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Relative contribution of rat cytochrome P450 isoforms to the metabolism of caffeine: The pathway and concentration dependence

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ABSTRACT

The aim of the present study was to estimate the relative contribution of rat P450 isoforms to the metabolism of caffeine and to assess the usefulness of caffeine as a marker substance for estimating the activity of P450 in rat liver and its potential for pharmacokinetic interactions in pharmacological experiments. The results obtained using rat cDNA-expressed P450s indicated that 8-hydroxylation was the main oxidation pathway of caffeine (70%) in the rat. CYP1A2 was found to be a key enzyme catalyzing 8-hydroxylation (72%) and substantially contributing to 3-*N*-demethylation (47%) and 1-*N*-demethylation (37.5%) at a caffeine concentration of 0.1 mM (relevant to "the maximum therapeutic concentration in humans"). Furthermore, CYP2C11 considerably contributed to 3-*N*-demethylation (31%). The CYP2C subfamily (66%) – mainly CYP2C6 (27%) and CYP2C11 (29%) – played a major role in catalyzing 7-*N*-demethylation. At higher substrate concentrations, the contribution of CYP1A2 to the metabolism of caffeine decreased in favor of CYP2C11 (*N*-demethylations) and CYP3A2 (mainly 8-hydroxylation). The obtained results were confirmed with liver microsomes (inhibition and correlation studies). Therefore, caffeine may be used as a marker substance for assessing the activity of CYP1A2 in rats, using 8-hydroxylation (but not 3-*N*-demethylation—like in humans); moreover, caffeine may also be used to simultaneously, preliminarily estimate the activity of CYP2C using 7-*N*-demethylation as a marker reaction. Hence caffeine pharmacokinetics in rats may be changed by drugs affecting the activity of CYP1A2 and/or CYP2C, e.g. by some antidepressants.

1. Introduction

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid, is the most universally used psychoactive substance as a component of coffee, tea and numerous drugs. It has multiple pharmacological effects. As an adenosine receptor antagonist, caffeine increases the release of various neurotransmitters, and at higher concentrations inhibits phosphodiesterase and GABA receptors [1]. Due to its ability to interact with neurotransmission in different regions of the brain, caffeine displays psychomotor stimulant properties, promoting behavioral functions such as vigilance, attention, mood and

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Abbreviations: CYP; P450, cytochrome P450; FMO, flavin-containing monooxygenase; HPLC, high performance liquid chromatography; αNF, α-naphthoflavone; SULF, sulfaphenazole; CIM, cimetidine; QUIN, quinine; *trans*-DCE, *trans*-1,2-dichloroethylene; ERY, erythromycin; K_m, the Michaelis constant; V_{max}, maximum velocity of the reaction.

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arousal [2]. In recent years caffeine and newly synthesized xanthine derivatives have been investigated as adenosine receptor antagonists and neuroprotective drugs in animal models of neurodegenerative diseases *in vivo* [3–6]. Since the disposition of caffeine *in vivo* depends on cytochrome P450, mutual drug interactions between caffeine and other centrally acting drugs at both the pharmacodynamic and pharmacokinetic level are feasible [7].

On the other hand, due to its natural character, caffeine is used as a marker substance for estimating phenotypes with regard to the activity of CYP1A2 in man. 3-*N*-Demethylation to paraxanthine is regarded as a reaction specifically catalyzed by CYP1A2 in humans [8–10]. However, it remains to be ascertained whether this reaction may also be used for testing the activity of CYP1A2 in laboratory rodents, since no detailed study on the contribution of individual P450 isoforms to the metabolism of caffeine has been carried out to date.

Interestingly, caffeine is oxidized in a few positions of its structure—apart from its 3-*N*-demethylation to paraxanthine catalyzed by CYP1A2, the compound undergoes 1-*N*-demethylation, 7-*N*-demethylation and 8-hydroxylation (to theobromine, theophylline and 1,3,7-trimethyluric acid, respectively), (Fig. 1). While in man 3-*N*-demethylation is quantitatively the main oxidation pathway, in the rat 8-hydroxylation seems to be a dominant metabolic reaction [8,11,12]. Some literature data suggest that other oxidation pathways of caffeine may be mediated by P450 isoforms different to CYP1A2, both in humans [9,13,14] and rats [8,11,12,15]. It seems that the CYP3A subfamily is the main isoenzyme catalyzing 8-hydroxylation to 1,3,7-trimethyluric acid [9,12]; however, some other data also suggest the contribution of CYP2B1 and CYP2E1 to the catalyzing of this reaction in the rat [11]. It has also been suggested that 1-*N*-demethylation and mainly 7-*N*-demethylation, which lead to the formation of theobromine and theophylline, respectively, are likely to engage CYP2B isoforms in rats [15]. Our results concerning the influence of psychotropic drugs on the metabolism of caffeine also indicate that more than one enzyme contributes to the oxidative metabolism of caffeine in the rat [16-17]; moreover, recent studies with selective P450 inducers suggest that CYP2C11 may be engaged in the 7-*N*-demethylation of caffeine in this species [18].

Although a number of studies have indicated that phase I of caffeine metabolism in the liver may proceed *via* a few cytochrome P450 isoforms, their identification and relative quantitative contribution to the specific metabolic pathways of the test drug has not been precisely estimated as yet, especially in laboratory rodents. So far, most studies into the involvement of P450s in the metabolism of caffeine in rats have been carried out using high (1–10 mM) substrate concentrations, predominantly unspecific P450 inducers or inhibitors [11,19], often not including all P450 isoforms [12,20] or all caffeine metabolic reactions [19]. Therefore it was difficult to identify all the P450 isoforms involved in caffeine metabolism; furthermore, quantitative estimation of the contribution of individual P450s to the four oxidative metabolic reactions of caffeine at concentrations relevant to those found in humans was practically impossible. According to the literature, the concentration value of ca. 100 μM seems to be the highest encountered in the clinic [21,22] and may be assumed as ''the maximum therapeutic concentration in humans''. Pharmacokinetic studies showed that caffeine concentrations found in the blood plasma of coffee drinkers were below 100 μM (e.g. 10–31 μM after consumption of 2–6 cups of coffee [23,24].

Since the latest achievements in biotechnology have facilitated the obtaining and availability of cDNA-expressed P450s, which at present constitute a modern model for testing drug metabolism, it was possible to carry out systematic studies into caffeine metabolism, including all individual rat P450s and the four oxidative metabolic reactions of caffeine.

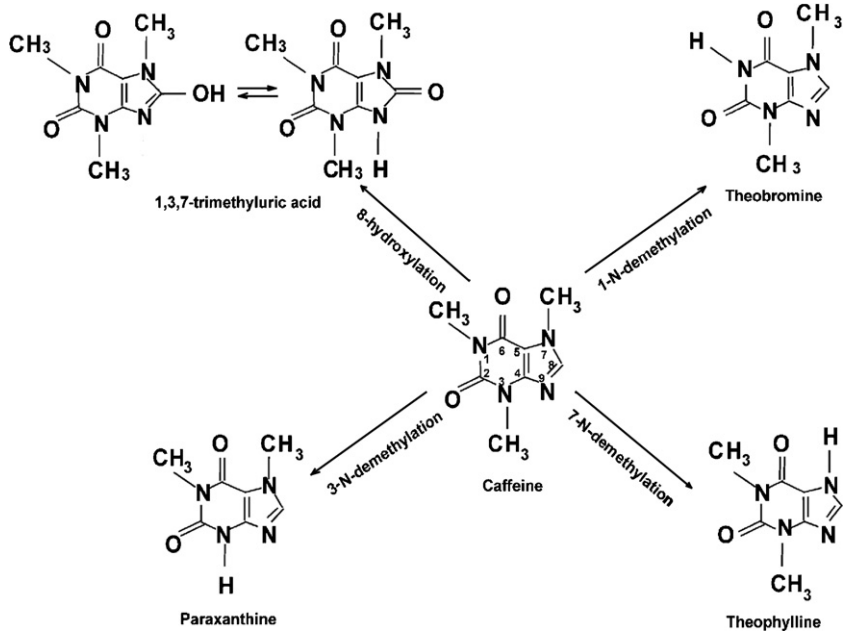

Fig. 1 – The main metabolic pathways of caffeine.

The aim of the present study was to qualitatively and quantitatively estimate the contribution of P450 isoforms to the metabolism of caffeine at different drug concentrations in rat liver and, on the basis of the obtained results, to assess the usefulness of caffeine as a marker substance for estimating the activity of P450 and its potential for pharmacokinetic interactions in pharmacological experiments with rats. To this aim, rat cDNA-expressed P450s were used, and the obtained results were confirmed with liver microsomes (inhibition and correlation studies).

2. Materials and methods

2.1. Drugs and chemicals

Caffeine and its metabolites—theobromine, paraxanthine, theophylline and 1,3,7-trimethyluric acid, phenacetin, as well as α-naphthoflavone, sulfaphenazole, cimetidine, quinine, *trans*-1,2-dichloroethylene, erythromycin, NADP, DL-isocitric acid (trisodium salt), isocitric dehydrogenase, NADPH were purchased from Sigma (St. Louis, USA). Acetamidophenol, $MgCl_2{\cdot}6H_2O$ and all organic solvents with HPLC purity were supplied by Merck (Darmstadt, Germany).

2.2. Animal procedures

All the experiments with animals were performed in accordance with the Polish governmental regulations (Animals Protection Act, DZ.U. 97.111.724, 1997). The experiments were carried out on male Wistar rats (230–260 g) kept under standard laboratory conditions. Liver microsomes were prepared by differential centrifugation in 20 mM Tris/KCl buffer (pH 7.4), including washing with 0.15 M KCl according to a conventional method.

The CYP2E1 inhibitor *trans*-1,2-dichloroethylene (*trans*-DCE) was administered intraperitoneally in a dose of 100 mg/kg to rats [25]. Control animals were injected with the same volume of water. After 5 h, untreated and *trans*-DCE-treated rats were sacrificed, and their livers were removed and homogenized. Liver microsomes were prepared as described above.

2.3. Caffeine metabolism in liver microsomes

Studies into caffeine metabolism in liver microsomes were carried out at the linear dependence of product formation on time, protein and substrate concentration. For determining enzyme kinetics (Eadie–Hofstee plots), the caffeine concentrations used ranged from 0.1 to 2.8 mM. For inhibition studies, the concentrations of caffeine were from 50 to 400 μM. Caffeine was incubated with liver microsomes in the absence or presence of one of the selective inhibitors added *in vitro*: 0.025–0.1 μM α-naphthoflavone (a CYP1A2 inhibitor), 0.5–5.0 μM sulfaphenazole (a CYP2C6 inhibitor), 25–100 μM cimetidine (a CYP2C6 + CYP2C11 inhibitor), 1.25–10 μM quinine (a CYP2D inhibitor), 50–400 μM erythromycin (a CYP3A2 inhibitor). The CYP2E1 inhibitor *trans*-1,2-dichloroethylene (*trans*-DCE) was administered intraperitoneally as described above. Caffeine metabolism was studied in the liver microsomes of untreated and *trans*-DCE-treated rats at the substrate concentration of 100 μM.

Incubations were carried out in a system containing the liver microsomes (ca. 1 mg of protein/ml), a phosphate buffer (0.15 M, pH 7.4), $MgCl_2{\cdot}6H_2O$ (6 mM), NADP (1.2 mM), DL-isocitric acid (6 mM) and isocitric dehydrogenase (1.2 U/ml). The final incubation volume was 1 ml. After a 3-min (with α-naphthoflavone), a 15-min (with cimetidine) or a 20-min (with erythromycin) preincubation at 37 °C, the reaction was initiated by adding caffeine (the other inhibitors were not preincubated). After a 50-min incubation, the reaction was terminated by adding 700 μl of a 2% $ZnSO_4$ and 50 μl of 2 M HCl.

To verify possible involvement of flavin-containing monooxygenase (FMO) in caffeine metabolism, thermal inactivation of FMO was applied [26,27]. To this end, control liver microsomes were preincubated with or without NADPH at 50 °C for 1.5 min before incubating the complete reaction mixture containing NADPH and caffeine (100 μM) at 37 °C. Caffeine and its metabolites were analyzed by a high-performance liquid chromatography method (HPLC) described below.

2.4. Correlation analysis of the data

Due to the dose-dependent contribution of the P450 isoforms studied to the oxidative metabolism of caffeine, a correlation study was carried out at a caffeine concentration of 100 μM (relevant to ''the maximum therapeutic concentration in humans'') and 800 μM (relevant to the K_m value for 3-*N*-demethylation in rat liver microsomes). The rates of caffeine 1-*N*-, 3-*N*- and 7-*N*-demethylation and 8-hydroxylation were correlated with the activity of P450 isoforms in liver microsomes.

The activity of CYP2C6 was studied by measuring the rate of warfarin 7-hydroxylation at the substrate concentration of 60 μM as described previously [28]. The concentrations of warfarin and its metabolite 7-hydroxywarfarin formed in liver microsomes were assessed by the HPLC method of Lang and Bocker [29]. The activities of CYP2A2, CYP2B, CYP2C11 and CYP3A2 were studied by measuring the rate of P450-specific reactions: the 7α-, 16β-, 2α- and 2β-hydroxylation of testosterone (respectively) at the substrate concentration of 100 μM as described previously [30]. The concentrations of testosterone and its metabolites formed in liver microsomes were assessed by the HPLC method of Sonderfan et al. [31]. The activity of CYP1A2 was studied by measuring the rate of phenacetin *O*-deethylation to acetamidophenol according to the method of Eagling et al. [32] at the substrate concentration of 10 μM [33].

Each pair of data was compared by a simple linear regression analysis using the statistical Prism 2.01 program (GraphPad Software Inc., San Diego, CA).

2.5. cDNA-expressed rat P450s

Microsomes from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP2D1, CYP2D2, CYP2E1 and CYP3A2 co-expressed with NADPH P450 oxidoreductase (Supersomes) were obtained from Gentest Co. (Woburn, MA, USA). CYP2A2, CYP2B1, CYP2C6, CYP2C13, CYP2E1 and CYP3A2 were also co-expressed with b_5. Microsomal protein expressing NADPH P450 oxidoreductase

and cytochrome b_5 was used as a control. Studies into caffeine metabolism in the Supersomes were carried out at the linear dependence of product formation on time, the amount of P450 isoform and substrate concentration. Caffeine metabolism was studied under experimental conditions similar to those described for liver microsomes, using 100, 200, 400, 800 and 1600 μM caffeine, except for the fact that the final concentration and incubation time of P450s was 100 pmol/ml and 50 min for CYP1A1, 50 pmol/ml and 30 min for CYP1A2, 60 pmol/ml and 30 min for CYP2A2, 70 pmol/ml and 30 min for CYP2B1, 50 pmol/ml and 60 min for CYP2C6, 60 pmol/ml and 30 min for CYP2C11, 70 pmol/ml and 30 min for CYP2C13, 60 pmol/ml and 20 min for CYP2D1, 60 pmol/ml and 45 min for CYP2D2, 100 pmol/ml and 60 min for CYP2E1, 50 pmol/ml and 15 min for CYP3A2.

2.6. Determination of caffeine and its metabolites

Caffeine and its four primary metabolites were assessed using the HPLC method based on Rasmussen et al. [34] as previously described [16]. Briefly, after incubation, samples were centrifuged and the water phase containing caffeine and its metabolites was extracted with 6 ml of an organic mixture consisting of ethyl acetate and 2-propanol (8:1, v/v). The residue obtained after evaporation of the microsomal extract was dissolved in 100 μl of the mobile phase described below. An aliquot of 20 μl was injected into the HPLC system. The Merck-Hitachi chromatograph, ''LaChrom'' (Darmstadt, Germany), equipped with a L-7100 pump, an UV detector and a D-7000 System Manager was used. The analytical column (Supelcosil LC-18, 15 cm × 4.6 mm, 5 μm) was from Supelco (Bellefonte, USA). The mobile phase consisted of 0.01 M acetate buffer (pH 3.5) and methanol (91:9, v/v). The flow rate was 1 ml/min (0–26.5 min), followed by 3 ml/min (26.6–35 min). The column temperature was maintained at 30 °C. The absorbance of caffeine and its metabolites was measured at a wavelength of 254 nm. The compounds were eluted in the following order: theobromine (9.7 min), paraxanthine (15.8 min), theophylline (16.9 min), 1,3,7-trimethyluric acid (23.4 min), caffeine (30.5 min). The sensitivity of the method allowed for quantification of theobromine as low as 0.001 nmol, paraxanthine as low as 0.004 nmol, theophylline as low as 0.005 nmol, 1,3,7-trimethyluric acid as low as 0.01 nmol and caffeine as low as 0.005 nmol in one sample. The accurracy of the method amounted to 1.2% (paraxanthine), 1.3% (theophylline), 2.1% (theobromine), 2.3% (1,3,7-trimethyluric acid) and 2.9% (caffeine). The intra- and inter-assay coefficients of variance were below 4 and 6%, respectively.

3. Results

3.1. Caffeine metabolism in rat liver microsomes

Fig. 2A–D shows the Eadie–Hofstee plots for caffeine oxidation processes in the pooled liver microsomes of five rats. All the plots were non-linear indicating for the multiple-enzyme

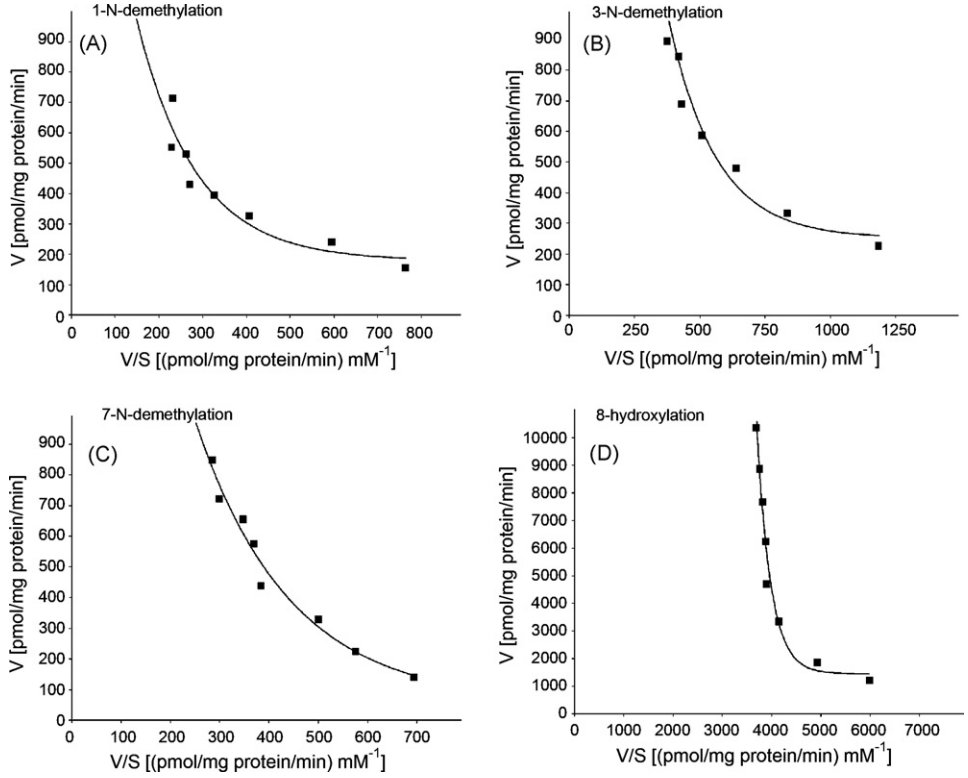

Fig. 2 – Eadie–Hofstee plots for caffeine 1-*N*-demethylation (A), 3-*N*-demethylation (B), 7-*N*-demethylation (C), and 8-hydroxylation (D) in rat liver microsomes. Pooled liver microsomes of five control rat were incubated in a 0.15 M phosphate buffer (pH 7.4) with caffeine (0.1–2.8 mM), 6 mM $MgCl_2 \cdot 6H_2O$ and the NADPH generating system (1.2 mM NADP, 6 mM DL-isocitric acid, 1.2 U/ml isocitric dehydrogenase) for 50 min.

Table 1 – Kinetic parameters of caffeine metabolism in cDNA-expressed rat CYPs (Supersomes)

P450	1-N-Demethylation (theobromine)			3-N-demethylation (paraxanthine)			7-N-Demethylation (theophylline)			C-8-hydroxylation (1,3,7-trimethyluric acid)		
	$^{a}K_m$	$^{b}V_{max}$	V_{max}/K_m	$^{a}K_m$	$^{b}V_{max}$	V_{max}/K_m	$^{a}K_m$	$^{b}V_{max}$	V_{max}/K_m	$^{a}K_m$	$^{b}V_{max}$	V_{max}/K_m
1A1 (not constitutive)	0.214	0.117	0.547	0.171	0.288	1.684	5.029	0.423	0.084	0.158	1.094	6.924
1A2	0.121	0.804	**6.645**	0.074	0.791	**10.689**	0.429	0.675	**1.573**	0.091	9.427	**103.593**
2A2	0.137	0.163	1.190	0.837	0.146	0.174	1.433	0.669	0.467	0.806	0.751	0.932
2B1	0.080	0.072	0.900	0.675	0.082	0.121	6.521	1.100	0.169	0.338	0.154	0.456
2C6	0.547	0.102	0.186	0.470	0.062	0.132	1.558	1.438	**0.923**	0.751	0.934	1.244
2C11	18.735	1.828	0.097	2.559	1.038	0.406	10.260	7.725	**0.753**	1.189	0.301	0.253
2C13	0.333	0.129	0.387	0.919	0.075	0.082	1.160	0.310	0.267	0.720	0.052	0.072
2D1	1.184	0.207	0.175	1.506	0.315	0.209	1.397	0.740	0.530	0.096	0.281	2.930
2D2	1.230	0.068	0.055	0.277	0.048	0.173	0.592	0.389	0.657	0.174	0.265	1.523
2E1	0.291	0.187	0.643	5.405	0.596	0.110	5.305	0.987	0.186	5.750	0.625	0.109
3A2	0.484	0.198	0.409	5.555	1.519	0.273	4.050	2.120	0.523	5.800	52.100	**8.983**

$^{a}K_m$ (mM), $^{b}V_{max}$ (pmol/pmol P450 isoform/min). The highest intrinsic clearances are shown in bold. Kinetic parameters were derived following non-linear analysis (program SigmaPlot 8.0; Enzyme Kinetics).

catalysis of both *N*-demethylations and 8-hydroxylation. There was no significant difference in the rate of caffeine metabolism (100 μM) between liver microsomes preincubated with and without NADPH at 50 °C (data not shown).

3.2. *A study with rat cDNA-expressed P450s*

Kinetic parameters showing the caffeine metabolism in rat cDNA-expresssed P450s, obtained using non-linear analysis (Program Sigma Plot 8.0; Enzyme Kinetics), are presented in Table 1. These kinetic parameters indicate distinct inter-isoform differences, which is consistent with the multi-enzyme Eadie–Hofstee plots derived from liver microsomes (Fig. 2A–D). CYP1A2 showed the highest intrinsic clearance (V_{max}/K_m) towards caffeine metabolism, its highest value being reached for 8-hydroxylation. The intrinsic clearance of CYP1A2 was also relatively high in the case of 3-*N*-demethylation and 1-*N*-demethylation. In the case of 7-*N*-demethylation,

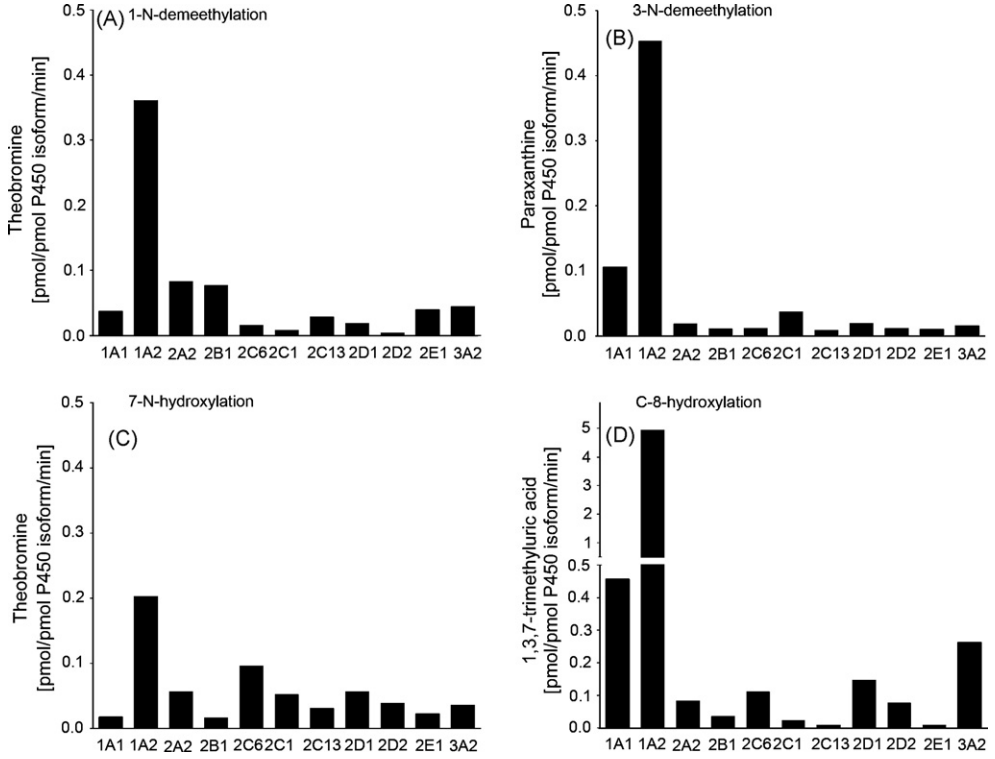

Fig. 3 – The biotransformation of caffeine *via* 1-*N*-demethylation (A), 3-*N*-demethylation (B), 7-*N*-demethylation (C), and C-8-hydroxylation (D) by rat cDNA-expressed P450s (Supersomes). Caffeine (100 μM) was incubated with Supersomes (50–100 pmol P450/ml), 6 mM $MgCl_2 \cdot 6H_2O$ and the NADPH generating system (1.2 mM NADP, 6 mM DL-isocitric acid, 1.2 U/ml isocitric dehydrogenase) for 15–60 min, depending on optimum conditions for a P450 isoform. Each bar represents the mean value of two independent analyses.

the highest intrinsic clearance was found for CYP1A2, CYP2C6 and CYP2C11.

The ability of rat cDNA-expressed P450s to metabolize caffeine at its therapeutic concentration (100 μM) is shown in Fig. 3A–D. According to the calculated intrinsic clearance values, the preference of P450 enzymes for catalyzing caffeine metabolism was as follows (pmol of product/pmol of P450 isoform/min):
CYP1A2 > CYP2A2 > CYP2B1 > CYP2E1 > CYP1A1 > CYP3A2 > CYP2C13 > CYP2C6 > CYP2D1 > CYP2C11 > CYP2D2 for 1-*N*-demethylation, CYP1A2 > CYP1A1 > CYP2C11 > CYP3A2 > CYP2D1 > CYP2A2 > CYP2D2 > CYP2C6 > CYP2B1 > CYP2E1 > CYP2C13 for 3-*N*-demethylation, CYP1A2 > CYP2C6 > CYP2C11 > CYP2D2 > CYP2D1 > CYP3A2 > CYP2A2 > CYP2-CYP2C13 > CYP2E1 > CYP2B1 > CYP1A1 for 7-*N*-demethylation, CYP1A2 > CYP3A2 > CYP1A1 > CYP2D1 > CYP2D2 > CYP2C6 > CYP2A2 > CYP2B1 > CYP2C11 > CYP2E1 > CYP2C13 for 8-hydroxylation.

3.3. *Quantitative estimation of the contribution of P450 enzymes to the specific metabolic pathways of caffeine*

We roughly estimated the contribution of the P450 isoforms studied to caffeine phase I oxidation pathways on the basis of the rate of those reactions in Supersomes and the contribution of each isoform to the total P450 content in the liver (Table 2).

CYP2B1, the only available CYP2B isoform, was assumed to be a representative of CYP2B1/2 isoforms, regarding their similar structure (a 97% amino acid sequence similarity); [35] and catalytic function [36,37]. The calculations done at a caffeine concentration of 100 μM (relevant to "the maximum therapeutic concentration in humans") indicated that CYP1A2 was the main isoform responsible for caffeine metabolism. CYP1A2 is a chief enzyme catalyzing 8-hydroxylation (72%) and substantially contributing to 3-*N*-demethylation (47%) and 1-*N*-demethylation (37.5%). Moreover, 8-hydroxylation was also visibly mediated by CYP3A2 (15%), while 3-*N*-demethylation – by CYP2C11 (31%) and CYP3A2 (7%), and 1-*N*-demethylation – by CYP3A2 (17%) and CYP2C13 (14%). The catalysis of 7-*N*-demethylation was predominantly governed by CYP2C11 (29%) and CYP2C6 (27%), and to lower extent – by CYP1A2 (14%), CYP2C13 and CYP3A2 (10% each). At a higher concentration of the substrate, CYP1A2 contribution to the metabolism of caffeine decreased – mostly in favor of CYP2C11 (in the case of *N*-demethylations) and/or CYP3A2 (mainly in the case of 8-hydroxylation) (Fig. 4).

3.4. *Inhibition of caffeine metabolism by specific P450 inhibitors in rat liver microsomes*

α-Naphtoflavone (a CYP1A2 inhibitor) exerted a strong inhibitory effect on the rate of the four oxidative caffeine pathways, being most effective towards 8-hydroxylation (to 34% of the control value; K_i = 0.048 μM), and the least active towards 7-*N*-demethylation (to 48% of the control value; K_i = 0.136 μM) (Fig. 5 and Table 3).

Sulfaphenazole (a CYP2C6 inhibitor) significantly decreased the rate of 7-*N*-demethylation (to 71% of the control value; K_i = 2.8 μM), not affecting other reactions. Cimetidine (a CYP2C6 + CYP2C11 inhibitor) reduced the rate of 7-*N*-demethylation (to 57% of the control value; K_i = 35.2 μM), and to a smaller extent that of 3-*N*-demethylation (to 76% of the control value; K_i = 138 μM). Erythromycin (a CYP3A inhibitor) significantly diminished the rate of 1-*N*-demethylation and 8-hydroxylation (to 69 and 86%, respectively, of the control value; K_i = 490 and 858 μM, respectively). Neither

Table 2 – Estimation of the contribution of P450 isoforms to the particular metabolic pathways of caffeine on the basis of the rates of these reaction in Supersomes and average P450 contents in the liver

P450	Relative contribution of the iso-form to the total P450 contents in liver microsomes (fraction)[a]	Relative contribution of the isoforms to caffeine metabolism in liver microsomes (percentage)[b]							
		Caffeine1-*N*-demethylation		Caffeine 3-*N*-demethylation		Caffeine 7-*N*-demethylation		Caffeine C-8-hydroxylation	
		Caffeine		Caffeine		Caffeine		Caffeine	
		100 μM	800 μM	100 μM	800 μM	100 μM	800 μM	100 μM	800 μM
CYP1A2	0.050[a]	**37.5**	**22.6**	**46.9**	**19.2**	**14.1**	**5.4**	**71.9**	**30.4**
CYP2A2	0.035[a]	6.9	3.5	1.6	1.2	3.1	1.7	1.0	0.6
CYP2B1	0.010[a]	1.6	0.6	0.2	0.2	0.2	0.3	0.1	0.02
CYP2C6	0.200[a]	6.6	6.7	5.2	2.8	26.8	24.5	6.5	6.1
CYP2C11	0.400[a]	**6.4**	**23.8**	**31.3**	**50.8**	**29.3**	**41.3**	2.7	2.8
CYP2C13	0.230[a]	13.8	14.3	4.2	3.9	10.0	6.9	0.7	0.4
CYP2D1	0.025[c]	1.0	1.1	1.0	1.5	2.0	1.5	1.1	0.4
CYP2D2	0.025[c]	0.2	0.4	0.7	0.4	1.4	0.6	0.6	0.3
CYP2E1	0.100[b]	9.2	9.5	2.2	4.1	3.0	3.1	0.3	0.7
CYP3A2	0.200[b]	16.8	17.4	**6.6**	**15.9**	10.1	14.6	**15.3**	**58.1**

[a] Data according to [a]Lewis [38]; [b]Shimojo et al. [39]; [c]Larrey et al. [40].
[b] Relative contribution of P450s to the particular metabolic pathways of caffeine was calculated as percentage of the sum of predicted velocities in liver microsomes. The predicted velocity in liver microsomes was calculated by multiplying the velocity in Supersomes (see Fig. 3) by the relative contribution of isoform to the total P450 content in liver microsomes. For details of calculations, see Wójcikowski et al. [41]. Essential differences between 100 and 800 μM concentration of caffeine are shown in bold.

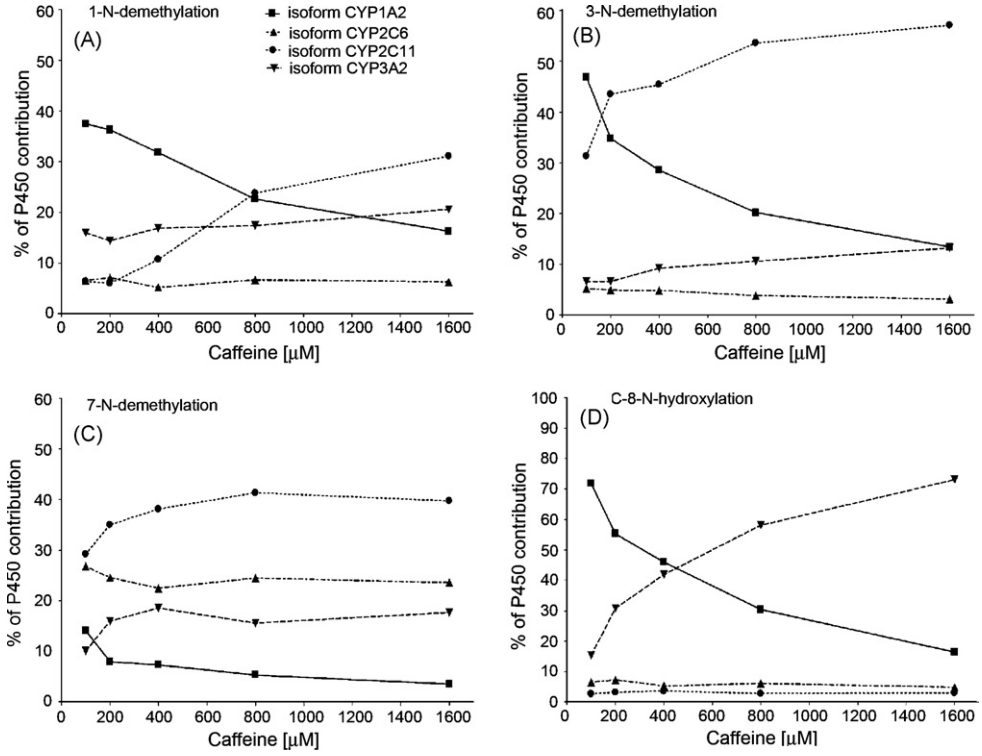

Fig. 4 – The concentration-dependent contribution of P450 isoforms to caffeine metabolism in rat liver (based on the rate of caffeine metabolism in Supersomes and the mean of P450s in the liver). For further explanations see Fig. 3.

quinine (a CYP2D inhibitor) nor *in vivo* administration of trans-DCE (a CYP2E1 inhibitor) significantly affected caffeine metabolism *in vitro*.

3.5. *Correlation study*

It was shown that the 8-hydroxylation of caffeine was a major metabolic reaction (~70%). An approximately sixfold inter-individual variability of the rates of caffeine metabolic reactions both at 100 and 800 μM concentrations of caffeine was found (data not shown). The rate of formation of caffeine metabolites by different preparations of rat liver microsomes, determined for two concentrations of caffeine (100 and 800 μM), was compared with the monooxygenase activity assessed in our laboratory for each liver preparation. The results of those analyses are shown in Table 4A (for 100 μM caffeine) and B (for 800 μM caffeine), where the correlation coefficient (r) and the p value are given for each pair of data.

At a caffeine concentration of 100 μM, the rates of all the caffeine metabolic reactions strongly correlated with the activity of CYP1A2. Moreover, the rate of 3-*N*-demethylation significantly correlated with the activity of CYP2B, the rate of 1-*N*-demethylation correlated with the activity of CYP2C6, whereas the rate of 8-hydroxylation strongly correlated with the activity of CYP3A2 (Table 4A).

At a caffeine concentration of 800 μM, the rates of all the caffeine metabolic reactions also significantly correlated with the activity of CYP1A2 (Table 4B), however, the correlations concerning 8-hydroxylation and in particular 7-*N*-demethylation were weaker than at 100 μM caffeine (Table 4A) and not as strong as those for 3-*N*- or 1-*N*-demethylation at 800 μM caffeine (Table 4B). Moreover, the rate of 3-*N*-demethylation positively correlated with the activities of CYP2B and CYP2C11, while the rate of 7-*N*-demethylation potently correlated with the activity of CYP2C11, as well as with the activities of CYP2B and CYP2C6. As observed previously for 100 μM caffeine, 8-hydroxylation in the case of 800 μM caffeine, was also strongly correlated with the activity of CYP3A2.

4. Discussion

The data presented above are the first comprehensive report on the qualitative and quantitative contribution of individual P450 isoforms to the four oxidation pathways of caffeine metabolism in the rat. The obtained results indicate that the main oxidation pathway of caffeine in the rat is 8-hydroxylation (70%), which is specifically catalyzed by CYP1A2 (72%) at a concentration of 100 μM (relevant to "the maximum therapeutic concentration in humans"), and may be used as a marker reaction for this enzyme in the above-mentioned species. The above results showing the importance of 8-hydroxylation for caffeine metabolism are consistent with those obtained by Berthou et al. [8] with rats, but disagree with those pertaining to humans which show that 3-*N*-demethylation is the main metabolic pathway of caffeine [8,9]. Since both these main reactions in the two species (8-hydroxylation in rats and 3-*N*-demethylation in humans) are specifically catalyzed by CYP1A2, they may imply species differences in CYP1A2 catalytic competence and kinetics, as well as in caffeine metabolism.

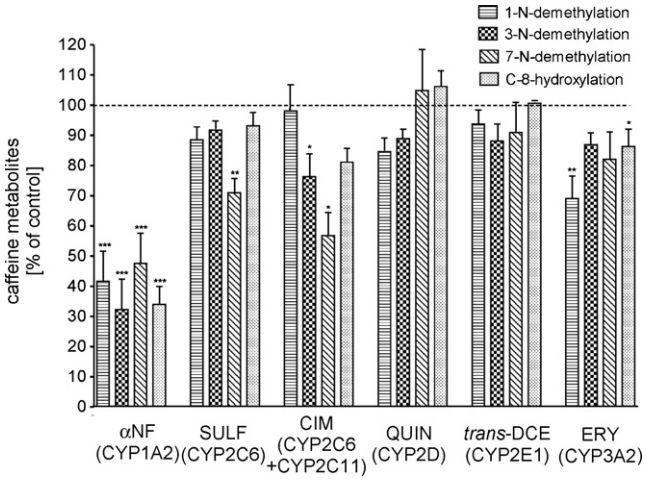

Fig. 5 – The effect of P450-specific inhibitors on the rate of caffeine 1-*N*-demethylation (A), 3-*N*-demethylation (B), 7-*N*-demethylation (C), and 8-hydroxylation (D) in rat liver microsomes. Microsomes were incubated with 100 μM caffeine in the absence (control) or presence of P450-specific inhibitors: 0.1 μM α-naphthoflavone (αNF), 5 μM sulfaphenazole (SULF), 50 μM cimetidine (CIM), 10 μM quinine (QUIN) or 400 μM erythromycin (ERY); *trans*-1,2-dichloroethylene (*trans*-DCE) was administered *in vivo* in a dose of 100 mg/kg i.p. Absolute control values were 0.0026 ± 0.0001 nmol of theobromine/mg of protein/min, 0.0036 ± 0.0001 nmol of paraxanthine/mg of protein/min, 0.0021 ± 0.0001 nmol of theophylline/mg of protein/min, 0.0144 ± 0.0001 nmol of 1,3,7-trimethyluric acid/mg of protein/min. Mean values ± S.E.M. ($n = 5$) are presented. Statistical significance was assessed using Student's *t* test and indicated with ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. For further explanation, see Fig. 2.

1-*N*- and 3-*N*-demethylations are also preferentially catalyzed by CYP1A2, to a lesser degree than 8-hydroxylation, though, while 7-*N*-demethylation is mainly mediated by isoforms of the subfamily CYP2C (66%). At higher substrate concentrations, the contribution of CYP1A2 to the metabolism of caffeine decreases in favor of the other P450s: CYP2C11 (*N*-demethylation reactions) and CYP3A2 (mainly 8-hydroxylation). The above conclusions are based on our results of the Eadie–Hofstee analysis (non-linear plots suggesting a multiple-enzyme catalysis) and the proven ability of cDNA-expressed P450s to metabolize caffeine; they are also confirmed by some further data derived from correlation and inhibition studies with liver microsomes.

The use of cDNA-expressed P450s revealed that CYP1A2 had the biggest intrinsic clearance (V_{max}/K_m) towards caffeine metabolism, which reached its highest value for 8-hydroxylation. Although lower than in the case of 8-hydroxylation, the clearance by CYP1A2 was also the highest in the case of 1-*N*- and 3-*N*-demethylation, compared to other P450s. Accordingly, the theoretically calculated contribution of P450 isoforms to caffeine oxidation pathways (on the basis of the rate of these reactions in Supersomes and the mean of P450s in the liver) indicates that CYP1A2 is a key enzyme that catalyzes 8-hydroxylation (72%) and substantially contributes to 3-*N*-demethylation (47%) and 1-*N*-demethylation (37.5%) at a caffeine concentration of 100 μM, corresponding to ''the maximum therapeutic concentration in humans''. Furthermore, also CYP2C11 considerably contributes to 3-*N*-demethylation (31%), while CYP3A2 facilitates 1-*N*-demethylation and 8-hydroxylation (17 and 15%, respectively).

The above results are in line with the observed inhibition of the four metabolic pathways of caffeine by the CYP1A2 inhibitor α-naphtoflavone (the strongest effect on 8-hydroxylation, and the weakest one on 7-*N*-demethylation) and with a significant correlation of the rate of these reactions with the activity of CYP1A2 in liver microsomes. The results obtained using cDNA-expressed P450s are also in line with the inhibition of 3-*N*-demethylation by cimetidine (a CYP2C6 + CYP2C11 inhibitor), and with the inhibition of 1-*N*-demethylation and 8-hydroxylation by erythromycin (a CYP3A inhibitor) in liver microsomes. It is noteworthy that the rate of 3-*N*-demethylation correlates with the activity of CYP2C11 (at 800 μM caffeine). Moreover, the relatively high clearance of CYP3A2 and its theoretically calculated contribution to caffeine 8-hydroxylation in rat liver correspond to the significant correlation of the rate of this reaction with the activity of CYP3A2 in liver microsomes. The contribution of CYP3A2 to 8-hydroxylation markedly increases at the higher substrate concentration of 100–800 μM (from 15 to 58%) at the expense of CYP1A2 (a decrease from 72 to 30%).

Table 3 – The influence of P450 inhibitors on the metabolism of caffeine in rat liver microsomes

P450 Inhibitors	Caffeine 1-*N*-demethylation (theobromine) K_i (μM)	Caffeine 3-*N*-demethylation (paraxanthine) K_i (μM)	Caffeine 7-*N*-demethylation (theophylline) K_i (μM)	Caffeine C-8-hydroxylation) (1,3,7-trimethyluric acid) K_i (μM)
α-Naphthoflavone (1A2)	0.083	0.085	0.136	0.048
Sulfaphenazole (2C6)	No effect	No effect	2.8	No effect
Cimetidine (2C6 + 2C11)	No effect	137.8	35.2	158
Quinine (2D)	No effect	No effect	No effect	No effect
trans-DCE (2E1)	No effect	No effect	No effect	No effect
Erythromycin (3A)	490	No effect	No effect	858

The presented inhibition constants (K_i) for inhibition of particular metabolic pathways were calculated using Dixon analysis. *Trans*-DCE, *trans*-1,2-dichloroethylene. For further explanation, see Fig. 5.

Table 4 – A correlation (*r* value) between the rate of caffeine metabolism at 100 μM (A) and 800 μM (B) caffeine concentration and the velocity of P450-specific reactions in rat liver microsomes ($n = 17$)

A Caffeine 100 μM	*Phenacetin* o-deethylation (CYP1A2)	Testosterone 7α-hydroxylation (CYP2A2)	Testosterone 16β-hydroxylation (CYP2B)	Warfarin 7-hydroxylation (CYP2C6)	Testosterone 2α-hydroxylation (CYP2C11)	Testosterone 2β-hydroxylation (CYP3A2)
Caffeine 1-N-deme-thylation	**0.7733*****	0.1592$^{N.S.}$	0.4254$^{N.S.}$	**0.4691***	0.4003$^{N.S.}$	-0.03637$^{N.S.}$
Caffeine 3-N-demethylation	**0.8241*****	0.0695$^{N.S.}$	**0.4880***	0.4307$^{N.S.}$	0.4632$^{N.S.}$	0.1294$^{N.S.}$
Caffeine 7-N-demethylation	**0.7424*****	0.1864$^{N.S.}$	0.2413$^{N.S.}$	0.3003$^{N.S.}$	0.2533$^{N.S.}$	0.0324$^{N.S.}$
Caffeine C-8-hydroxylation	**0.7910*****	0.1545$^{N.S.}$	0.2262$^{N.S.}$	0.0661$^{N.S.}$	0.0400$^{N.S.}$	**0.8767*****
B Caffeine 800 μM	*Phenacetin* o-deethylation (CYP1A2)	Testosterone 7α-hydroxylation (CYP2A2)	Testosterone 16β-hydroxylation (CYP2B)	Warfarin 7-hydroxylation (CYP2C6)	Testosterone 2α-hydroxylation (CYP2C11)	Testosterone 2β-hydroxylation (CYP3A2)
Caffeine 1-N-demethylation	**0.7775*****	0.0062$^{N.S.}$	0.3532$^{N.S.}$	0.3705$^{N.S.}$	0.2587$^{N.S.}$	0.2431$^{N.S.}$
Caffeine 3-N-demethylation	**0.8833*****	−0.0409$^{N.S.}$	**0.5472***	0.4319$^{N.S.}$	**0.5198***	0.3466$^{N.S.}$
Caffeine 7-N-demethylation	**0.5663***	−0.1525$^{N.S.}$	**0.7084****	**0.6289****	**0.8066*****	−0.0974$^{N.S.}$
Caffeine C-8-hydroxylation	**0.6391****	0.0513$^{N.S.}$	0.2538$^{N.S.}$	0.0571$^{N.S.}$	0.1124$^{N.S.}$	**0.8849*****

Each pair of data was compared by a simple linear regression analysis using the statistical program Prism 2.01 (GraphPad Software, Inc.). Statistical significance was indicated with $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$; N.S. ($p > 0.05$) is statistically not significant. Significant correlations between the rate of caffeine metabolism and the velocity of P450-specific reactions are shown in bold.

In contrast to 8-hydroxylation, the CYP1A2 clearance related to 7-*N*-demethylation is relatively low and comparable to that of CYP2C6 and CYP2C11. However, the amount of the metabolite formed by an individual P450 isoform in the liver depends not only on enzyme kinetics (intrinsic clearance), but also on the contribution of an isoform to the total P450 content. The roughly estimated contribution of P450 isoforms to the 7-*N*-demethylation of caffeine indicates that the CYP2C isoforms, mainly CYP2C6 (27%) and CYP2C11 (29%), play a major role in catalyzing this reaction. The contribution of CYP2C11 to 7-*N*-demethylation increases from 29 to 41% at a higher substrate concentration, while this of CYP1A2 shows a decreasing tendency (from 14 to 5%). The theoretically calculated contribution of the CYP2C isoforms to the 7-*N*-demethylation of caffeine can be supported by the results obtained with liver microsomes, namely those concerning inhibition of the rate of this reaction by sulfaphenazole (CYP2C6 inhibitor) and cimetidine (CYP2C6 + CYP2C11 inhibitor), as well as the positive correlation between the rate of 7-*N*-demethylation of caffeine (800 μM) and the activity of the two above-mentioned CYP2C isoforms. On the other hand, the correlation between the rate of 7-*N*-demethylation of caffeine (800 μM) and the activity of CYP1A2 is relatively weak compared to that concerning CYP2C isoforms. The substantial contribution of CYP2C11 to 7-*N*-demethylation and – as has been mentioned elsewhere – to 3-*N*-demethylation is in line with the results of our previous study obtained using P450 inducers [18] and with the findings of Bienvenu et al. [42] who observed a decrease in the rate of these reactions after a continuous infusion of the growth hormone to hypophysectomized male rats.

The correlation between the rate of 7-*N*-demethylation and the activity of CYP2B appears to be accidental due to the ability of CYP2C11 (constituting ~40% of the total P450) to catalyze the 16β-hydroxylation of testosterone, which is a marker reaction of CYP2B activity (our unpublished results). An analogous miscorrelation seems to occur between the rate of 3-*N*-demethylation and the activity of CYP2B. As has been mentioned elsewhere, the contribution of CYP2C11 to 3-*N*-demethylation reaches 31 and 50% at caffeine concentrations of 100 and 800 μM, respectively.

The above results are only partly in line with the conclusions drawn by other authors. One of the causes may be that caffeine metabolism was studied in different experimental models and/or that conclusions were drawn from one experimental model only [20,43,44]. Other possible causes are the use of unspecific inducers (or inhibitors) and relating the rates of caffeine reactions to the activity of only one (mainly an induced one) P450 isoform, as well as the application of high (1–10 mM) caffeine concentrations and not taking account of all P450 isoforms in both experimentation and data interpretation [11,12,15,19].

The obtained data only partly agree with the conclusions drawn by Morita et al. [12] which indicate that CYP3A2 is responsible for the 8-hydroxylation of caffeine. The latter authors concentrated exclusively on CYP3A2 and performed their studies on liver microsomes at a high (10 mM) caffeine concentration. As shown in our present work, CYP1A2 is the major enzyme catalyzing caffeine 8-hydroxylation at a concentration of 100 μM (relevant to "the maximum

therapeutic concentration in humans''), but the contribution of CYP3A2 to 8-hydroxylation markedly rises at higher substrate concentrations at the expense of CYP1A2. Our results do not closely correspond, either, to the findings of Chung et al. [11,19] who proposed – on the basis of unspecific P450 and FMO inducers/inhibitors [45] and using the very high 10 mM concentration of caffeine – that caffeine 8-hydroxylation was catalyzed mainly by CYP2B1, CYP3A1, CYP2E1, while *N*-demethylation reactions by CYP1A2 and FMO. Our present study indicates that FMO does not significantly contribute to caffeine metabolism measured *in vitro* at the substrate concentration of 100 μM, as shown by the thermal inactivation of FMO. On the other hand, our experiment with cDNA-expressed P450s shows that CYP2B1 and CYP2E1 only insignificantly contribute to the 8-hydroxylation and total metabolism of caffeine, which is in line with the conclusions of our recent study with selective P450 inducers [18].

Our results correspond to the hypothesis of Bienvenu et al. [44] that the *N*-demethylation reactions of caffeine are catalyzed by polycyclic hydrocarbon-inducible P450s; on the other hand, however, they do not support the hypothesis put forward by Berthou et al. [15] who – on the basis of the auto-induction of caffeine metabolism accompanied with increases in CYP1A and CYP2B protein levels and activities and in the formation of theophylline – inferred that the 7-*N*-demethylation of caffeine (1 mM) was predominantly mediated by CYP2B. In all the above-cited studies the authors did not take account of P450 isoforms of the CYP2C subfamily, which are abundantly expressed in rat liver and constitute about 80% of the total P450.

Strain differences may influence the relative contribution of P450 isoforms to caffeine metabolism in the case of Dark Agouti rats with an increased CYP3A level [12,46,47]. However, researches use mainly Wistar or Sprague–Dawley rats [11,12,15] and differences between studies have been observed within the same strain (e.g. Wistar in Morita et al. [12], and our study). Therefore it seems that different substrate concentrations (0.1–10 mM) and unspecific tests used by the authors are chiefly responsible for the observed discrepancies in the involvement of individual P450 isoforms in caffeine metabolism in rats.

The results obtained in the present research suggest that caffeine pharmacokinetics may be changed by drugs which affect the activity of CYP1A2 and/or CYP2C isoforms. Accordingly, our recent study into the effect of antidepressants on the metabolism of caffeine showed that fluoxetine given chronically increased the rate of 7-*N*-demethylation only, while sertraline and mirtazapine enhanced the rate of all caffeine oxidation pathways [48]. The latter results indicated that chronic treatment with fluoxetine-induced caffeine metabolism by enhancing the activity of CYP2C only, while administration of sertraline and mirtazapine—mainly by elevating the activity of CYP2C and CYP1A2. Indeed, our previous studies showed that the investigated antidepressants-induced CYP2C isoforms [49,28,50].

In conclusion, the results obtained in the present study show that (1) the 1-*N*- and 3-*N*-demethylation of caffeine is predominantly catalyzed by CYP1A2 and CYP2C (early studies did not take account of CYP2C); (2) 7-*N*-demethylation is governed by P450s of the CYP2C subfamily (and not by CYP2B as suggested previously); (3) 8-hydroxylation, the main oxidation pathway of caffeine in the rat, is specifically mediated by CYP1A2 (and not by CYP3A as suggested previously) at a concentration of 100 μM, corresponding to ''the maximum therapeutic concentration in humans''. At higher substrate concentrations, the contribution of CYP1A2 to the metabolism of caffeine decreases in favor of CYP2C11 (*N*-demethylations) and CYP3A2 (mainly 8-hydroxylation). Therefore, caffeine may be applied as a marker substance for assessing the activity of CYP1A2 in the rat, using 8-hydroxylation (but not 3-*N*-demethylation—like in humans) as a specific reaction. Furthermore, caffeine can be used to preliminarily and simultaneously estimate CYP2C activity using 7-*N*-demethylation as a marker reaction. The above-presented results indicate species-differences in caffeine metabolism and show for the first time the contribution of the CYP2C subfamily to this metabolism. Moreover, they suggest that caffeine pharmacokinetics in rats may be changed by drugs affecting the activity of CYP1A2 and/or CYP2C, e.g. by some antidepressants.

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