor metabolizer phenotype; mDA, male Dark Agouti rats; EM, human CYP2D6 extensive metabolizer phenotype; pHLM, pooled human liver microsomes; PM HLM, poor metabolizer genotype human liver microsomes; BM, body mass; IS, internal standard; GC-MS, gas chromatography-mass spectrometry; EI, electron ionization; SIM, selected-ion monitoring; PAR, peak area ratio; K_m, Michaelis–Menten constant; V_{max}, maximal turnover rate; PA, peak area; RAF, relative activity factor; LC-MS, liquid chromatography-mass spectrometry; APCI, atmospheric pressure chemical ionization.

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In 2002, the increasing use of TFMPP in the United States of America led to the temporary placement of these compounds into Schedule I of the Controlled Substance Act [2].

TFMPP is known as a centrally active compound with serotonergic properties [13–19]. For this reason, it has widely been used as a pharmacological probe drug for drug discrimination procedures in animals [20–23]. On the internet, drug abusers describe its effects to be similar to those of the classical designer drugs methylenedioxymethamphetamine (MDMA, ecstasy) or methylenedioxyethylamphetamine (MDEA), which could partly be supported by animal studies [21,23,24]. The reported anxiogenic effects and panic reactions could also be supported by animal studies [25–27] and human studies on structurally related compounds [28,29]. Further pharmacological effects of TFMPP and other arylpiperazines have been extensively reviewed by Murphy *et al*. [28].

The involvement of particular CYPs in the biotransformation of a new drug has to be thoroughly investigated before it can be marketed. Such investigations allow to predict possible drug–drug interactions, inter-individual variations in pharmacokinetic profiles and increased appearance of side effects and serious poisonings [30]. However, such risk assessment is typically performed for substances intended for therapeutic use, but not for drugs of the illicit market. In addition, there is good evidence that genetic variations in drug metabolism have important behavioral consequences that can alter the risk of drug abuse and dependence [31].

Previous *in vivo* studies in WI showed that TFMPP was mainly metabolized by aromatic hydroxylation to hydroxy TFMPP (HO-TFMPP) followed by partial glucuronidation or sulfatation [32]. Aromatic hydroxylation of the structurally related 1-(3-chlorophenyl)piperazine (mCPP) was described to be catalyzed by the polymorphically expressed CYP2D6 [33,34]. Therefore, the first aim of the work presented here was to study, whether TFMPP hydroxylation may be catalyzed by this CYP. For this purpose a rat model was chosen. Female Dark Agouti rats (fDA) have been proposed as a model of the PM allowing a preliminary screening for CYP2D6 substrates [35,36] and WI as the corresponding model of the human CYP2D6 extensive metabolizer phenotype (EM) [36,37]. In addition, male Dark Agouti rats (mDA) were used as an intermediate model between the PM and EM models [38]. The second aim was to identify the human hepatic CYPs catalyzing TFMPP hydroxylation, to determine the kinetic constants for this reaction, to draw conclusions concerning the intrinsic clearances, and to compare the metabolite formation in pooled human liver microsomes (pHLM) and in single donor human liver microsomes with PM genotype (PM HLM). Finally, plasma levels in the above mentioned rats were compared in order to get hints for possible differences in pharmacokinetics in human PM and EM subjects relevant for risk assessment.

2. Materials and methods

2.1. Materials

TFMPP-HCl and mCPP-HCl were obtained from Lancaster Synthesis, quinine and quinidine were obtained from Promochem, $NADP^+$ was obtained from Biomol, isocitrate and isocitrate dehydrogenase from Sigma, all other chemicals and reagents from Merck. The following microsomes were from Gentest and delivered by NatuTec: baculovirus-infected insect cell microsomes containing 1 nmol/mL human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 (Supersomes), wild-type baculovirus-infected insect cell microsomes (control Supersomes), pHLM (20 mg microsomal protein/mL, 400 pmol total CYP/mg protein) and PM HLM (20 mg microsomal protein/mL). After delivery, the microsomes were thawed at 37°, aliquoted, snap-frozen in liquid nitrogen and stored at −80° until use.

2.2. Animals, treatments and collection of urine and blood samples

The investigations were performed using urine or blood of male WI (Charles River), of mDA or of fDA (Harlan-Winckelman) for toxicological diagnostic reasons according to the corresponding German law. The animals were housed in groups under controlled humidity and temperature (23 ± 1°) and a 12-hr light:12-hr dark cycle (lights on 7.00 a.m. to 7.00 p.m.) with food and water *ad libitum*. A single dose of TFMPP (20 mg/kg body mass, BM) in aqueous solution was administered to rats by gastric intubation (N = 8, for urine samples; N = 4–6 for blood samples). Furthermore, WI were pretreated with quinine (80 mg/kg BM) [39] before administration of TFMPP (20 mg/kg BM). The rats were housed in metabolism cages for 24 hr, having water *ad libitum*. Urine was collected separately from the faeces over a 24 hr period. Blood samples were taken from the tail vein 1, 3, 5, 7, and 9 hr after administration. All samples were directly analyzed as described below.

2.3. Sample preparation of rat urine and rat plasma samples

The rat urine and blood plasma samples were prepared according to a validated procedure with modifications [40]. A 0.5-mL portion of urine was adjusted to pH 5.2 with acetic acid (1 mol/L) and incubated at 37° for 12 hr with 100 μL of a mixture (100,000 Fishman units/mL) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1) before extraction. The enzymatically hydrolyzed rat urine samples (dosage 20 mg/kg BM) were then diluted with 2 mL of purified water. Plasma samples (0.1 mL) were also diluted with 2 mL of purified water after addition

of 0.05 mL of a methanolic solution of mCPP with a concentration of 1 μg/mL as internal standard (IS). The diluted samples were shortly mixed (15 s) on a rotary shaker, centrifuged for 3 min at 1000 *g* and loaded on mixed-mode HCX SPE cartridges previously conditioned with 1 mL of methanol and 1 mL of purified water. After extraction, the cartridges were washed with 1 mL of purified water, 1 mL of 0.01 M aqueous hydrochloric acid, and 2 mL of methanol. Reduced pressure was applied until the cartridges were dry, and the analytes were eluted with 1 mL of methanol-aqueous ammonia (98:2 v/v) into 1.5 mL polypropylene reaction vials. The eluates were evaporated to dryness under a stream of nitrogen at 56°. After addition of 20 μL of heptafluorobutyric anhydride, the reaction vials were sealed and left on a rotary shaker (15 s). Derivatization was carried out under microwave irradiation (440 W, 5 min). After cooling, the derivatized extracts were shortly mixed (15 s) with 0.1 mL of *n*-hexane, and the mixtures were centrifuged for 15 s at 10,000 *g*. Then, 0.2 mL of an aqueous 0.5 M Na_3PO_4 solution was added and the vials were shaken on a rotary shaker for 3 min and centrifuged for 2 min at 10,000 *g*. The organic layers were transferred to autosampler vials and 2 μL were injected into the gas chromatograph.

2.4. Gas-chromatography-mass spectrometry (GC-MS) analysis of rat urine and plasma samples

2.4.1. Apparatus

The samples were analyzed using a Hewlett-Packard (Agilent) HP 6890 Series GC system combined with an HP 5972 Series mass selective detector, an HP 6890 Series injector and an HP Chem Station G1701AA version A.03.00.

2.4.2. GC-MS conditions

GC conditions were as follows: splitless injection mode; column, HP-5MS capillary (30 m × 0.25 mm i.d.), 5% phenyl methyl siloxane, 250 nm film thickness; injection port temperature, 280°; carrier gas, helium; flow rate, 0.6 mL/min; column temperature, 100° increased to 250° at 10°/min, to 310° at 30°/min, and was held at this temperature for 1 min. MS conditions were as follows: transfer line heater, 280°; source temperature, 140°; electron ionization (EI) mode; ionization energy, 70 eV; selected-ion monitoring (SIM) with the following program for analysis of rat urine extracts: solvent delay, 4 min; window A, 9.50–11.00 min, *m/z* 200, 229, 426 for heptafluorobutyrylated TFMPP, time window B 12.00–13.50 min, *m/z* 412, 441, 638 for bis-heptafluorobutyrylated HO-TFMPP and for analysis of rat plasma extracts: solvent delay, 4 min; time window A, 9.50–11.00 min, *m/z* 200, 229, 426 for heptafluorobutyrylated TFMPP, time window B 11.50–13.50 min, *m/z* 195, 392, 394 for heptafluorobutyrylated mCPP. In rat urine samples, the peak area ratios (PAR) between TFMPP and its metabolite HO-TFMPP were determined. In rat plasma samples the PAR between TFMPP and mCPP (IS) were determined.

2.5. Microsomal incubations

Typical 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 $NADP^+$, 2 U/mL isocitrate dehydrogenase, 200 U/mL superoxide dismutase and substrate at 37°. The substrate was added after dilution of a 250 mM methanolic stock solution in buffer. The methanol concentration did not exceed 0.4%. Reactions were started by addition of the ice-cold microsomes and terminated with 5 μL of 60% (w/w) perchloric acid. After addition of 1 μL of 0.5 mM mCPP in methanol as internal standard, the samples were centrifuged and the supernatants were transferred to autosampler vials.

2.6. Initial screening studies

In order to investigate the involvement of particular CYPs in TFMPP metabolism, 200 μM TFMPP and 50 pmol/mL CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 were incubated for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 or 90 mM Tris-buffer, according to the Gentest manuals.

2.7. Enzyme kinetic studies

Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations (N = 2 each) with the following TFMPP concentration ranges, incubation times and protein concentrations: 1–1500 μM TFMPP with 50 pmol CYP1A2/mL for 20 min; 0.5–600 μM TFMPP with 50 pmol CYP2D6/mL for 20 min; 10–1300 μM TFMPP with 80 pmol CYP3A4/mL for 20 min and 1–1500 μM TFMPP with 0.5 mg pHLM protein/mL for 20 min. Less than 20% of substrate were metabolized in all incubations. Apparent K_m and V_{max} values for single isoenzymes were estimated by nonlinear regression according to the Michaelis–Menten equation:

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

Eq. (2) for a two-site binding model was applied to the data of the pHLM experiments [41].

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

Unfortunately, no reference substances of the metabolites were available. Therefore, only relative estimations of V_{max} values, expressed as dimensionless peak areas (PA)/min and mg pHLM protein or pmol CYP for cDNA-expressed CYPs, could be obtained.

2.8. Calculation of relative activity factors and of intrinsic clearance

Taking into account differences in functional levels of redox partners between the two enzyme sources, the relative activity factor (RAF) approach was used [42–47].

Reaction activities of probe substrates (phenacetin deethylation for CYP1A2, bufuralol 1′-hydroxylation for CYP2D6, and testosterone 6β-hydroxylation for CYP3A4) in both, insect cell microsomes and pHLM were taken from the supplier's data sheets. The RAFs were calculated according to Eq. (3):

$$\mathrm{RAF}_{\mathrm{isoform}} = \frac{\text{turnover rate for probe substrate in HLM [pmol/min/mg protein]}}{\text{turnover rate or probe substrate in cDNA-expressed CYP [pmol/min/mg protein]}} \quad (3)$$

V_{max} values of TFMPP hydroxylation obtained from incubations with cDNA-expressed CYPs were then multiplied with the corresponding RAF leading to a value, which is defined as "contribution":

$$\mathrm{Contribution}_{\mathrm{CYP\,enzyme}} = (V_{max} \text{ for the test reaction with cDNA-expressed CYP}) \times \mathrm{RAF}_{\mathrm{CYP\,enzyme}} \quad (4)$$

The percentage of intrinsic clearance by a particular enzyme was derived from calculations according to Eq. (5), where intrinsic clearance equals contribution/apparent K_m:

$$\text{Percentage of clearance by isoform (\%)} = \frac{\mathrm{clearance}_{\mathrm{isoform}}}{\sum \mathrm{clearances}_{\mathrm{isoform}}} \times 100 \quad (5)$$

2.9. Chemical inhibition studies

The effect of 3 μM quinidine on HO-TFMPP formation was assessed in incubations containing 0.5 mg pHLM protein/mL and 10 μM TFMPP. Controls contained no quinidine, but the same amount of methanol to control for any solvent effects (N = 6, each). Significance of inhibition was tested by a one-tailed unpaired *t*-test.

2.10. Comparative studies between pHLM and PM HLM

Incubations were carried out at 10 μM TFMPP for 20 min using either 0.5 mg pHLM or PM HLM protein/mL. Significance of differences in metabolite formation was tested by a one-tailed unpaired *t*-test.

2.11. Statistical analysis

All statistics were calculated using GraphPad Prism 3.02 software designed for nonlinear regression analysis. The Michaelis–Menten parameters K_m and V_{max} were calculated by fitting kinetic data to a one- or two-site binding model. The data sets of the animal experiments were tested for outliers using the Nalimov test (outlier criterion $P = 99\%$). Significance of inhibition and differences in metabolite formation was determined using one-tailed unpaired *t*-test ($P < 0.05$).

2.12. Liquid chromatography-mass spectrometry (LC-MS) conditions and quantification in microsomal incubations

TFMPP, HO-TFMPP and mCPP were separated and quantified using an Agilent Technologies AT 1100 series atmospheric pressure chemical ionization (APCI) LC-MSD, SL version and an LC-MSD ChemStation using the A.08.03 software.

2.12.1. LC conditions

Gradient elution was achieved on a Merck LiChroCART column (125 mm × 2 mm i.d.) with Superspher60 RP Select B as stationary phase and a LiChroCART10-2 Superspher60 RP Select B guard column. The mobile phase consisted of ammonium formate (5 mM, adjusted to pH 3.0 with formic acid; eluent A) and acetonitrile (eluent B) according to Maurer *et al*. [48]. The gradient and the flow rate were programmed as follows: 0–3 min 15% B (flow: 0.4 mL/min), 3–5 min 40% B (flow: 0.4 mL/min), 5–8 min 90% B (flow: 0.6 mL/min), 8–8.75 min 90% B (flow: 0.9 mL/min), 8.75–10 min 15% B (flow: 0.4 mL/min). The injection volume was 2 μL.

2.12.2. APCI conditions

The following APCI inlet conditions were applied: drying gas (7000 mL/min, 300°) and nebulizer pressure (25 psi), both nitrogen; capillary voltage, 4000 V; drying gas temperature set at 300°, vaporizer temperature set at 400°; corona current was 5.0 μA; positive SIM mode; fragmentor voltage 100 V.

2.12.3. MS conditions

For quantification, the following target ions (*m*/*z*) were used in the SIM mode: 247 for HO-TFMPP; 197 for mCPP and 231 for TFMPP.

3. Results

3.1. Analysis of rat urine samples

In rat urine samples, the PAR of TFMPP vs. HO-TFMPP was determined. Figure 1 shows that the lowest ratio was determined in urine of WI and the highest in urine of fDA. The ratio determined in urine of mDA lay between these two groups. The means of metabolic ratios of the three rat groups were significantly different among each other (comparison in pairs, $P < 0.0001$).

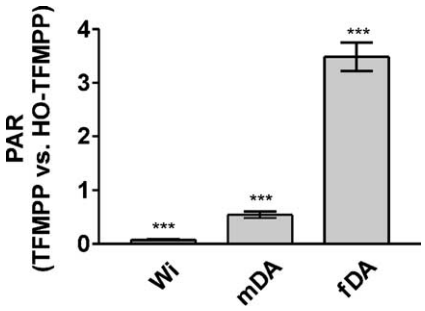

Fig. 1. Peak area ratios (PAR) of TFMPP vs. HO-TFMPP determined in urine of WI (left), mDA (middle) and fDA (right) after solid phase extraction and heptafluorobutyrylation. Each bar represents the mean of the results of eight analyses ±SEM ($^{***}P < 0.0001$).

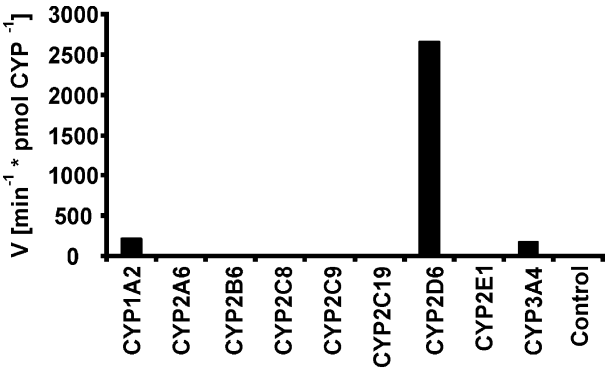

Fig. 3. Formation rates (V) of TFMPP hydroxylation (50 μM TFMPP) with 200 pmol/mL of the given individual CYPs (V given as dimensionless PA/min and pmol CYP) and with insect cell control microsomes.

3.2. LC-MS procedures

The mass fragmentograms in Fig. 2 show that the applied LC-MS conditions provided baseline separation of HO-TFMPP, mCPP and TFMPP. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and IS) and zero samples (control microsomes without substrate, but with IS; data not shown).

3.3. Initial screening studies

Figure 3 illustrates that among the nine CYPs tested only CYP1A2, CYP2D6 and CYP3A4 catalyzed the hydroxylation of TFMPP. HO-TFMPP was not detectable in incubations with the remaining cDNA-expressed CYPs or with insect cell control microsomes.

3.4. Kinetic studies

All incubations were carried out at initial rate conditions, a prerequisite for Michaelis–Menten kinetics. All of the kinetics of the investigated reactions with single cDNA-expressed CYPs showed a typical hyperbolic profile, as shown in Fig. 4. The kinetic parameters (apparent K_m, V_{max}, RAF, contribution and intrinsic clearance) for these reactions are listed in Table 1. They were estimated using Michaelis–Menten Eq. (1) for incubations with cDNA-expressed CYPs. Eq. (2) was used for incubations with pHLM.

In general, V_{max} values could only be expressed as arbitrary units, because the metabolites could not be quantified without reference substance. The V_{max} values in Table 1 are expressed as dimensionless PA/min and pmol CYP for cDNA-expressed CYPs or as PA/min and mg microsomal protein for pHLM.

3.5. Chemical inhibition studies

In order to underline the importance of CYP2D6 in TFMPP hydroxylation, the CYP2D6 specific inhibitor quinidine (3 μM) was added to incubation mixtures and the metabolite formation rate compared with incubations without inhibitor. Figure 5 shows that in presence of

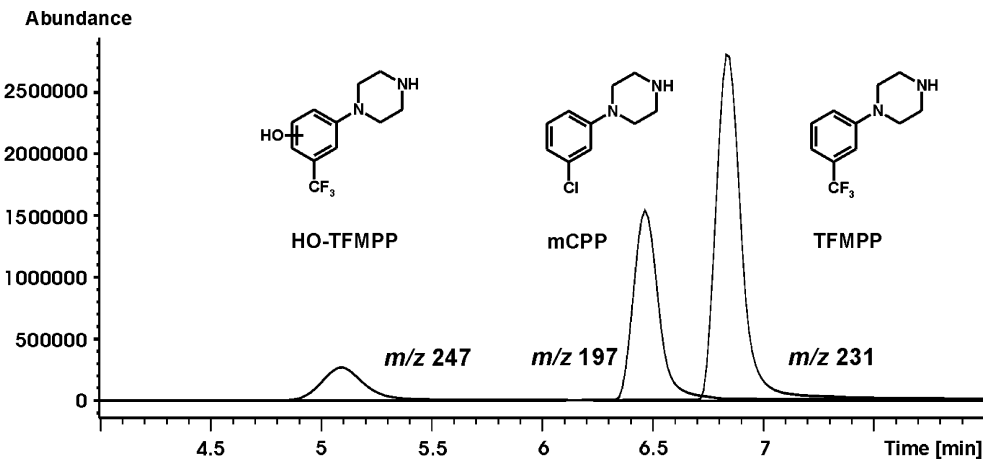

Fig. 2. Typical mass fragmentograms of a supernatant of an incubation mixture of 10 μM TFMPP with cDNA-expressed CYP2D6 with the following ions: m/z 247, 197 or 231 for HO-TFMPP, mCPP (IS) or TFMPP, respectively.

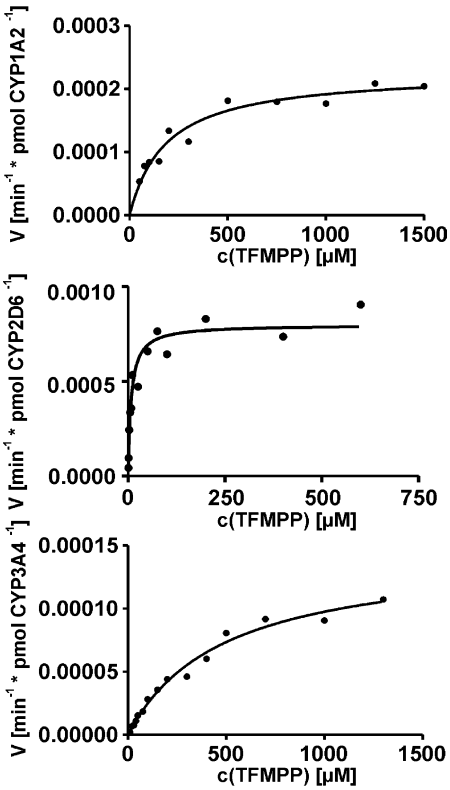

Fig. 4. Michaelis–Menten plots for TFMPP hydroxylation catalyzed by CYP1A2 (upper part), CYP2D6 (middle part), and CYP3A4 (lower part). Values represent the mean of duplicate incubations. V given as dimensionless PA/min and pmol CYP. Curves were calculated by nonlinear regression according to Eq. (1) (one-site binding model).

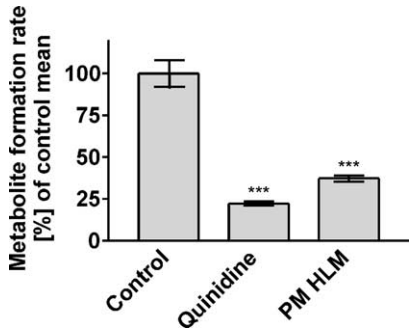

Fig. 5. Metabolite formation in PM HLM (right bar) and effect of 3 µM quinidine on metabolite formation in pHLM (middle bar) in incubation mixtures containing 10 µM TFMPP. Controls with pHLM without inhibitor (left bar) were set to 100%. Each bar represents the mean of six incubations ±SEM ($^{***}P < 0.0001$).

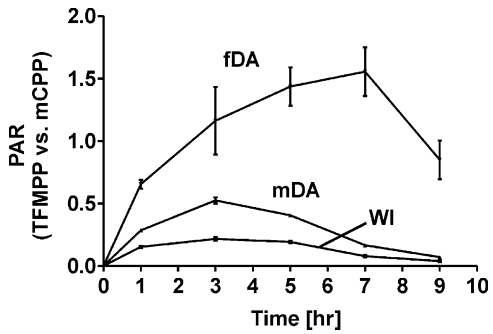

Fig. 6. Peak area ratios (PAR) of TFMPP vs. mCPP (IS) determined in blood plasma, after solid phase extraction and heptafluorobutyrylation, of fDA (upper curve), mDA (middle curve) and WI (lower curve) taken 1, 3, 5, 7, and 9 hr after administration. Each point represents the mean of four samples ±SEM (P-values between 0.0164 and <0.0001).

inhibitor and 10 µM TFMPP the metabolite formation was inhibited significantly by about 78% ($P < 0.0001$).

3.6. Comparative studies between pHLM and PM HLM

In order to further underline the importance of CYP2D6 in TFMPP hydroxylation and to demonstrate differences in PM and EM subjects, the metabolite formation rate of pHLM was compared to that of PM HLM. The metabolite formation rate of PM HLM was about 63% lower ($P < 0.0001$) than that of pHLM (Fig. 5).

3.7. Analysis of rat plasma samples

In rat plasma samples, the PAR of TFMPP vs. mCPP were determined. As shown in Fig. 6, the blood plasma levels in fDA were significantly higher than those of WI ($P \leq 0.0013$) and of mDA ($P \leq 0.0164$). The blood plasma levels in mDA always lay in between fDA and WI and were always significantly higher than those of WI ($P \leq 0.004$). Pretreatment of WI with quinine led to significantly higher blood plasma levels at all time points ($P \leq 0.01$) compared to untreated WI (Fig. 7).

Table 1
Kinetic data of TFMPP hydroxylation by CYP1A2, CYP2D6, CYP3A4 and pHLM

	CYP1A2	CYP2D6	CYP3A4	pHLM[a]
Apparent K_m (best fit value ± standard error)	186.2 ± 32.3	7.8 ± 1.8	487.7 ± 98.9	$K_{m,1}$: 11.2 ± 11.6[a]
V_{max} (best fit value ± standard error)	2.3 ± 0.1	8.0 ± 0.4	1.5 ± 0.1	$V_{max,1}$: 60.7 ± 20.9
RAF	0.11	0.01	0.30	
Contribution	0.25	0.01	0.44	
Intrinsic clearance	0.0014	0.0096	0.0009	
Percentage of net clearance	11.5	80.9	7.6	

Units are: apparent K_m in µM, V_{max} and contribution in dimensionless PA/min and pmol CYP (cDNA-expressed CYPs) or PA/min and mg protein (pHLM), inhibition of enzymes with specific chemical inhibitor in pHLM in %.

[a]Kinetic data estimated according to Eq. (2).

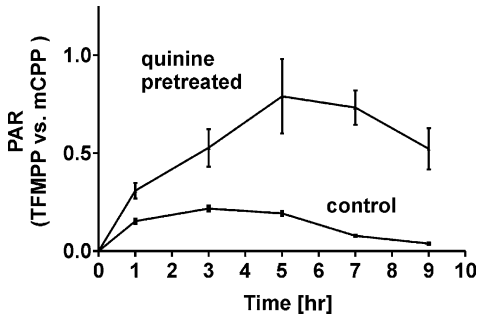

Fig. 7. Peak area ratios (PAR) of TFMPP and mCPP (IS) determined in blood plasma, after solid phase extraction and heptafluorobutyrylation, of WI after pretreatment with 80 mg/kg BM of quinine (upper curve) and without pretreatment (control, lower curve) taken 1, 3, 5, 7, and 9 hr after administration. Each point represents the mean of four samples ±SEM (*P*-values between 0.01 and <0.0001).

4. Discussion

Metabolism studies of new designer drugs are commonly conducted using rat models, due to ethical reservation about human studies [32,49–57]. In the current study, it was examined whether TFMPP hydroxylation may be catalyzed by CYP2D6 by comparing PAR of TFMPP vs. HO-TFMPP in urine from fDA, mDA and WI. These animals were proposed as a model allowing a preliminary screening for CYP2D6 substrates [35,36], with fDA as a PM model, WI as an EM model [36,37] and mDA as an intermediate model between the PM and EM models [38]. The results showed that WI excreted TFMPP mainly as the corresponding hydroxy metabolite. Compared to that, mDA rats excreted TFMPP significantly less metabolized, with HO-TFMPP still being the main analyte. In contrast to these two findings, fDA mainly excreted the unmetabolized parent compound TFMPP. These results suggested that TFMPP hydroxylation should be catalyzed by CYP2D6 in humans.

For confirmation, the human hepatic CYPs involved in TFMPP hydroxylation were identified using microsomal preparations from different sources, cDNA-expressed CYP enzymes, pHLM and PM HLM. This method has already been described for other new designer drugs [46,47]. The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in TFMPP hydroxylation. According to the supplier's advice, the incubation conditions chosen were adequate to make a statement on a general involvement of a particular CYP. The data revealed that CYP1A2, CYP2D6 and CYP3A4 were capable of catalyzing the monitored reaction. For this reason, the kinetic profiles of the reactions by these particular CYPs and pHLM were further investigated. Kinetic assays with these single CYPs and pHLM were performed under initial rate conditions, a prerequisite for Michaelis–Menten kinetics [58].

As no reference substance of the monitored metabolite was available for its exact quantification, only PAs could be determined instead of absolute metabolite concentrations. However, this neither affected the conclusions drawn from the kinetic estimations, nor those from the inhibition studies. Linearity of the mass spectrometer response over the concentration range could be shown for TFMPP and for hydroxy phenylpiperazine, which is structurally closely related to the monitored metabolite (data not shown), so that linearity of the mass spectrometer response of HO-TFMPP could be assumed.

As expected, classical hyperbolic Michaelis–Menten plots (Fig. 4) were found using cDNA-expressed CYPs. The apparent K_m and V_{max} values of the investigated CYPs were calculated by nonlinear regression fit according to Eq. (1). As more than one enzyme was involved in TFMPP hydroxylation, Eq. (2) was used to fit into the data points of pHLM experiments.

The RAF approach [42–47] and the use of immunologically determined CYP liver content [59,60] have been the two main strategies to correct recombinant CYP formation rates for native human liver enzyme activity. In contrast to immunoquantified CYP levels, a RAF does not only depend on the liver abundance of the CYP, but also reflects the specific activity of the cDNA-expressed CYP preparation used. Stormer *et al*. showed that applying RAFs, provided by the manufacturer, yielded more reliable results than using the immuno-quantification approach [61]. As summarized in Table 1, CYP2D6 accounted for 81% of predicted total TFMPP hydroxylation clearance by all individual CYPs in pHLMs. CYP1A2 and CYP3A4 were responsible for about 10% each.

In order to confirm the role of CYP2D6 in TFMPP hydroxylation, inhibition studies with the CYP2D6 specific chemical inhibitor quinidine [37] were performed. The concentration of the inhibitor (3 μM) was based on average literature data [33,34,46,47,58,62,63]. The substrate concentration examined was 10 μM, which corresponds to the calculated $K_{m,1}$ value in pHLM. The results showed that at this substrate concentration the overall turnover was significantly inhibited by about 77%. This notable inhibition was consistent with the observation, that CYP2D6 accounted for about 81% of the net intrinsic clearance of TFMPP. Usually, the accuracy of predictions of CYP contribution to a particular reaction can be readily assessed by comparison with chemical inhibition data in pHLM. At a given substrate concentration, a CYP specific inhibitor should reduce the formation rate of the metabolite in pHLM by approximately the same fraction that the particular CYP is estimated to account for. RAF-corrected kinetic studies, as well as inhibition experiments demonstrated that CYP2D6 contributed predominantly to the clearance of TFMPP. In order to study whether this finding resulted in significant differences between CYP2D6 PM and EM, comparative studies using pHLM and PM HLM were conducted. The metabolite formation rate of PM HLM was about 63% lower than that of pHLM (Fig. 5).

Our findings on the CYPs involved in TFMPP hydroxylation are in accordance with the findings on the metabolism

of mCPP reported by Rotzinger *et al*. [34] and von Moltke *et al*. [33]. Hydroxylation of this structurally closely related compound was reported to be catalyzed only by CYP2D6. However, von Moltke *et al*. reported that the monitored reaction was consistent with a two enzyme Michaelis–Menten process, which indicates that more than one enzyme was involved. Our studies on TFMPP showed that, additionally to CYP2D6, CYP1A2 and CYP3A4 were able to catalyze the reaction. The fact that we used much more sensitive LC-MS detection compared to the ultraviolet detection used by the above mentioned authors may be an explanation for these differences.

As the described *in vivo* and *in vitro* studies showed that TFMPP hydroxylation was mainly catalyzed by polymorphically expressed CYP2D6, it was of particular interest to know whether the TFMPP plasma levels vary in PM and EM possibly resulting in different toxicological risks. Therefore, differences in plasma levels in the different rat models were examined, because corresponding human studies were not possible for ethical reasons. The deficiency of fDA of hydroxylating TFMPP should led to increased blood plasma levels of the parent compound. In fact, fDA showed the highest and WI showed the lowest TFMPP blood plasma levels at all sample times. As expected, the plasma levels in mDA lay between these two groups. For further confirmation, that these differences could be attributed to the differences in CYP2D activity, TFMPP hydroxylation was altered in WI using the CYP2D specific inhibitor quinine [37,64]. Actually, pretreatment with quinine resulted in significantly higher TFMPP plasma levels.

In summary, the studies showed that TFMPP hydroxylation is mainly catalyzed by CYP2D6. The animal studies indicated that CYP2D6 PM, who account for about 7% of the Caucasian population [65,66], might exhibit a lower clearance than EM. Furthermore, simultaneous intake of potent CYP2D6 inhibitory drugs, such as fluoxetine or paroxetine [67], might also lead to a decreased clearance of TFMPP and, consequently, lead to elevated plasma concentrations. Whether this genetic polymorphism and/or drug interactions are of relevance for TFMPP pharmacokinetics and/or clinical outcome of intoxications, cannot be assessed at the moment due to lack of sufficient authentic human data.

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