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The relative contribution of human cytochrome P450 isoforms to the four caffeine oxidation pathways: An *in vitro* comparative study with cDNA-expressed P450s including CYP2C isoforms

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ABSTRACT

The aim of the present study was to estimate the relative contribution of cytochrome P450 isoforms (P450s), including P450s of the CYP2C subfamily, to the metabolism of caffeine in human liver. The experiments were carried out *in vitro* using cDNA-expressed P450s, liver microsomes and specific P450 inhibitors. The obtained results show that (1) apart from the 3-N-demethylation of caffeine – a CYP1A2 marker reaction and the main oxidation pathway of caffeine in man – 1-N-demethylation is also specifically catalyzed by CYP1A2 (not reported previously); (2) 7-N-demethylation is catalyzed non-specifically, mainly by CYP1A2 and, to a smaller extent, by CYP2C8/9 and CYP3A4 (and not by CYP2E1, as suggested previously); (3) C-8-hydroxylation preferentially involves CYP1A2 and CYP3A4 and, to a smaller degree, CYP2C8/9 and CYP2E1 (and not only CYP3A, as suggested previously) at a concentration of 100 μM corresponding to the maximum therapeutic concentration in humans. At a higher caffeine concentration, the contribution of CYP1A2 to this reaction decreases in favour of CYP2C8/9. The obtained data show for the first time the contribution of CYP2C isoforms to the metabolism of caffeine in human liver and suggest that apart from 3-N-demethylation, 1-N-demethylation may also be used for testing CYP1A2 activity. Moreover, they indicate that the C-8-hydroxylation is not exclusively catalyzed by CYP3A4.

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1. Introduction

Caffeine (1,3,7-trimethylxanthine), the most widely used psychoactive substance [1,2], recently found to possess neuroprotective properties [3–5], is an established marker substrate for testing CYP1A2 activity using 3-N-demethylation in humans [6,7] and C-8-hydroxylation in rats [8].

Numerous studies show that 3-N-demethylation to paraxanthine (the main oxidation pathway) in humans is specifically catalyzed by CYP1A2; moreover, they suggest that other oxidation pathways of caffeine may be mediated – at least partly – by P450 isoforms different from CYP1A2. Using liver microsomes and CYP1A specific inhibitors or antibodies, it has been shown that human CYP1A2 plays a pivotal role in caffeine metabolism, especially in catalyzing N-demethylation reactions [9–12]. Further studies with selected cDNA-expressed CYP isoforms or CYP1A2 and CYP2E1 cell lines have indicated that caffeine 3-N-demethylation is most efficiently

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Abbreviations: CYP, cytochrome P450; HPLC, high performance liquid chromatography; DDC, diethyldithiocarbamate; FUR, furafylline; SULF, sulfaphenazole; KET, ketoconazole; P450, cytochrome P450.

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catalyzed by CYP1A2, while the CYP3A subfamily is the main isoenzyme catalyzing C-8-hydroxylation to 1,3,7-trimethyluric acid. In addition, CYP2E1 may also contribute to 1-N- and 7-N-demethylation [13–18]. However, CYP2C isoforms (CYP2C8/9/18/19) which constitute over 20% of total human liver P450 have not been studied in this respect so far. For this reason precise qualitative and relative quantitative estimation of the contribution of individual P450 isoforms to the specific metabolic pathways of caffeine was not possible.

Since our earlier studies with rats revealed that P450s of the CYP2C subfamily were important for caffeine metabolism (in particular for 7-N-hydroxylation) and that the contribution of these isoforms to caffeine metabolism was concentration-dependent [8,19], we attempted to carry out a complementary investigation comprising all human drug-metabolizing P450s, including CYP2C isoforms. The present comparative study carried out on a full set of human cDNA-expressed P450s allowed us to estimate for the first time the relative contribution of individual P450s to the four oxidative metabolic reactions of caffeine. The obtained results show that CYP2C8/9 isoforms substantially contribute to the 7-N-demethylation and C-8-hydroxylation of caffeine (Fig. 1).

2. Materials and methods

2.1. Drugs and chemicals

Caffeine and its metabolites—theobromine, paraxanthine, theophylline and 1,3,7-trimethyluric acid, as well as furafylline, sulfaphenazole, ketoconazole and NADPH were purchased from Sigma (St. Louis, USA). All the organic solvents with HPLC purity were supplied by Merck (Darmstadt, Germany).

2.2. Human liver microsomes–kinetic and inhibition studies

The liver microsomes from individual patients HK23, HK37, HG56, HG89, HG93 and the pooled liver microsomes from patients HH02, HH03, HH04, HH10, HH12, HH16, HH24, HH25, HH32, HH33, HH41, HH49, HH55, HH64, HH68, HH72, HH74, HH89 were obtained from Gentest Co. (Woburn, MA, USA) (Table 1).

Studies into caffeine metabolism in human liver microsomes were carried out at the linear dependence of product formation on time, protein and substrate concentration. To determine enzyme kinetics (Eadie–Hofstee plots), the caffeine concentrations used ranged from 0.05 to 1.6 mM. For inhibition studies, 100 μM caffeine was incubated with pooled liver microsomes in the absence or presence of one of the selective inhibitors added *in vitro* : 10 μM furafylline (a CYP1A2 inhibitor), 10 μM sulfaphenazole (a CYP2C9 inhibitor), 2 μM ketoconazole (a CYP3A inhibitor). Incubation was carried out in a system containing liver microsomes (ca. 0.5 mg of protein/ml), a phosphate buffer (0.15 M, pH 7.4) and NADPH (1 mM). The final incubation volume was 1 ml. After a 2-min (with furafylline) or 3-min (with sulfaphenazole or ketoconazole) preincubation at 37 °C, the reaction was initiated by adding caffeine. After a 60-min incubation, the reaction was terminated by adding 700 μl of a 2% $ZnSO_4$ and 50 μl of 2 M HCl. Caffeine and its metabolites were analyzed by the high-performance liquid chromatography method (HPLC) described below.

2.3. cDNA-expressed human P450s

Microsomes from baculovirus-infected insect cells expressing CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6,

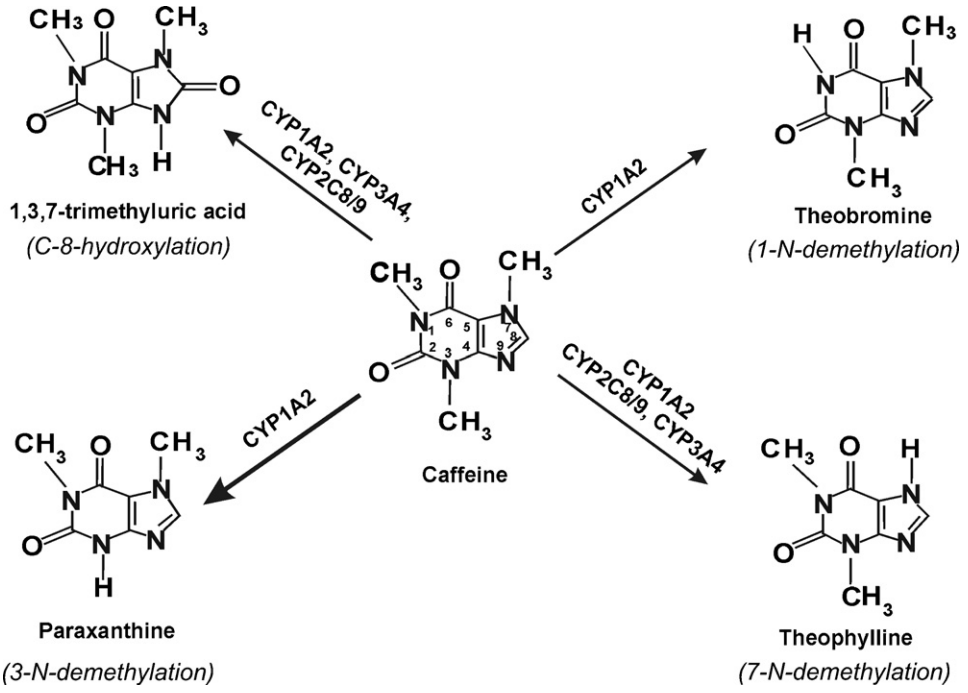

Fig. 1 – The main metabolic pathways of caffeine and the contribution of P450 isoforms (based on the present study).

Table 1 – Clinical characteristics of liver specimen donors

Patient	Age (years)	Gender	Cause of death	Medical history	Drugs
HH02	54	Male	Ascending aorta aneurysm	Not available	Not available
HH03	38	Male	Head injury	Allergy based asthma	Not available
HH04	66	Female	Intracranial bleeding	Hypertension	Not available
HH10	84	Male	Intracranial hemorrhage	Hypertension, asthma, arthritis	Heparin, dopamine, pitressin
HH12	66	Female	Cardiac arrest	Coronary artery disease, peripheral vascular disease, hypertension, bilateral endarectomy, ischemic cardiomyopathy, diabetes mellitus	Dopamine, neosynephrine, T4
HH16	54	Male	Anoxia	Myasthenia-gravis, appendectomy	Levoquin, clindamycin, mannitol, dopamine, esmolul, nipride, T4
HK23	24	Male	Head injury	Not available	Not available
HH24	53	Female	Cerebral vascular accident	St. John's wort	Ancef, heparin
HH25	66	Female	Intracerebral hemorrhage	Hypertension, stomach ulcer, rheumatoid arthritis	Not available
HH32	77	Female	Intracranial hemorrhage	Diabetes, hypertension, hypothyroid, angina	Glucotrol, synthroid, digoxin, levostatin, ticlid, glipizide, lopressor
HH33	65	Female	Intracranial bleeding	Not available	Not available
HK37	56	Female	Brain hemorrhage	Not available	Not available
HH41	58	Male	Pulmonary hypertension	Chronic heart failure, emphysema, cardiac and respiratory problems	Not available
HH49	44	Female	Intracranial bleeding	Hysterectomy, occasional depression	Welburtin
HH55	52	Male	Cerebral vascular accident	Hypertension	Nipride, dopamine, atropine, ancef
HG56	57	Female	Aneurysm	Not available	Not available
HH64	77	Male	Cerebral vascular accident	Hypertension, mild diabetes, gallbladder removed	Heparin, levophed, insulin, propofol, lopressor, zantac, vasopresson
HH68	47	Male	Head trauma	Not available	Dilantin, vadopressin, neosynepione
HH72	54	Male	Intracranial bleeding	Not available	Not available
HH74	55	Male	Intracranial bleeding	Not available	Not available
HH89	33	Male	Stroke	Not available	Not available
HG89	71	Female	Intracranial hemorrhage	Not available	Not available
HG93	45	Female	Closed head trauma	Not available	Dopamine, pitressin, levofed, synthroid

CYP2E1 and CYP3A4/5 co-expressed with NADPH P450 oxidoreductase (Supersomes) were obtained from Gentest Co. (Woburn, MA, USA). CYP2E1 was also co-expressed with cytochrome 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 and the amount of P450 isoforms and substrate concentration. Caffeine metabolism was studied under experimental conditions similar to those described for liver microsomes using 50, 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 60 min for CYP1A2, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1 and CYP3A4, but 200 pmol/ml and 15 min for CYP2A6 and CYP3A5.

2.4. *Determination of caffeine and its metabolites*

Caffeine and its four primary metabolites were assessed using the HPLC method based on the procedure of Rasmussen et al. [20], as described previously [21]. 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 yielded 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).

3. Results

3.1. *Caffeine metabolism in human liver microsomes*

It was shown that the 3-N-demethylation of caffeine was a major metabolic reaction in human liver microsomes (~70%)

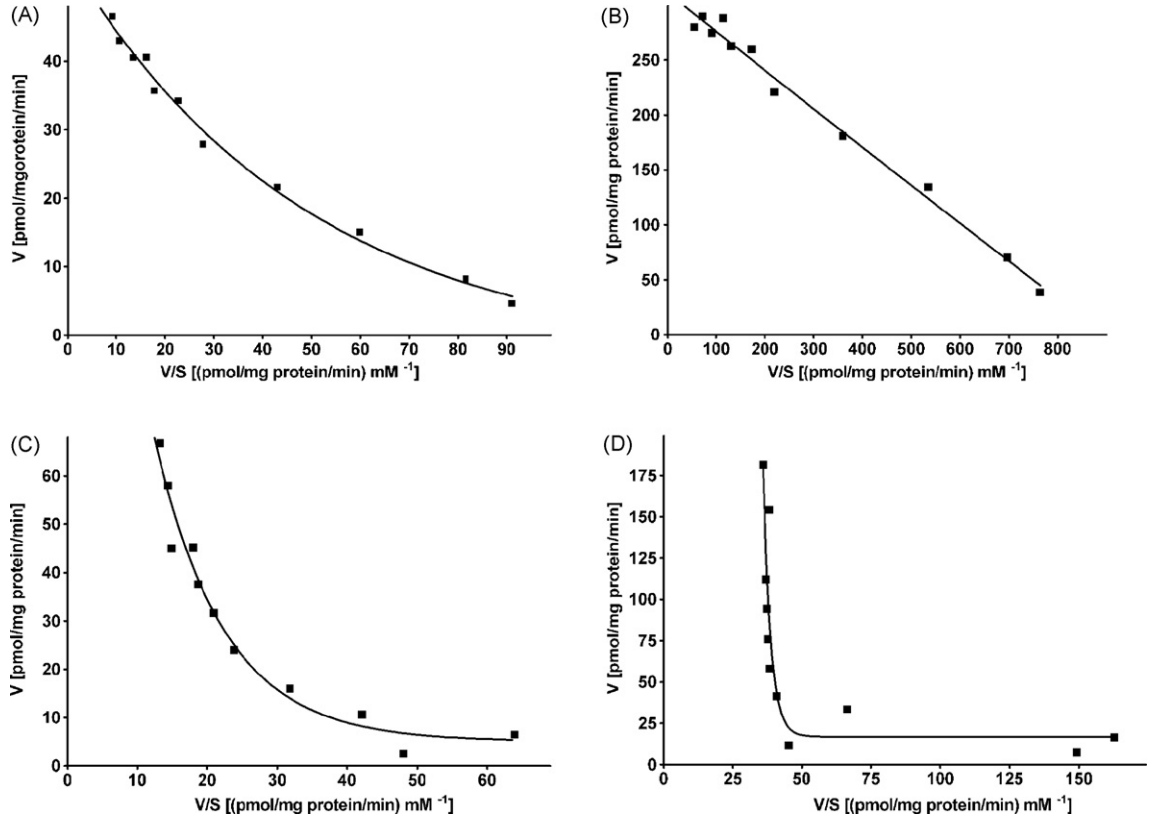

Fig. 2 – The Eadie–Hofstee plots for caffeine 1-N-demethylation (A), 3-N-demethylation (B), 7-N-demethylation (C), C-8-hydroxylation (D) in human liver microsomes. Human liver microsomes of donor HG56 were incubated in a 0.15 M phosphate buffer (pH 7.4) with caffeine (0.05–1.6 mM) and NADPH (1 mM) for 60 min.

compared to 1-N- and 7-N-demethylation (7–8%) and C-8-hydroxylation (~15%), as measured at the substrate concentration of 100 μM. Fig. 2A–D shows the Eadie–Hofstee plots for caffeine oxidation processes in liver microsomes of donor HG56. The plot of 3-N-demethylation was linear, which suggests a one-enzyme catalysis of the reaction. All the other plots were non-linear (the curvature of the 1-N-demethylation plot being very gentle, though), which indicates a multiple-enzyme catalysis. Similar results (with regard to the form of the curvature and quantitative results) were observed with microsomes of donors HK23, HK37, HG89 and HG93.

3.2. *Study with human cDNA-expressed P450s*

Kinetic parameters showing caffeine metabolism in human cDNA-expressed P450s, obtained by a non-linear analysis (Program Sigma Plot 8.0; Enzyme Kinetics), are presented in Table 2. These kinetic parameters indicate distinct inter-isoform differences and are consistent with the Eadie–Hofstee plots derived from liver microsomes (Fig. 2). CYP1A2 showed the biggest intrinsic clearance (V_{max}/K_m) towards caffeine metabolism, its highest value being reached for 3-N-demethylation.

According to the calculated intrinsic clearance values, the preference of P450 enzymes for the catalysis of caffeine metabolism was as follows (pmol of product/pmol of P450 isoform/min):

CYP1A2 > CYP2D6 > CYP2C8 > CYP2C19 > CYP3A5 > CYP2C9 > CYP2B6 = CYP2C18 > CYP3A4 > CYP2A6 for 1-N-demethylation;

CYP1A2 > CYP3A4 > CYP2C9 > CYP2C8 > CYP2D6 > CYP2E1 > CYP2C19 > CYP2C18 > CYP2B6 > CYP2A6 > CYP3A5 for 3-N-demethylation;

CYP1A2 > CYP2D6 > CYP2C9 > CYP2E1 > CYP2A6 > CYP3A5 > CYP2C19 > CYP2C8 > CYP2B6 > CYP2C18 > CYP3A4 for 7-N-demethylation;

CYP1A2 > CYP2B6 > CYP2E1 > CYP2D6 > CYP2C18 > CYP3A4 > CYP3A5 > CYP2C19 > CYP2A6 > CYP2C8 > CYP2C9 for C-8-hydroxylation. The ability of human cDNA-expressed P450s to metabolize caffeine at the 100 μM concentration is shown in Fig. 3A–D.

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 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 3). The calculations done at the therapeutic concentration of caffeine (100 μM) indicated that CYP1A2 was the main isoform responsible for caffeine metabolism. CYP1A2 was a chief enzyme catalyzing 1-N- and 3-N-demethylation (75 and 85%, respectively) and substantially

Table 2 – Kinetic parameters of caffeine metabolism in cDNA-expressed human CYPs (Supersomes)

P450	1-N-demethylation (theobromine)			3-N-demethylation (paraxanthine)			7-N-demethylation (theophylline)			C-8-hydroxylation (1,3,7-trimethyluric acid)		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
1A2	2.56	0.341	0.133	2.96	3.34	1.128	1.87	0.377	0.201	0.39	0.187	0.479
2A6	40.64	0.068	0.002	1.14	0.039	0.034	0.60	0.042	0.070	1.72	0.172	0.100
2B6	2.31	0.019	0.008	0.64	0.027	0.042	4.13	0.194	0.047	0.55	0.195	0.354
2C8	0.92	0.014	0.015	0.20	0.016	0.080	3.56	0.172	0.048	3.37	0.319	0.095
2C9	2.17	0.021	0.010	0.13	0.014	0.108	1.03	0.087	0.084	3.88	0.318	0.082
2C18	4.93	0.042	0.008	0.60	0.026	0.043	337.3	13.180	0.039	0.97	0.170	0.175
2C19	11.93	0.154	0.013	1.42	0.069	0.048	6.05	0.327	0.054	2.37	0.294	0.124
2D6	74.74	1.242	0.017	0.755	0.050	0.066	24.70	3.75	0.152	1.93	0.441	0.228
2E1	n.d.	n.d.	n.d.	1.37	0.072	0.052	1.77	0.141	0.080	0.90	0.299	0.332
3A4	16.04	0.107	0.007	0.09	0.014	0.156	3.14	0.098	0.031	0.91	0.157	0.172
3A5	0.88	0.011	0.012	2.07	0.033	0.016	1.32	0.079	0.060	2.15	0.340	0.158

n.d.—not detected. K_m [mM], V_{max} [pmol/pmol P450 isoform/min].

contributing to 7-N-demethylation (38.7%) and C-8-hydroxylation (28.7%). Moreover, 7-N-demethylation was also visibly catalyzed by CYP2C8 (12.8%), CYP2C9 (12.2%) and CYP3A4 (13.6%), while C-8-hydroxylation was substantially mediated by CYP3A4 (30%) and, to a lesser extent by CYP2C8 (10%), CYP2C9 (8%) and CYP2E1 (11%). At a higher concentration of the substrate (1 mM), the contribution of CYP1A2 to caffeine C-8-hydroxylation decreased (to 16%) mostly in favour of CYP2C8 and CYP2C9 (to 17 and 11.6%, respectively) (Table 3 and Fig. 4).

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

Furafylline (a CYP1A2 inhibitor) exerted a strong inhibitory effect on the rate of the four oxidative caffeine reactions, being most effective for 1-N- and 3-N-demethylation (a drop to 5 and 2%, respectively, of the control value), and the least active towards C-8-hydroxylation (a decrease to 45% of the control value) (Fig. 5). Sulfaphenazole (a CYP2C9 inhibitor) significantly decreased the rate of 7-N-demethylation and C-8-

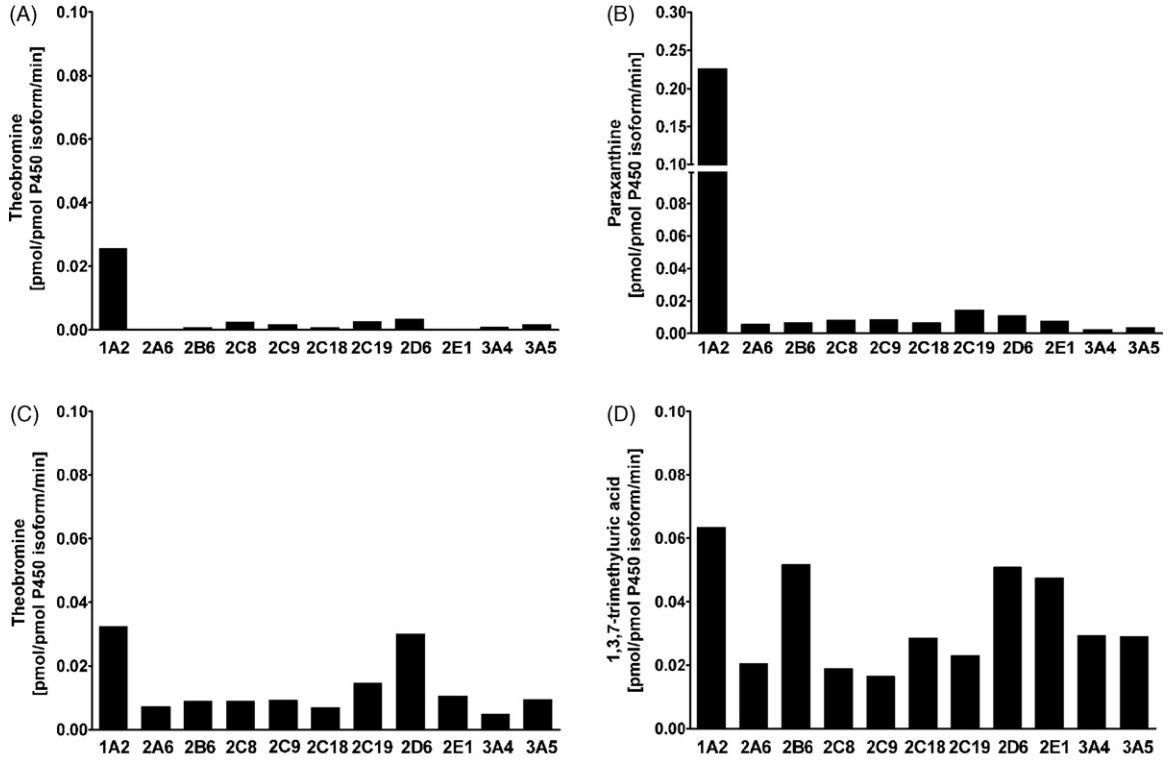

Fig. 3 – The biotransformation of caffeine *via* 1-N-demethylation (A), 3-N-demethylation (B), 7-N-demethylation (C), C-8-hydroxylation (D) by human cDNA-expressed P450s (Supersomes). Caffeine (100 μM) was incubated with the Supersomes (100–200 pmol P450/ml) and NADPH (1 mM) for 60 min. Each bar represents the mean value of two independent analyses.

Table 3 – 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 isoform to the total P450 contents in liver microsomes (Fraction)	Relative contribution of the isoforms to caffeine metabolism in liver microsomes (percentage)#							
		Caffeine 1-N-demethylation		Caffeine 3-N-demethylation		Caffeine 7-N-demethylation		Caffeine C-8-hydroxylation	
		100 μM	1000 μM	100 μM	1000 μM	100 μM	1000 μM	100 μM	1000 μM
CYP1A2	0.127[a]	**75.12**	**66.88**	**85.16**	**92.00**	**38.74**	**35.28**	**28.75**	**15.98**
CYP2A6	0.040[a]	0.30	0.55	0.67	0.32	2.76	1.56	2.91	2.17
CYP2B6	0.002[a]	0.02	0.06	0.04	0.03	0.17	0.19	0.35	0.22
CYP2C8	0.150[b]	8.31	5.14	3.69	1.18	**12.80**	**13.46**	**10.06**	**17.44**
CYP2C9	0.140[b]	5.50	5.85	3.57	1.01	**12.21**	**11.93**	**8.20**	**11.63**
CYP2C18	0.005[c]	0.08	0.13	0.10	0.06	0.33	0.23	0.51	0.45
CYP2C19	0.061[c]	3.66	3.31	2.62	1.14	8.41	5.80	5.00	7.02
CYP2D6	0.015[a]	1.20	2.01	0.49	0.30	4.24	5.10	2.71	2.25
CYP2E1	0.066[a]	n.d.	7.46	1.47	1.70	6.56	8.74	**11.13**	**9.26**
CYP3A4	0.288[a]	5.73	8.55	2.18	2.25	**13.60**	**17.60**	**30.16**	**33.27**
CYP3A5	0.002[d]	0.07	0.06	0.02	0.01	0.18	0.10	0.20	0.31

n.d.—not detected. Data according to [a]Shimada et al. [22], [b]Edwards et al. [23], [c]Jung et al. [24] and [d]Rodrigues [25]. #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. [26]. Essential differences between 100 and 1000 μM concentration of caffeine are shown in bold.

hydroxylation (to 75 and 80%, respectively, of the control value), not affecting other reactions. Ketoconazole (a CYP3A4 inhibitor) reduced the rate of 1-N- and 7-N-demethylation and C-8-hydroxylation (to 85, 70 and 45%, respectively, of the control value).

4. Discussion

The data presented above constitute a complete report on the qualitative and quantitative contribution of all the individual drug-metabolizing P450 isoforms to the four oxidation pathways of caffeine metabolism in humans. The obtained results

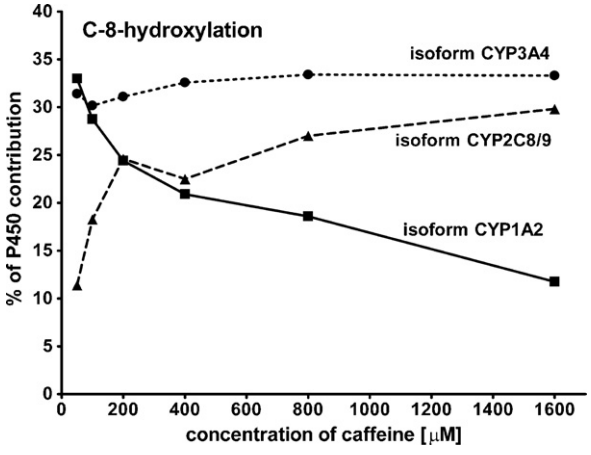

Fig. 4 – The concentration-dependent contribution of P450 isoforms to caffeine 8-hydroxylation in human liver (based on the rate of caffeine metabolism in the Supersomes and the mean of P450s in the liver). For further explanations see Fig. 3.

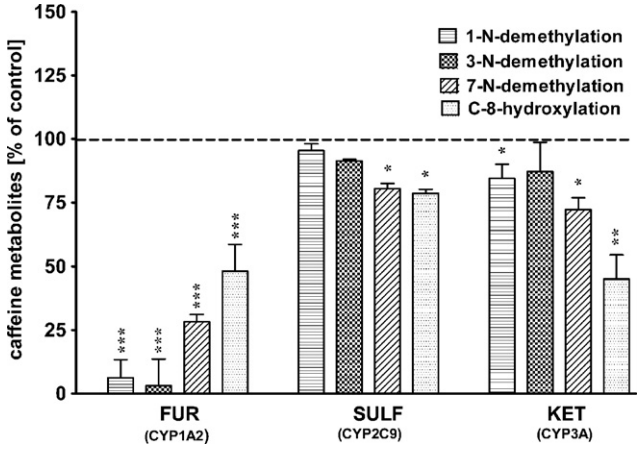

Fig. 5 – The effect of P450-specific inhibitors on the rate of caffeine 1-N-demethylation, 3-N-demethylation, 7-N-demethylation and C-8-hydroxylation in pooled human liver microsomes. The microsomes were incubated with 100 μM caffeine in the absence (control) or presence of P450-specific inhibitors: 10 μM furafylline (FUR), 10 μM sulfaphenazole (SULF), 2 μM ketoconazole (KET). The absolute control values were 0.0023 ± 0.00001 nmol of theobromine/mg of protein/min, 0.0240 ± 0.001 nmol of paraxanthine/mg of protein/min, 0.0048 ± 0.0002 nmol of theophylline/mg of protein/min, 0.0026 ± 0.0003 nmol of 1,3,7-trimethyluric acid/mg of protein/min. The mean values ± S.E.M. ($n = 6$) are presented. Statistical significance was assessed using Student's test, and was indicated with ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$. For further explanations, see Fig. 2.

confirm the principal role of the CYP1A2 isoform in the metabolism of caffeine. They show that the main oxidation pathway of caffeine in humans – 3-N-demethylation (70%) – is specifically catalyzed by CYP1A2 (85%) at a concentration of 100 μM, which corresponds to the maximum therapeutic concentration in humans [27,28] and supports the earlier results of Berthou et al. [11,12] and Tassaneeyakul et al. [17]. Moreover, our study presents a few new findings indicating that also 1-N-demethylation is governed to a great extent by CYP1A2 (75%), while 7-N-demethylation and C-8-hydroxylation are catalyzed less specifically–by CYP1A2 and other CYP isoforms, mainly CYP3A4 and CYP2C8/9. The above conclusion is based on our consistent results of the Eadie–Hofstee analysis, demonstration of the ability of cDNA-expressed P450s to metabolize caffeine and inhibition studies.

Kinetic parameters showing caffeine metabolism in human cDNA-expressed P450s indicate distinct inter-isoform differences, which is consistent with the Eadie–Hofstee plots derived from liver microsomes, the latter being indicative of the one-enzyme catalysis of 3-N-demethylation (and the fairly similar catalysis of 1-N-demethylation) on one hand, and the visibly multiple-enzyme catalysis of 7-N-demethylation and C-8-hydroxylation on the other.

The results obtained using cDNA-expressed P450s showed that CYP1A2 displayed the highest intrinsic clearance towards caffeine metabolism, its highest value being reached for 3-N-demethylation (Table 2). The intrinsic clearance of CYP1A2 was also the highest among the P450s tested in the case of other oxidation pathways, and it descended in the following order: 3-N-demethylation > C-8-hydroxylation > 7-N-demethylation > 1-N-demethylation. We roughly estimated the contribution of the P450 isoforms studied to caffeine 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 3). The calculations done at a caffeine concentration of 100 μM, which corresponds to the maximum therapeutic concentration in humans indicate that CYP1A2 is the main isoform responsible for caffeine metabolism. CYP1A2 is the chief enzyme catalyzing 1-N- and 3-N-demethylation (75 and 85%, respectively) and substantially contributing to 7-N-demethylation (38.7%) and C-8-hydroxylation (28.7%). Moreover, 7-N-demethylation and C-8-hydroxylation are also considerably mediated by CYP3A4 (13.6 and 30%, respectively) and CYP2C8/9 (25 and 18%, respectively) and, to a lesser extent by CYP2E1 in the case of C-8-hydroxylation (11%). At a higher concentration of the substrate, the contribution of CYP1A2 to caffeine C-8-hydroxylation decreases, mostly in favour of CYP2C8/9 (from 18 to 29%). It is noteworthy that the present results, based on cDNA-expressed P450s and the average values of P450s in the liver, are theoretical, since actual results should depend on the inter-individual variability of P450s and the diverse contribution of individual P450s to the total content of P450 protein in the liver. We based our calculations mainly on the mean values of P450s collected from 60 donors (30 Caucasians and 30 Japanese), provided by Shimada et al. [22]. Moreover, we included the results obtained by Edwards et al. [23] who quantitatively determined CYP2C8 and CYP2C9 in 30 individuals, and the data obtained by Jung et al. [24] who estimated separately CYP2C18 and CYP2C19. In general, the values of the above-discussed contribution of individual P450 isoforms to the total P450 content [22–24] and to caffeine metabolism (Table 3) are similar to those calculated on the basis of the meta-analysis of data on P450s abundance in Caucasians [29], the latter values concerning CYP2C8 being lower and those referring to CYP2C9 and CYP2E1 higher, though. Importantly, however, in the case of the meta-analysis [29] based on a large number data from various sources, it should be kept in mind that P450 contribution values for individual isoforms were obtained from different groups of subjects (as regards their number and the examined individuals themselves), which accounts for the qualitative difference in estimating particular enzymes.

The above results obtained using human cDNA-expressed P450s are in line with the data on inhibition of the four metabolic pathways of caffeine by the CYP1A2 inhibitor furafylline, which had the strongest effect on 1-N- and 3-N-demethylation, and the weakest on C-8-hydroxylation (Fig. 5). Furthermore, the above findings agree with the inhibition of 7-N-demethylation and C-8-hydroxylation by sulfaphenazole (a CYP2C9 inhibitor) and ketoconazole (a CYP3A4 inhibitor) in human liver microsomes.

Hence the results on the relative contribution of P450 isoforms to the metabolism of caffeine, obtained in the present study, confirm the high CYP1A2-selective dependence of caffeine 3-N-demethylation [11,17,18], but do not support the formerly suggested involvement of CYP2E1 in 1-N- and 7-N-demethylation [13,17,18], or the predominant role of CYP3A4 in caffeine C-8-hydroxylation [17,18] in human liver. The above-discussed differences between our present results and those of other authors may stem from the different *in vitro* model used (liver microsomes and selected P450 inhibitors; selected cDNA-expressed P450 isoforms or CYP1A2 and CYP2E1 cell lines) [14,15,17], the high caffeine concentrations (above 0.1 mM) used in '*in vitro* experiments' as reviewed by Ha et al. [18], the use of unspecific P450 inhibitors [17], such as α-naphtoflavone or diethyldithiocarbamate—DDC [30,31], and the fact that the CYP2C isoforms were not taken into account. The latter fact allowed us to calculate the relative contribution of individual isoforms to the metabolism of caffeine in human liver.

In conclusion, the obtained data show that (1) apart from the 3-N-demethylation of caffeine–a CYP1A2 marker reaction and the main oxidation pathway of caffeine in man–1-N-demethylation is also specifically catalyzed by CYP1A2 (not described previously); (2) 7-N-demethylation is non-specifically catalyzed, mainly by CYP1A2 and, to a lesser extent by CYP2C8/9 and CYP3A4 (and not by CYP2E1, as suggested previously); (3) C-8-hydroxylation preferentially involves CYP1A2 and CYP3A4 and to a lesser degree CYP2C8/9 and CYP2E1 (but not only CYP3A, as suggested previously) at a concentration of 100 μM, which corresponds to the maximum therapeutic concentration in humans. At a higher caffeine concentration, the contribution of CYP1A2 to this reaction decreases in favour of CYP2C8/9.

These data show for the first time the contribution of CYP2C isoforms to the metabolism of caffeine in human liver and suggest that, apart from 3-N-demethylation, 1-N-demethylation may also be used for testing CYP1A2 activity.

Moreover, they indicate that the C-8-hydroxylation is not exclusively catalyzed by CYP3A4.

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