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Investigations on the cytochrome P450 (CYP) isoenzymes involved in the metabolism of the designer drugs N-(1-phenyl cyclohexyl)-2-ethoxyethanamine and N-(1-phenylcyclohexyl)-2-methoxyethanamine

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ABSTRACT

Investigations using insect cell microsomes with cDNA-expressed human cytochrome P450 (CYP)s and human liver microsomes (HLM) are reported on the CYP isoenzymes involved in the metabolism of the designer drugs *N*-(1-phenylcyclohexyl)-2-ethoxyethanamine (PCEEA) to *O*-deethyl PCEEA and *N*-(1-phenylcyclohexyl)-2-methoxyethanamine (PCMEA) to *O*-demethyl PCMEA. Gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry was used for the analysis of the incubation samples. PCEEA *O*-deethylation was catalyzed by CYP2B6, CYP2C9, CYP2C19, and CYP3A4, while PCMEA *O*-demethylation was catalyzed only by CYP2B6 and CYP2C19. Considering the relative activity factor approach, these enzymes accounted for 53%, 25%, 4%, and 18% of net clearance for PCEEA and 91% and 9% of net clearance for PCMEA, respectively. The chemical CYP2B6 inhibitor 4-(4-chlorobenzyl)pyridine (CBP) reduced the metabolite formation in pooled HLM by 63% at 1 μM PCEEA. At 10 μM PCEEA, CBP reduced metabolite formation by 61%, while inhibition of CYP3A4 by ketoconazole and inhibition of CYP2C9 by sulfaphenazole showed no inhibitory effect. At 1 μM PCMEA, CBP reduced metabolite formation in pooled HLM by 70% and at 10 μM PCMEA by 78%, respectively. In conclusion, the main metabolic step of both studied drugs was catalyzed by different CYPs.

1. Introduction

Enzymes of the cytochrome P450 (CYP) family catalyze more than 90% of oxidative metabolic reactions of xenobiotics [1]. The involvement of particular CYP enzymes in the biotransformation of a new drug is usually thoroughly investigated before it can be marketed. Such studies are important to assess the risk of increased side effects in poor metabolizer subjects [2] and of drug–drug or drug–food interactions [3]. However, for drugs of the illicit market, such data are typically not acquired.

A considerable number of new synthetic drugs of abuse from various drug classes were seized in the German Federal State of Hesse and surrounding federal states during the late 1990s. One of these substances was *N*-(1-phenylcyclohexyl)-propanamine (PCPR), a phencyclidine (PCP)-derived compound. After a short time, further members of this new class of PCP-derived designer drugs appeared on the illicit drug

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market, namely N-(1-phenylcyclohexyl)-3-methoxy-propanamine (PCMPA), N-(1-phenylcyclohexyl)-2-methoxyethanamine (PCMEA) and N-(1-phenylcyclohexyl)-2-ethoxyethanamine (PCEEA). The seized preparations contained either one compound alone or in mixture with other designer drugs [4]. In expectance of its appearance on the illicit drug market, a further homologue, namely N-(1-phenylcyclohexyl)-3-ethoxypropanamine (PCEPA), was synthesized as reference substance for scientific purposes.

Unfortunately, only little information on the pharmacological properties of these compounds is available [5]. Due to structural similarities, they might be assumed to be similar to those of PCP or ketamine, which both act as antagonists at N-methyl-D-aspartate (NMDA) receptors and have psychotomimetic as well as anesthetic properties [6]. Furthermore, it has been reported that (1-phenylcyclohexyl)-amine, a known metabolite of PCP and of the above-mentioned PCP-derived compounds [7–10], produced a long-lasting dose-dependent effect on the efflux of dopamine in the rat [11]. A similar pharmacological profile of the above-mentioned PCP-derived compounds would clearly be in line with their abuse as designer drugs.

Studies on the metabolism and toxicological detection of PCPR, PCEPA, PCMPA, PCEEA, and PCMEA in the rat have recently been described [7–10]. Sauer et al. [9] have found that PCEEA and PCMEA are mainly metabolized by O-dealkylation followed by oxidation to the corresponding acid, N-dealkylation, hydroxylation of the cyclohexyl ring at different positions, aromatic hydroxylation, and finally combinations of those. O-Dealkylation of PCEEA and PCMEA lead to the same O-dealkyl metabolite [N-(1-phenylcyclohexyl)-2-hydroxyethanamine, PCHEA]. As so far no data are available on the CYP mediated metabolism of PCEEA and PCMEA, the aim of the presented study was to study the involvement of human CYP isoenzymes in the main metabolic step.

2. Materials and methods

2.1. Chemicals and reagents

Hydrochlorides of PCEEA, PCMEA and PCEPA were provided by the Hessian State Criminal Office (Wiesbaden, Germany) for research purposes. PCHEA was biotechnologically synthesized as described previously by Peters et al. [12]. $NADP^+$ was obtained from Biomol (Hamburg, Germany), isocitrate and isocitrate dehydrogenase from Sigma (Taufkirchen, Germany), 4-(4-chlorobenzyl)pyridine (CBP) was obtained by ABCR (Karlsruhe, Germany), ketoconazole (Janssen, Beerse, Belgium), sulfaphenazole (Labotest, Niederschöna, Germany). All other chemicals and reagents were obtained by 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), and pooled human liver microsomes (pHLM 20 mg microsomal protein/mL, 400 pmol total CYP/mg protein).

2.2. Microsomal incubations and workup for initial activity screening

According to Ref. [13], 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 5000 μM aqueous stock solution for PCEEA and PCMEA, respectively, in the above-mentioned phosphate buffer. Reactions were started by addition of ice-cold microsomes and terminated with 50 μL of acetonitrile. After addition of 5 μL of internal standard (IS) solution (100 μM PCEPA in water for PCEEA and 100 μM PCEEA in water for PCMEA), the samples were centrifuged. In the initial screening experiments, the supernatants were diluted with 1 mL of water and worked-up by solid-phase extraction and acetylation as described for urine samples in Ref. [8]. Aliquots (2 μL) of the derivatized extracts were analyzed by GC–MS as described below. In all other experiments, the supernatants were directly transferred to autosampler vials and 5–30 μL were analyzed by LC–MS as described below.

2.3. Initial screening studies

Incubations with 250 μM PCEEA or PCMEA and 50 pmol/mL CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 were performed for 30 min. According to the Gentest manual, phosphate buffer was replaced by 45 mM or 90 mM Tris buffer, respectively, for incubations with CYP2A6 or CYP2C9.

2.4. Kinetic studies

Kinetic constants of PCHEA formation were derived from incubations with the following PCEEA concentrations, protein concentrations and incubation times: 10, 25, 50, 100, 200, 300, 400, 500, 700, 1000 1500, and 2000 μM PCEEA with 10 pmol CYP2B6/mL for 10 min; 10, 25, 50, 100, 200, 300, 400, 500, 600, 700, 1000, and 1500 μM PCEEA with 15 pmol CYP2C19/mL for 10 min; 10, 25, 50, 100, 200, 300, 400, 500, 600, 700, 1000, and 1500 μM PCEEA with 20 pmol CYP2C9/mL for 25 min; 25, 50, 100, 200, 300, 400, 500, 600, 700, 1000, 1500, 2000, 2500, and 3000 μM PCEEA with 15 pmol CYP3A4/mL for 20 min; 10, 25, 50, 100, 200, 300, 400, 500, 600, 700, 1000, 1500, and 2000 μM PCEEA with 0.2 mg HLM protein/mL for 10 min. For determination of the kinetic constants of PCHEA formation by incubations with PCMEA, following PCMEA concentrations, protein concentrations and incubation times were used: 25, 50, 100, 200, 300, 400, 500, 600, 700, 1000, 1500, 2000, and 2500 μM PCMEA with 10 pmol CYP2B6/mL for 10 min; 7, 8.4, 10.5, 17.5, 35, 70, 140, 210, 280, 350, 490, 700, 1050, 1400, and 1750 μM with 15 pmol CYP2C19/mL for 15 min; 7, 17.5, 35, 70, 140, 210, 280, 350, 490, 700, 1050, 1400, and 1750 μM PCMEA with 0.2 mg HLM protein/mL for 15 min.

By non-linear regression using GraphPad Prism 3.02 software (San Diego, CA) the enzyme kinetic constants were estimated. The Michaelis–Menten equation (Eq. (1)) was used

to calculate apparent V_{max} and K_m values for single-enzyme systems and HLM:

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

Eadie–Hofstee plots were used to check for biphasic kinetics [14]. If the Eadie–Hofstee plot indicated biphasic kinetics, Eq. (1) and the alternative Eq. (2) for a two site binding model [14] were applied to the respective data. For Eq. (2), $CL_{int,2}$ represents the intrinsic clearance or V_{max}/K_m of the low affinity component [14]. If Eq. (2) was found to fit the data significantly better (*F*-test, $P < 0.05$), biphasic kinetics were assumed.

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

2.5. Calculation of relative activity factors, contributions, and percentages of net clearance

To account for differences in functional levels of redox partners between the two enzyme sources, the relative activity factor (RAF) approach [15,16] was used. The turnover rates (TR) of CYP2B6 [probe substrate (PS) 7-ethoxy-4-trifluoromethylcoumarin], CYP2C19 (PS *S*-mephenytoin), CYP2C9 (PS Diclofenac), and CYP3A4 (PS testosterone) in ICM and HLM were taken from the supplier's data sheets. The RAFs were calculated according to Eq. (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 *O*-dealkylation of PCEEA/PCMEA obtained from incubations with cDNA-expressed CYPs were then multiplied with the corresponding RAF leading to a value, which is defined as 'contribution':

$$\text{contribution}_{enyzme} = \text{RAF} \times V_{max} \text{ of PCHEA formation in ICM} \quad (4)$$

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

$$\text{clearance}_{enzyme}(\%) = \frac{\text{clearance}_{enzyme}}{\sum \text{clearances}_{enzymes}} \quad (5)$$

2.6. Chemical inhibition studies

The effect of 0.1 μM CBP (CYP 2B6), 0.3 μM sulfaphenazole (CYP 2C9), and 0.08 μM ketoconazole (CYP 3A4) on PCHEA formation of PCEEA was assessed in incubations containing 0.3 mg HLM protein/mL for 10 min at a PCEEA concentration of 1 μM and 10 μM ($n = 6$ each). The effect of 0.1 μM CBP (CYP 2B6) on PCHEA formation of PCMEA was assessed in incubations containing 0.2 mg HLM protein/mL for 10 min for PCMEA at a concentration of 1 μM and 10 μM of the drug ($n = 6$ each). Control incubations were performed without the respective inhibitor ($n = 6$ each). Significance of inhibition was tested by one-tailed unpaired *t*-test using GraphPad Prism 3.02 software.

2.7. GC–MS apparatus for identification of metabolites

A Hewlett Packard (HP, Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software version C03.00 was used for the analysis of the incubation extracts. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m × 0.2 mm i.d.), cross-linked methyl silicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 mL/min; column temperature, programmed from 100 to 310 °C at 30 °C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full-scan mode, *m/z* 50–800 u; EI mode, ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface, heated at 260 °C.

2.8. Liquid chromatography–mass spectrometry (LC–MS) conditions and quantification of metabolite

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 was used for the determination of PCEEA, PCMEA, PCEPA, and PCHEA.

2.8.1. LC conditions

An Alltech Mixed-Mode/Cation exchange column (150 mm × 4.6 mm i.d.) was used with an isocratic mobile phase consisting of 50 mM ammonium formate buffer pH 3.5 and acetonitrile at a flow rate of 1.0 mL/min. The analytes were quantified by positive APCI-MS in the selected-ion monitoring mode.

2.9. 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* 248 for PCEEA, *m/z* 234 for PCMEA, *m/z* 262 PCEPA, and *m/z* 220 for PCHEA; fragmentor voltage 50 V.

2.10. Metabolite quantification

Plotting peak area ratios (PCHEA vs. IS) of spiked calibrators vs. their concentrations (0.5, 1.0, 10.0, 30.0, 50.0, 100.0 μM) calibration curves were constructed. Quantification was carried out using a weighted ($1/x^2$) linear regression model.

3. Results

3.1. Initial screening studies

GC–MS analysis of the supernatants of the initial screening experiments allowed identification of formed metabolites by library search as described by Sauer et al. [9]. Except for the common metabolite PCHEA, no further metabolite of PCEEA

Table 1 – Kinetic data of PCEEA O-deethylation by CYP2B6, CYP2C9, CYP2C19, CYP3A4 and HLM calculated according to Eq. (1). Units are: K_m in μM, V_{max} and contribution in pmol/min/pmol P450 (CYP2B6, CYP2C9, CYP2C19, and CYP3A4) or pmol/min/mg protein (pHLM).

	CYP2B6	CYP2C9	CYP2C19	CYP3A4	pHLM
K_m (best fit value ± standard error)	121 ± 7	334 ± 22	47 ± 4	550 ± 48	97 ± 7
V_{max} (best fit value ± standard error)	253 ± 4	14 ± 0.3	39 ± 0.7	27 ± 0.9	4 ± 0.1
Goodness of fit R^2	0.9906	0.9926	0.9763	0.9805	0.9836
RAF	0.027	0.669	0.005	0.384	
Contribution (V_{max} × RAF)	6.819	9.187	0.209	10.3	
Clearance (contribution/K_m)	0.056	0.028	0.004	0.019	
Percentage of net clearance	53	25	4	18	

and PCMEA was detected in any of the incubations with human liver microsomes or with ICM.

Among the 10 CYPs tested, only CYP2B6, CYP2C9, CYP2C19, and CYP3A4 were markedly capable of catalyzing the O-dealkylation of PCEEA. Only CYP2B6 and CYP2C19 showed relevant metabolite formation in the case of PCMEA. In incubations of the other CYPs, only very little (CYP1A2 and CYP2C9 in case of PCMEA) or no formation of PCHEA was observed.

3.2. LC–MS procedure

LC–MS analysis allowed direct injection of the supernatants without further workup. The mass fragmentograms showed that the applied LC–MS conditions provided sufficient separation of PCHEA, PCEEA, PCMEA, and PCEPA. As proven with blank samples (control microsomes without substrate and standard) and zero samples (control microsomes without substrate, but with standard), the chosen target ions were selective for the analytes under conditions applied. The method showed good linearity in a range of 0.5–100.0 μM PCHEA ($R^2 = 1.000$), and acceptable intra- and inter-day precision (relative S.D. ≤8.5%). Matrix effect studies gave no indication of ion suppression comparing the peak areas of PCHEA in neat standard solutions with those in spiked incubation mixtures containing the same concentrations of PCHEA.

3.3. Kinetic studies

Only the CYPs involved in the O-dealkylation of both drugs, namely CYP2B6, CYP2C19, CYP2C9, and CYP3A4 for PCEEA and CYP2B6 and CYP2C19 for PCMEA were characterized by their kinetic profiles. Duration and protein content of all incubations in these studies were within 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 (data not shown).

CYP2B6, CYP2C9, CYP3A4, and pHLM showed typical hyperbolic metabolite formation profiles allowing use of Eq. (1) for estimation of the kinetic constants. The resulting K_m and V_{max} values for PCEEA O-deethylation and PCMEA O-demethylation are listed in Tables 1 and 2, respectively. In case of PCEEA O-deethylation, CYP2C19 also showed a classical hyperbolic profile (Table 1). However, visual inspection of the data for PCMEA O-demethylation by this enzyme gave evidence of biphasic kinetics. This was confirmed by the corresponding Eadie–Hofstee plot (data not shown) and by significantly better fit when using Eq. (2) for a two-site binding model (F-test, $p < 0.05$). The resulting $K_{m,1}$ and $V_{max,1}$ data are reported in Table 2.

CYP2C19 turned out to have a 2.5-fold higher affinity towards PCEEA than CYP2B6 and approximately the same affinity as CYP2B6 towards PCMEA, whereas the capacity of CYP2B6 for O-dealkylation of both compounds was considerably higher than the capacities of all of the other involved CYPs.

3.4. Calculation of relative activity factors, contributions, and percentages of net clearance

The turnover rates (provided by the supplier) of the specific probe substrates in the used batches of ICM and pHLM, respectively, were as follows: 1486 pmol/min/mg protein and

Table 2 – Kinetic data of PCMEA O-demethylation by CYP2B6, CYP2C19, and HLM. Units are: K_m in μM, V_{max} and contribution in pmol/min/pmol P450 (CYP2B6 and CYP2C19) or pmol/min/mg protein (pHLM).

	CYP2B6	CYP2C19 (1)	CYP2C19 (2)	pHLM
K_m (best fit value ± standard error)	86 ± 8[a]	$K_{m,1}$ 61 ± 11[b]		103 ± 6[a]
V_{max} (best fit value ± standard error)	324 ± 7[a]	$V_{max,1}$ 16 ± 1[b]		3 ± 0.1[a]
Goodness of fit R^2	0.9785	0.9826	0.9826	0.9899
RAF	0.027	0.011		
Contribution (V_{max} × RAF)	8.716	0.175		
Clearance (contribution/K_m)	0.101	0.0029	0.0078	
Percentage of net clearance	91	2	7.0	

[a] Kinetic data estimated according to Eq. (1).
[b] Kinetic data estimated according to Eq. (2).

Table 3 – Inhibitory effect of 0.1 μM CBP on PCHEA formation in incubation mixtures containing 1 and 10 μM PCEEA or PCMEA. Controls were set to 100%. Each box represents the mean of six incubations ± standard error of the mean.

	1 μM PCEEA	10 μM PCEEA
	CBP (P450 2B6)	CBP (P450 2B6)
Metabolite formation, % of control	37 ± 7	39 ± 4
	1 μM PCMEA	10 μM PCMEA
	CBP (P450 2B6)	CBP (P450 2B6)
Metabolite formation, % of control	30 ± 12	22 ± 7

40 pmol/min/mg protein for 7-hydroxy-4-trifluoromethylcoumarin formation (CYP2B6), 4783 pmol/min/mg protein and 3200 pmol/min/mg protein for 4′-hydroxydiclofenac formation (CYP2C9), 5814 pmol/min/mg protein for PCEEA, 2857 pmol/min/mg protein for PCMEA and 31 pmol/min/mg protein for 4′-hydroxy-mephenytoin formation (CYP2C19), 16,667 pmol/min/mg and 6400 pmol/min/mg for 6β-testosterone formation (CYP3A4). For the incubation with (CYP2C19), different charges of ICM were used for PCEEA and PCMEA so that different turnover rates had to be taken into account. The RAFs, contribution and intrinsic clearance data, and percentages of net clearance calculated from these and the above-mentioned kinetic data are reported in Tables 1 and 2.

3.5. *Inhibition studies with chemical inhibitors using pHLM*

In Table 3, the results of the experiments with the chemical inhibitors CBP for CYP2B6 are presented for PCEEA (top) and for PCMEA (bottom). All observed inhibition effects were statistically significant ($p < 0.05$). Concerning the experiments with the chemical inhibitors sulfaphenazole for CYP2C9 and ketoconazole for CYP3A4, no inhibitory effect could be observed (data not shown).

4. Discussion

Former studies on the metabolism of PCEEA and PCMEA in the rat had shown that both drugs are extensively metabolized *in vivo* [9]. However, in consideration of possible inter-species variability in metabolism, it first had to be clarified which of the metabolites were formed in incubations with HLM and ICM with cDNA-expressed human CYPs. PCHEA, the common *O*-dealkyl metabolite of PCEEA and PCMEA was the only metabolite detected in these incubations. Therefore, we assumed the *O*-dealkylation of PCEEA and PCMEA to be the main metabolic step in humans and the initial reaction for formation of further metabolites of these designer drugs.

CYP2B6, CYP2C19, and HLM were markedly capable of catalyzing the *O*-dealkylation of both drugs and *O*-deethylation of PCEEA was further catalyzed by CYP2C9 and CYP3A4. Therefore, the kinetics of PCHEA formation by these enzymes were determined and used to estimate the percentages of net clearance of each individual CYP (Tables 1 and 2). The RAF approach [16–18] was used for this purpose, because it is an accepted strategy to correct recombinant CYP formation rates for native human liver enzyme activity. According to the results of the RAF approach, CYP2B6 should be the most relevant CYP for *O*-dealkylation of both compounds accounting for 53% (PCEEA) and 91% (PCMEA) of the net clearance.

It is interesting to note that the shorter side chain of PCMEA was associated with a higher contribution of CYP2B6, which is in good accordance with our previously published findings for the related drugs PCEPA and PCMPA [13], and PCPR [19]. In the case of the PCPR, the compound with the shortest side-chain, CYP2B6 was the only enzyme involved in side-chain hydroxylation (i.e. 100% net clearance). For PCMEA with a slightly longer side-chain the net clearance of CYP2B6 is somewhat lower with about 90%. This trend continued with PCMPA and PCEEA which have a similar side-chain length but longer than that of PCMEA. Here the percentage of net clearance of CYP2B6 was about 50%. Finally, in the case of the derivative with the longest side-chain, PCEPA, it was lowest with only about 20%. In accordance with the decreasing involvement of CYP2B6, the number of CYPs involved and their contributions increased with increasing side-chain length.

A further approach for confirming the role of CYP2B6 and also CYP3A4 in *O*-dealkylation of PCEEA and PCMEA was inhibition of these isoenzymes in pHLM using the chemical inhibitor CBP, sulfaphenazole, and ketoconazole, respectively. CBP is a new selective inhibitor for CYP2B6 [20]. For inhibition of CYP2C9 sulfaphenazole and for CYP3A4 ketoconazole were used, their concentration and the concentration of CBP were selected according to the literature in Refs. [14,20–27]. No data of plasma levels of PCEEA and PCMEA in humans are available. Considering the plasma levels of the related drug PCP to estimate the approximate plasma level of PCEEA and PCMEA after a common drug user's dose, the expected plasma levels should be approximately 1 μM or lower [28,29]. Therefore, the first substrate concentration in the inhibition experiments was 1 μM. This concentration also represents a middle range of PCP plasma concentration described for 15 cases of nonfatal intoxication in literature [28,29]. The second concentration (10 μM) should represent much higher plasma levels as might be expected in severe or lethal intoxications. Furthermore the higher concentration was chosen to estimate the role of the enzymes with lower affinity, which should become increasingly evident at this concentration. The results of the CYP2B6 inhibition study (Table 3) showed that at both substrate concentrations the overall turnover was inhibited significantly ($p < 0.05$). The inhibition of CYP2B6 both for *O*-dealkylation of PCEEA and PCMEA showed an approximate correlation to the RAF approach and confirmed that CYP2B6 is the most relevant of the involved CYPs.

Considering the above-described major involvement of CYP2B6 in the *O*-dealkylation of PCMEA in humans, simultaneous intake of potent CYP2B6 inhibitory drugs, e.g. triethylenethiophosphoramide (Thiotepa), ticlopedine, clopidogrel [30,31] or several antidepressants [32] might lead to a decreased clearance of PCMEA and, consequently, to elevated

plasma concentrations. However, the clinical relevance of such interactions remains to be established.

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