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EFFECTS OF ANTIDEPRESSANT DRUGS ON THE ACTIVITY OF CYTOCHROME P-450 MEASURED BY CAFFEINE OXIDATION IN RAT LIVER MICROSOMES

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Caffeine is a marker drug for testing the activity of CYP1A2 (3-N-demethylation) in humans and rats. Moreover, it is also a relatively specific substrate of CYP3A (8-hydroxylation). In the case of 1-N- and in particular 7-N-demethylation of caffeine, apart from CYP1A2, other cytochrome P-450 isoenzymes play a considerable role. The aim of the present study was to investigate the influence of imipramine, amitriptyline and fluoxetine on cytochrome P-450 activity measured by caffeine oxidation in rat liver microsomes. The obtained results showed that imipramine exerted a most potent inhibitory effect on caffeine metabolism. Imipramine decreased the rate of 3-N-, 1-N- and 7-N-demethylations, and 8-hydroxylation of caffeine, the effect on 3-N-demethylation being most pronounced (K_i = 33 μM). Amitriptyline showed distinct inhibition of 3-N- and 1-N-demethylation of caffeine, though its effect was less potent than in the case of imipramine (K_i = 57 and 61 μM, respectively). The influence of amitriptyline on 8-hydroxylation and especially on 7-N-demethylation of caffeine was weaker (K_i = 108 and 190 μM, respectively) than on 3-N- or 1-N-demethylation, suggesting a narrower spectrum of cytochrome P-450 inhibition by amitriptyline than by imipramine, involving mainly the subfamily CYP1A2, and – to a lesser degree – CYP3A. In contrast to the tested tricyclic antidepressants, fluoxetine did not exert any considerable effect on the 3-N- or 1-N-demethylation of caffeine (K_i = 152 and 196 μM, respectively), which indicates its low affinity for CYP1A2. However, fluoxetine displayed a clear inhibitory effect on caffeine 7-N-demethylation (K_i = 72 μM), the reaction which is catalyzed mainly by other than CYP1A2 isoenzymes. Fluoxetine diminished markedly the 8-hydroxylation of the marker drug; as reflected by K_i values, the potency of inhibition of rat CYP3A by fluoxetine was similar to that of imipramine (K_i = 40 and 45 μM, respectively). In summary, CYP1A2 was distincly inhibited by imipramine and amitriptyline, CYP3A by imipramine and fluoxetine, while other CYP isoenzymes (CYP2B and/or 2E1) by imipramine and fluoxetine.

Key words: *caffeine metabolism, rat, cytochrome P-450 activity, imipramine, amitriptyline, fluoxetine*

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INTRODUCTION

The cytochrome P450 1A subfamily (CYP1A) contains two members, CYP1A1 and CYP1A2 which catalyze the activation of procarcinogens such as polycyclic aromatic hydrocarbons and aromatic N-aryl amines, respectively. Moreover, CYP1A2 catalyzes the metabolism of certain drugs, e.g. phenacetin O-deethylation, propranolol side chain hydroxylation, and N-demethylation of tricyclic antidepressants and xanthine derivatives [10, 13]. There is also evidence for the implication of CYP1A enzymes in the metabolism of various steroids [10]. The 3-N-demethylation of caffeine is considered as a specific reaction for testing CYP1A2 activity in humans and laboratory animals. Expression of CYP1A2 occurs exclusively in the liver, whereas CYP1A1 is expressed in the liver and extrahepatic tissues, such as the lungs, skin and placenta. CYP1A2 is constitutively present in human liver while CYP1A1 is considered as a non-constitutive enzyme, though low levels of CYP1A1 mRNA and CYP1A1 activity have been found in human liver [14, 17] and minor immunoreactive bands of CYP1A1 were detected in the rat and rabbit [3].

Caffeine (1,3,7-trimethylxanthine) is extensively metabolized in humans and laboratory animals. The primary metabolic pathways of caffeine are 3-N-demethylation to paraxanthine (1,7-dimethylxanthine), 1-N-demethylation to theobromine, and 7-N-demethylation to theophylline (1,3-dimethylxanthine) which account for about 80, 11 and 4%, respectively, of caffeine metabolism in humans *in vivo* and *in vitro* [3, 12]. The three demethylation pathways are about equal in the mouse, rabbit and rat *in vitro* [3]. 8-Hydroxylation to 1,3,7-trimethyluric acid, C8-C9 bond scission to form 6-amino-5--[N-formylmethylamino]-1,3-dimethyluric acid, and renal elimination of unchanged drug account for the rest of caffeine elimination in man.

Experiments with use of V79 Chinese hamster cells engineered for stable expression of single forms of rat CYP1A1, CYP1A2, CYP2B1 and human CYP1A2 as well as rat liver epithelial cells expressing murine CYP1A2 showed that caffeine was metabolized by human, rat and murine CYP1A2 resulting in the formation of the four primary demethylated and hydroxylated metabolites [11]. However, there were differences in the relative amounts of the metabolites. The human and the mouse CYP1A2 isoforms predominantly mediated 3-N-demethylation. The rat CYP1A2 mediated 3-N- and 1-N-demethylation to a similar extent; the rates of the reactions were ca. twice as high as 7-N-hydroxylation and ca. 5 times higher than 8-hydroxylation. All cell lines were able to catalyze 8-hydroxylation, with highest activities in cell lines expressing rat CYP1A2. On the other hand, studies with cDNA expressed human CYP1A1 and CYP1A2 in yeast showed that both isoenzymes formed paraxanthine and minor amount of theobromine, while theophylline was not formed by either enzyme [10]. 1,3,7-Trimethyluric acid was exclusively formed by CYP1A1. Berthou et al. [1, 2, 3] also suggested the involvement of both CYP1A2 and other isoenzymes in the first step of caffeine metabolism in human and rat liver microsomes. They stated that CYP1A2 catalyzed the 3-N- and 1-N-demethylation in human and rats while 7-N-demethylation leading to the formation of theophylline engaged other than CYP1A subfamily. They suggested CYP2B1/B2 as being involved in 7-N-demethylation of caffeine in rats [2]. Recent results of Chung et al. [4] obtained by using inducer-treated rat liver microsomes that were characterized with testosterone metabolic pattern pointed to the involvement of CYP3A1, CYP2B1 and CYP2E1 in 8-hydroxylation, and CYP1A2 and flavin containing monooxygenase in the three N-demethylations of caffeine.

Studies of caffeine metabolism carried out on human liver microsomes characterized with cytochrome P-450 (CYP) isoform-specific activities and immunoreactive CYP protein contents as well as investigation using cDNA-expressed human CYP isoforms (COS-7 cells) indicated major involvement of CYP1A2 in the high affinity components of all three human hepatic N-demethylations and of CYP2E1 in the low affinity components of 1-N- and 7-N-demethylation of caffeine [19]. 8-Hydroxylation to 1,3,7-trimethyluric acid seemed to be catalyzed predominantly by CYP3A with a minor contribution of CYP2E1 and CYP1A. The literature facts presented above indicate that caffeine is a marker substrate for measurement of CYP1A2 activity (3-N-demethylation) and a relatively specific substrate of CYP3A (8-hydroxylation), while the contribution of CYP isoenzymes to 1-N- and 7-N-demethylation of caffeine requires further studies.

The aim of our studies was to estimate a possible inhibitory effect of the three frequently used antidepressant drugs, imipramine, amitriptyline and fluoxetine on the CYP activity measured by the rate of caffeine metabolism *in vitro*.

MATERIALS and METHODS

Chemicals

Imipramine hydrochloride was obtained from Polfa, Jelenia Góra, Poland. Amitriptyline hydrochloride was provided by H. Lundbeck A/S, Copenhagen, Denmark. Fluoxetine hydrochloride was obtained from Eli Lilly, Indianapolis, USA. Caffeine and its metabolites, NADP, DL-isocitric acid (trisodium salt) and isocitric dehydrogenase were purchased from Sigma, St. Louis, USA. All organic solvents with HPLC purity were supplied by Merck, Darmstadt, Germany.

Animals

The experiment was 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.

In vitro studies into caffeine metabolism in rat liver microsomes

Pooled liver microsomes from six control rats were used. Each sample was prepared in duplicate. The caffeine metabolism in liver microsomes was studied at linear dependence of the product formation on time, and protein and substrate concentrations. The rates of 3-N-, 1-N- and 7-N-demethylation and 8-hydroxylation of caffeine (caffeine concentrations: 100, 400, 800 nmol/ml) were assessed in the absence and presence of one of the antidepressants added *in vitro* (antidepressant concentrations: 200, 400, 800 nmol/ml). Incubations were carried out in a system containing liver microsomes (ca. 1 mg of protein/ml), phosphate buffer (0.1 M, pH = 7.4), $MgCl_2 \times 6\ H_2O$ (6 mM), NADP (1.2 mM), DL-isocitric acid (6 mM) and isocitric dehydrogenase (1.2 U/ml). The final incubation volume was 0.5 ml. After a 2-min preincubation, the reaction was initiated by adding NADPH generating system and the incubation lasted for 50 min. Afterwards, the reaction was stopped by adding 350 µl of 2% $ZnSO_4$ and 25 µl of 2 M HCl.

Determination of caffeine and its metabolites

Caffeine and its four primary metabolites were assessed using the HPLC method based on Rasmussen and Brøsen [16]. After incubation samples were centrifuged for 10 min at 2000 × g. A water phase containing caffeine and its metabolites was extracted with 6 ml of organic mixture consisting of ethyl acetate and 2-propanol (8:1, v/v). The residue obtained after evaporation of microsomal extract was dissolved in 100 µl of the mobile phase described below. An aliquot of 20 µl was injected into the HPLC system. A 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 calculated recovery, intra-day precision, inter-day reproducibility and accuracy were similar to the respective values obtained by Rasmussen and Brøsen [16].

The obtained results were evaluated using Dixon analysis [9].

RESULTS and DISCUSSION

As mentioned in the Introduction, caffeine is a marker drug for testing the activity of CYP1A2 (3-N-demethylation) in humans and rats. Moreover, it seems also to be a relatively specific substrate of CYP3A (8-hydroxylation). In the case of 1-N- and in particular 7-N-demethylation of caffeine, apart from CYP1A2, other CYP isoenzymes play a considerable role, probably CYP2B and/or CYP2E1 [2, 19]. For this reason, caffeine was used in our studies to show a direct interactions of most frequently used antidepressant drugs with CYP.

The obtained results showed that all the investigated antidepressants had an inhibitory effect on

caffeine metabolism in rat liver microsomes, though their potency towards particular metabolic pathways was different. Dixon analysis of caffeine metabolism carried out on control liver microsomes, in the absence and presence of the antidepressants showed that imipramine exerted a most potent inhibitory effect on caffeine metabolism. Imipramine decreased the rate of both the three N-demethylations and 8-hydroxylation of caffeine (Fig. 1A, B, C, D), the effect on 3-N-demethylation

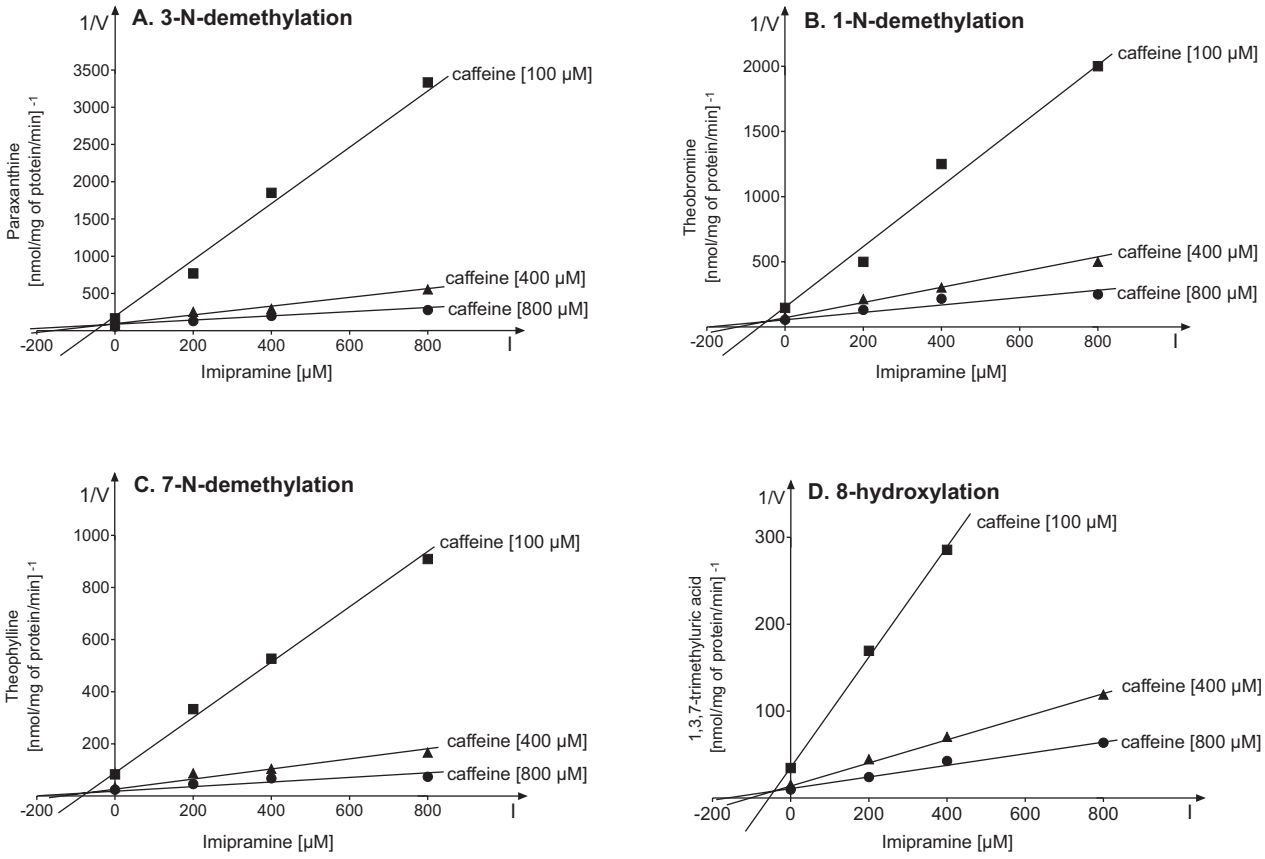

Fig. 1. The influence of imipramine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation: K_m = 242 μM, V_{max} = 0.02 nmol/mg protein/min, K_i = 33 μM (A); 1-N-demethylation: K_m = 260 μM, V_{max} = 0.0018 nmol/mg protein/min, K_i = 47 μM (B); 7-N-demethylation: K_m = 351 μM, V_{max} = 0.054 nmol/mg protein/min, K_i = 73 μM (C); 8-hydroxylation: K_m = 332 μM, V_{max} = 0.11 nmol/mg protein/min, K_i = 45 μM (D); V = velocity of the reaction, I = concentration of inhibitor

Table 1. The influence of antidepressant drugs on the metabolism of caffeine. The presented inhibition constants (K_i) for competitive inhibition of particular metabolic pathways were calculated using Dixon analysis

Antidepressants (inhibitors)	Inhibition of caffeine metabolism			
	Paraxanthine (caffeine 3-N-demethylation)	Theobromine (caffeine 1-N-demethylation)	Theophylline (caffeine 7-N-demethylation)	1,3,7-trimethyluric acid (caffeine C-8-hydroxylation)
	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]
Imipramine	33	47	73	45
Amitriptyline	57	61	190	108
Fluoxetine	152	196	72	40

being most pronounced (Tab. 1). This indicates inhibition of CYP1A2 (inhibition of 3-N- and 1-N--demethylation), 3A (inhibition of 8-hydroxylations), and possibly other CYP isoenzymes (inhibition of 7-N-demethylation), e.g. CYP2B2 and/or 2E1, by the antidepressant.

Amitriptyline showed distinct inhibition of CYP1A2 activity expressed as a decrease in the rates of 3-N- and 1-N-demethylations of caffeine. The above effect was less potent than in the case of imipramine (Fig. 2A, B). The influence of amitriptyline on 8-hydroxylation and especially on 7-N--demethylation of caffeine was weaker than on 3-N- or 1-N-demethylation (Fig. 2B, C), suggesting a narrower spectrum of CYP inhibition by amitriptyline than by imipramine, involving mainly CYP1A2, and – to a lesser degree – CYP3A (Tab. 1). Thus, tricyclic antidepressants of tertiary amine structure, which are N-demethylated by CYP1A2 and CYP3A [6, 13], behave not only as substrates but also as inhibitors of the mentioned isoenzymes.

In contrast to the tested tricyclic antidepressants, fluoxetine did not exert any considerable effect on the 3-N- or 1-N-demethylation of caffeine (Fig. 3A, B), which indicates its low affinity to CYP1A2. However, fluoxetine displayed a clear inhibitory effect towards caffeine 7-N-demethylation, the reaction which is suggested to be catalyzed mainly by other than CYP1A2 isoenzymes (CYP2B and/or CYP2E1) [2, 19]. On the other hand, fluoxetine diminished markedly the 8-hydroxylation of the marker drug; as reflected by K_i values, the potency of inhibition of rat CYP3A by fluoxetine was similar to that of imipramine (Tab. 1). Thus, fluoxetine is an inhibitor of CYP3A in rats (inhibition of 8-hydroxylation), which is consistent with its ability to inhibit CYP3A in humans [15, 18].

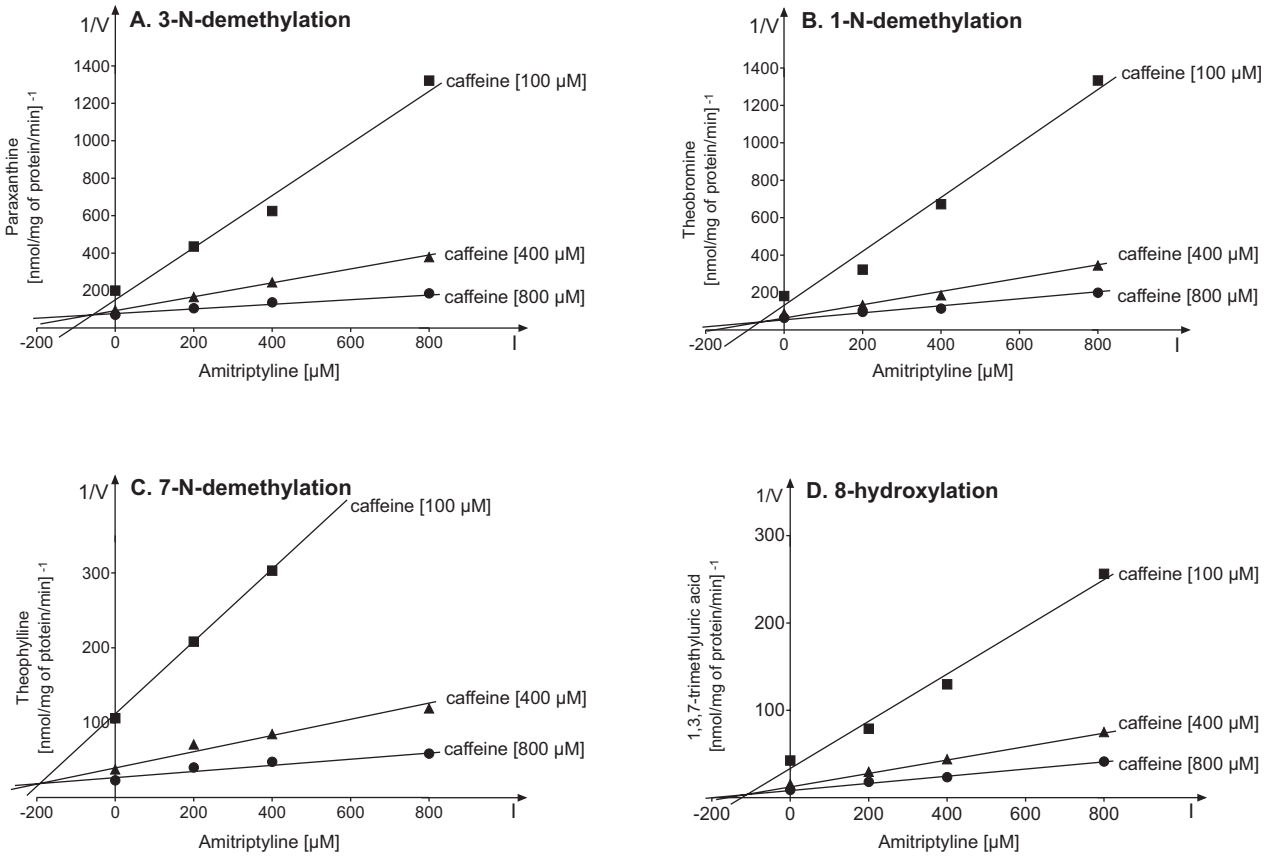

Fig. 2. The influence of amitriptyline on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation: K_m = 254 μM, V_{max} = 0.017 nmol/mg protein/min, K_i = 57 μM (A); 1-N-demethylation: K_m = 257 μM, V_{max} = 0.018 nmol/mg protein/min, K_i = 61 μM (B); 7-N-demethylation: K_m = 590 μM, V_{max} = 0.066 nmol/mg protein/min, K_i = 190 μM (C); 8-hydroxylation: K_m = 560 μM, V_{max} = 0.133 nmol/mg protein/min, K_i = 108 μM (D); V = velocity of the reaction, I = concentration of inhibitor

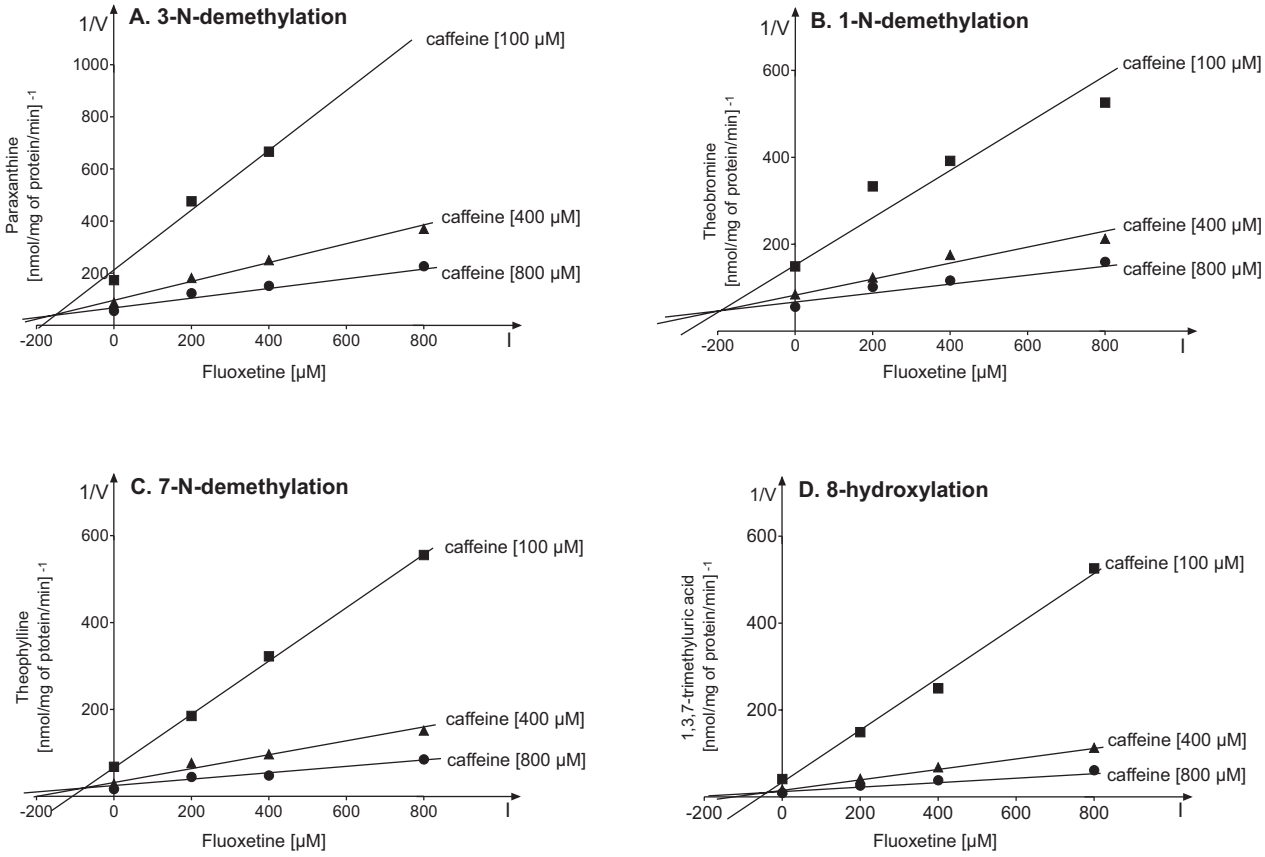

Fig. 3. The influence of fluoxetine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation: K_m = 286 μM, V_{max} = 0.02 nmol/mg protein/min, K_i = 152 μM (A); 1-N-demethylation: K_m = 222 μM, V_{max} = 0.02 nmol/mg protein/min, K_i = 196 μM (B); 7-N-demethylation: K_m = 455 μM, V_{max} = 0.08 nmol/mg protein/min, K_i = 72 μM (C); 8-hydroxylation: K_m = 667 μM, V_{max} = 0.136 nmol/mg protein/min, K_i = 40 μM (D); V = velocity of the reaction, I = concentration of inhibitor

In summary, CYP1A2 was distinctly inhibited by imipramine and amitriptyline, CYP3A by imipramine and fluoxetine, while other CYP isoenzymes (CYP2B and/or 2E1) by imipramine and fluoxetine. The observed interactions of antidepressants with CYP *in vitro* should be important in situations *in vivo* since the calculated K_i values were lower than the respective K_m values (Figs. 1–3), and were within the presumed concentration range of the inhibitors in the liver *in vivo* (i.e. below 100 μM), both in pharmacological experiments and in psychiatric patients [5, 7, 8, 15]. Considering contribution of the investigated CYP isoenzymes (CYP1A2 and CYP3A) to the metabolism of endogenous substances (e.g. steroids), drugs (xanthine derivatives, phenacetin, propranolol) and carcinogenic compounds, the inhibition of the mentioned CYP isoenzymes by prolonged administration of the tested antidepressants may be of physiological, pharmacological and toxicological importance. Therefore, the obtained results require further clinical consideration.

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