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Review

Caffeine as a marker substrate for testing cytochrome P450 activity in human and rat

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Abstract:
The current knowledge on the involvement of cytochrome P450 (P450, CYP) isoforms in the metabolism of caffeine in rat and human liver is reviewed. Attention is also paid to species- and concentration-dependent metabolism of caffeine. Finally, we discuss the P450-mediated metabolism of caffeine in relation to coffee addiction and drug interactions.
Due to its safety, favorable pharmacokinetic properties, and P450 isoform-selective metabolism, caffeine has great potential as a metabolic marker substance in both humans and rats, and as a more universal metabolic tool in the latter species. However, the qualitative and relative quantitative contribution of P450 isoforms to the metabolism of caffeine is species- and concentration-dependent. While 3-N-demethylation is quantitatively the main oxidation pathway in human, 8-hydroxylation is the dominant metabolic pathway in rat. Both of these main reactions in the two species are specifically catalyzed by CYP1A2. Caffeine may be applied as a marker substance for assessing the activity of CYP1A2 in human and rat liver, but by using different reactions: 3-N-demethylation in humans and C-8-hydroxylation in rats. In addition, caffeine can be used to preliminarily and simultaneously estimate CYP2C activity in rat liver using 7-N-demethylation as a marker reaction. On the other hand, CYP3A4-catalyzed 8-hydroxylation in humans is not sufficiently isoform-specific to mark the activity of CYP3A4. Caffeine pharmacokinetics may be changed by drugs affecting the activity of CYP1A2 (human and rat) or CYP2C (rat), e.g. *via* autoinduction or by treatment with certain antidepressants or neuroleptics. Therefore, patients taking caffeine-containing medicine or coffee drinkers taking drugs that interact with CYP1A2 may require proper dosage adjustments upon caffeine ingestion and cessation.

Key words:
caffeine, pharmacokinetics, pharmacodynamics, cytochrome P450, N-demethylation, 8-hydroxylation, drug interactions, coffee addiction

Abbreviations: log P – log octanol/water partition coefficient, P450, CYP – cytochrome P450, pK_a – ionization constant, SSRI – selective serotonin reuptake inhibitors

Introduction

A marker substrate is devised to provide information on the *in vivo* cytochrome P450 (P450, CYP) isoform activity (phenotyping), its level of induction, inhibition or drug interactions. It must be characterized by safety, wide availability, assay reliability in bodily fluids and, of course, a metabolic pathway specific for one P450 isoform. Currently, different marker substrates for various isoforms of cytochrome P450 are widely used to assess genetic, ethnic, and environmental differences in the *in vivo* metabolism of drugs. Interestingly, caffeine is the first-choice substrate for CYP1A2 phenotyping in a selective (CYP isoform-

specific) phenotyping method, as well as in a 'cocktail' phenotyping method with simultaneous administration of multiple types of CYP isoform-specific marker substrates [11, 19, 38, 63, 65, 71]. In addition, caffeine has proven to be useful for simultaneous estimation of N-acetyltransferase-2 (NAT-2) and xanthine oxidase (XO) activities [42, 47, 59]. This is not surprising since caffeine is relatively safe and possesses many favorable pharmacokinetic characteristics for a marker substrate. Several caffeine-based approaches for assessing CYP1A2 activity *in vivo* have been described. These approaches have utilized caffeine concentrations in plasma or saliva (caffeine clearance), as well as plasma, saliva, or urinary caffeine metabolite ratios. Moreover, a ^{14}C-caffeine 'breath test' which serves well for the *in vivo* estimation of CYP1A2 activity has been described [18, 32, 47, 61].

In this article, we review the current knowledge on the involvement of P450 isoforms in the metabolism of caffeine in rat and human liver, in order to critically evaluate caffeine as a marker substance for estimating the activity of cytochrome P450. We also give attention to species- and concentration-dependent metabolism of caffeine in regard to the degree of oxidation in particular positions, as well as the qualitative and quantitative contributions of P450 isoforms to particular oxidation pathways. Finally, we discuss the P450-mediated metabolism of caffeine in relation to coffee addiction and drug interactions.

Pharmacokinetic profile of caffeine

The ionization constant (pK_a) of caffeine is 14 and the lipid partition coefficient (log P) is 0.85. As a consequence, the molecule exists predominantly as a weak base in the gastric fluid (pH = 2–3) [60]. The moderate lipophilic character of caffeine allows its passage through all biological membranes [9, 10]. Following oral administration, gastrointestinal absorption of caffeine is rapid and complete, and reaches 100% bioavailability [1]. No significant first-pass effect occurs after oral administration [9, 10]. Caffeine is rather poorly bound to plasma albumin (10–30%) [8, 25] and its volume of distribution ranges from 0.5 to 0.75 l/kg in human and 0.9 l/kg in rat, which indicates that the substance distributes into the total body water [1, 9, 10, 14]. It seems that no physiological 'barriers' limit the passage of caffeine throughout tissues; consequently, easy and rapid equilibrium is reached between mother and fetus, blood and all tissues, including the brain [13]. Caffeine may enter the brain by simple diffusion and carrier-mediated transport [55]. It has been found that caffeine and its metabolites are strong inhibitors of the human organic anion transporter [64] which is expressed in human lungs, kidneys, and testes [53], as well as along the blood-brain-barrier, as shown in cultured human brain endothelial cells [40]. Caffeine has an elimination half-life of 4 to 5 h, but may have a prolonged elimination in patients with hepatic diseases, in neonates (to 100 h), or during pregnancy [21, 36, 48]. Its biotransformation is mainly restricted to the liver with minimal extrahepatic metabolism (connected with the presence of CYP1A2) or renal elimination of the unchanged parent compound (about 3%) [18, 47, 56].

Pharmacodynamic properties of caffeine

Caffeine, a component of coffee, tea, and numerous drugs, is a purine alkaloid and the most universally used psychoactive substance. Caffeine acts through multiple mechanisms, the most important of which is the antagonism of adenosine receptors (A_1 and A_{2A}). Caffeine blocks adenosine receptors and competitively inhibits the action of adenosine at the therapeutic concentrations of caffeine (10–100 μM), with K_i of 29 and 48 μM at the A_1 and A_{2A} adenosine receptors, respectively. As an adenosine receptor antagonist, caffeine increases the release of various neurotransmitters [36]. Moreover, due to a negative interaction between adenosine and dopaminergic receptors, caffeine increases responses from dopaminergic receptors [37]. Because of its ability to affect neurotransmission in different regions of the brain, caffeine displays psychomotor stimulant properties and promotes behavioral functions such as vigilance, attention, mood, and arousal [35]. At a higher substrate concentration (0.1–1 mM), caffeine inhibits the phoshodiesterase which converts cyclic AMP (cAMP) in cells to its noncyclic form with a K_i of 0.48 mM. Moreover, some observations indicate that caffeine at broad-range concentrations of 0.1 to 2 mM (IC_{50} = 0.35–0.5 mM) may interact with the γ-aminobutyric $acid_A$ receptor complex and inhibit benzodiazepine

binding. Additionally, caffeine in a wide range of concentrations (from 0.1 to 1 mM) inhibits the activity of 5'-nucleotidase in a competitive manner. Sequential actions of caffeine, such as Ca^{2+} mobilization, require concentrations of caffeine from 5 to 15 mM [17, 18, 24, 36].

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* [16, 20, 69, 70]. Therefore, the detailed knowledge of caffeine metabolism is very important, since caffeine represents not only a metabolic marker substance, but also a drug with a very interesting pharmacological spectrum. Both the metabolic and pharmacodynamic properties of caffeine must be considered in predicting drug-drug interactions in pharmacological experiments with laboratory animals and in clinical conditions. Since the metabolism of caffeine *in vivo* depends on cytochrome P450, mutual drug interactions between caffeine and other centrally acting drugs at both the pharmacodynamic and pharmacokinetic levels are feasible [33].

Metabolism of caffeine

Caffeine (1,3,7-trimethylxanthine) is oxidized at a few positions of its molecular structure; apart from its 3-N-demethylation to paraxanthine, it undergoes 1-N-demethylation, 7-N-demethylation, and 8-hydroxylation (to theobromine, theophylline, and 1,3,7-trimethyluric acid, respectively) (Fig. 1). It was shown that the C-8-hydroxylation of caffeine is the major metabolic reaction in rat liver microsomes (~70%) and liver slices compared to 1-N- and 7-N-demethylation (8–9%) and 3-N-demethylation (~13%), when measured at a substrate concentration of 100 μM [5, 7, 50, 51]. In contrast, 3-N-demethylation was the main oxidation pathway of caffeine in human liver microsomes (~70%) compared to 1-N- and 7-N-demethyla-

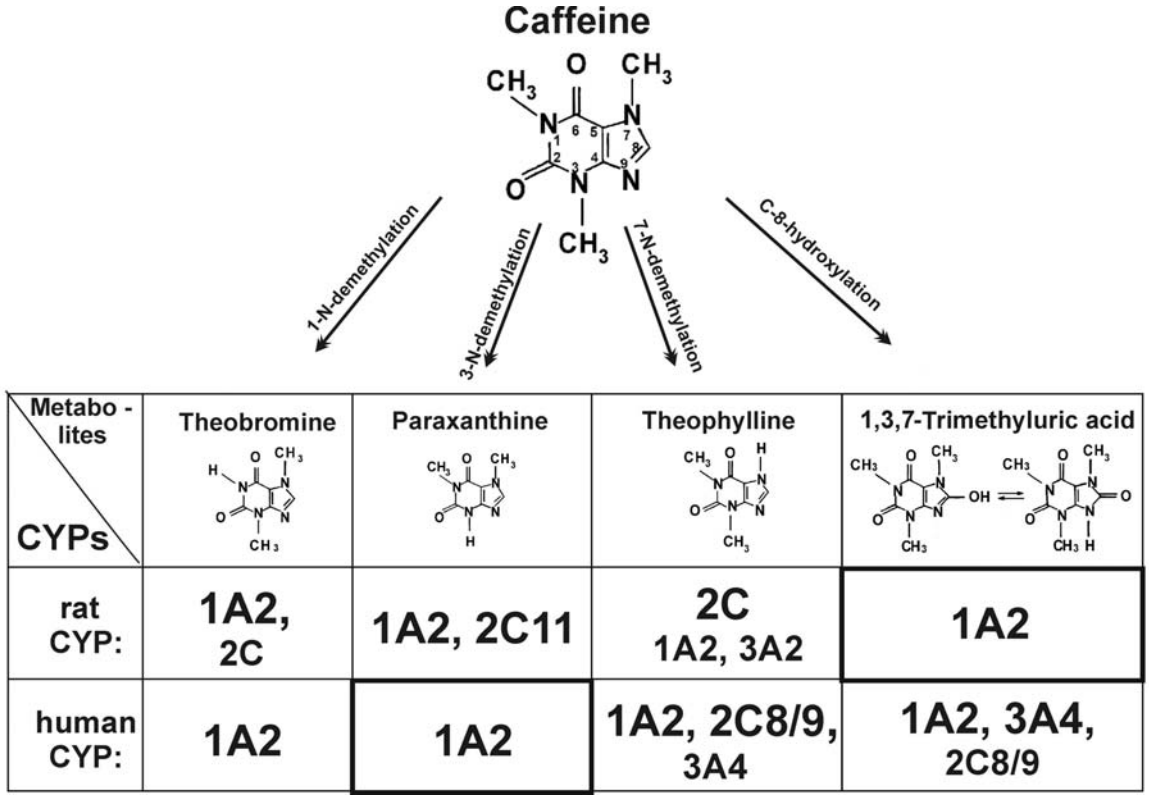

Fig. 1. Contribution of cytochrome P450 isoforms to caffeine metabolism in rat and human liver, based on the rate of caffeine metabolism in cDNA-expressed P450 isoforms and the mean of P450s in the liver [50, 51]. Bold frames show the dominant metabolic pathways of caffeine in the two species. The main contributing P450 isoforms are presented in a bigger font

tion (7–8%) and 3-N-demethylation (~15%), when measured at a substrate concentration of 100 μM [5, 50, 51].

The contribution of P450 isoforms to the metabolism of caffeine in rat liver

For many years, studies on the involvement of P450s in the metabolism of caffeine in rat were carried out using high (1–10 mM) substrate concentrations, predominantly unspecific P450 inducers or inhibitors [22, 23], and often without including all P450 isoforms [39, 57] or all caffeine metabolic reactions [22]. Therefore, it was difficult to identify all the P450 isoforms involved in caffeine metabolism; furthermore, quantitative estimations of the contribution of individual P450s to the four oxidative metabolic reactions of caffeine at concentrations relevant to those found in humans were practically impossible.

Nevertheless, the above studies suggested that 3-N-demethylation of caffeine to paraxanthine was catalyzed by CYP1A2 [5, 23, 57], while other oxidation pathways of caffeine might be mediated by P450 isoforms different from CYP1A2 [4, 5, 23, 57]. The CYP3A subfamily was proposed as the main isoenzyme catalyzing 8-hydroxylation to 1,3,7-trimethyluric acid [58]. However, other data also suggested the contribution of CYP2B1 and CYP2E1 to the catalysis of this reaction in rat [23]. It was also suggested that 1-N-demethylation and mainly 7-N-demethylation, which led to the formation of theobromine and theophylline, respectively, were likely to engage CYP2B isoforms in rat [4].

According to the literature, the concentration value of approximately 100 μM seems to be the highest encountered in the clinic situation [18, 48] and may be considered "the maximum therapeutic concentration in humans". Pharmacokinetic studies showed that caffeine concentrations found in the blood plasma of coffee drinkers were below 100 μM (usually 10–31 μM) after the consumption of 2–6 cups of coffee [31, 54]. Using a full set of CYP isoforms (baculovirus cDNA-expressed rat CYPs), the contribution of individual CYP isoforms to the metabolism of caffeine, at different drug concentrations in rat liver, has recently been thoroughly investigated and calculated (considering the relative amount of each isoform in rat liver) [49, 50]. It has become clear that CYP1A2 is a key enzyme that catalyzes C-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, CYP2C11 considerably contributes to 3-N-demethylation (31%), while CYP3A2 facilitates 1-N-demethylation and 8-hydroxylation (17 and 15%, respectively). The estimated contribution of P450 isoforms to the 7-N-demethylation of caffeine indicates that the CYP isoforms, mainly CYP2C6 (27%) and CYP2C11 (29%), play a major role in catalyzing this reaction [50]. At a higher substrate concentration (100–800 μM), the participation of CYP isoforms in the above described metabolic pathways of caffeine is changed. The contribution of CYP3A2 to C-8-hydroxylation markedly increases (from 15 to 58%) at the expense of CYP1A2 (a decrease from 72 to 30%). The contribution of CYP2C11 to 7-N-demethylation increases from 29 to 41%, while that of CYP1A2 shows a decrease (from 14 to 5%). 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 [49] and with the findings of Bienvenu et al. [6] who observed a decrease in the rate of these reactions after a continuous infusion of the growth hormone to hypophysectomized male rats. Similarly the contribution of CYP2C11 to the catalysis of 3-N-demethylation and 1-N-demethylation is distinctly enhanced (from 31–51% and 6–24%, respectively), while that of CYP1A2 is significantly decreased (from 47 to 19% and 37.5 to 22.6%, respectively) [50].

The significance of individual P450 isoforms in the metabolism of caffeine in rat liver observed using cDNA-expressed P450 isoforms has been confirmed in a study with rat liver microsomes in the presence of specific P450 inhibitors, or with liver microsomes after the treatment of rats with selective inducers [49, 50]. Generally, the results from correlation and inhibition studies, as well as *in vivo* studies with selective P450 inducers, support the conclusions drawn from the studies with recombinant rat CYP isoforms [49, 50]. In addition, recent investigation indicates that flavin-containing monooxygenase (FMO) does not contribute significantly to caffeine metabolism measured *in vitro* at a substrate concentration of 100 μM, as shown by the thermal inactivation of FMO [50].

The role of P450 isoforms in the metabolism of caffeine in human liver

Although numerous studies on caffeine metabolism show that 3-N-demethylation to paraxanthine in human (the main oxidation pathway) is specifically catalyzed by CYP1A2, they have also suggested that other oxidation pathways of caffeine may be mediated, at least partly, by P450 isoforms different from CYP1A2. In experiments with 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 [3, 5, 15, 41]. Further studies with selected cDNA-expressed CYP isoforms, or CYP1A2 and CYP2E1 cell lines, have indicated that caffeine 3-N-demethylation is most efficiently 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 [34, 39, 42, 43, 66, 67]. However, CYP2C isoforms (CYP2C8/9/18/19), which constitute over 20% of total human liver P450 and play an important role in drug metabolism, were not studied in this respect for a long time. Recently, a panel of recombinant human CYP isoforms (baculovirus cDNA-expressed human CYPs) has allowed both precise qualitative and relative quantitative estimations of the contribution of individual P450 isoforms to the specific metabolic pathways of caffeine [51]. The results of the above-mentioned studies confirmed that CYP1A2 was the main isoform responsible for caffeine metabolism at the therapeutic concentration of caffeine (100 μM). It has also become evident that CYP1A2 is the chief enzyme catalyzing 1-N- and 3-N-demethylation (75 and 85%, respectively). It also contributes substantially to 7-N-demethylation (38.7%) and C-8-hydroxylation (28.7%). Moreover, 7-N-demethylation is also clearly catalyzed by CYP2C8 (12.8%), CYP2C9 (12.2%), and CYP3A4 (13.6%), while C-8-hydroxylation is substantially mediated by CYP3A4 (30%) and to a lesser extent by CYP2C8 (10%), CYP2C9 (8%), and CYP2E1 (11%) [51]. Similar to the results concerning caffeine metabolism in rat, concentration-dependent oxidation of caffeine by human cytochrome P450 is also observed, though such an effect is mainly observed for C-8-hydroxylation. The contribution of CYP1A2 to C-8-hydroxylation of caffeine is clearly decreased (to 16%), mostly in favor of CYP2C8 and CYP2C9 (to 17 and 11.6%, respectively) at a higher concentration of the substrate (1 mM). These findings are also confirmed by inhibition studies with human liver microsomes and specific inhibitors [51].

Interspecies comparison of caffeine metabolism

The main differences between the metabolism of caffeine in rats and humans, lie in the efficiency of 3-N-demethylation and 8-hydroxylation, as well as quantitative and qualitative contributions of P450 isoforms to particular oxidation pathways. While 3-N-demethylation is quantitatively the main oxidation pathway in human, C-8-hydroxylation is the dominant metabolic pathway in rat. Both of these main reactions in the two species are specifically catalyzed by CYP1A2. Accordingly, the efficiency of CYP1A2 in metabolizing caffeine was highest for 3-N-demethylation in human and 8-hydroxylation in rat, compared to the other P450 isoforms [50, 51].

For the above reasons, caffeine may be applied as a marker substance for assessing the activity of CYP1A2 in both human and rat liver using different reactions: 3-N-demethylation in human and C-8-hydroxylation in rat [50, 51]. Another aspect worth considering is that caffeine can be used to preliminarily and simultaneously estimate CYP2C activity in rat liver using 7-N-demethylation as a marker reaction [50]. On the other hand, CYP3A4-catalyzed 8-hydroxylation in humans is not sufficiently isoform-specific (30%) to mark the activity of CYP3A4 [51].

The above-mentioned species differences in the metabolism of caffeine in rat and human liver may stem from the diverse contribution of individual P450s to the total content of P450 protein in rat and human liver, as well as the species variability of P450 isoforms and their catalytic competence.

Induction and inhibition of caffeine metabolism and drug interactions

Beverages containing caffeine, such as coffee, tea, and energy drinks enjoy great popularity. A caffeine

concentration value of approximately 100 μM may be considered "the maximum therapeutic concentration in humans". However, some individuals may consume more than 1 g/day (about 15 mg/kg/day) and even up to 3.5 g/day (about 50 mg/kg/day) of caffeine in a caffeinism syndrome which leads to caffeine concentrations above 100 μM in their blood plasma [2, 18, 48, 62]. There are some observations that point to the autoinduction of caffeine metabolism. Studies carried out in rats indicate that at high dosages, caffeine increases its own metabolism through induction of CYP1A [4, 49].

As mentioned above, CYP1A2 is the main isoform responsible for caffeine metabolism at the therapeutic concentration of caffeine (up to 100 μM). Therefore, pharmacokinetic interactions between caffeine and drugs affecting CYP1A2 activity may occur. For example, fluvoxamine, a substrate and potent inhibitor of CYP1A2 (K_i below 1 μM), decreases the activity of CYP1A2 [12, 45, 46, 68] which may lead to caffeine intoxication [46]. Other selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine, and sertraline exert a weaker inhibitory effect on human CYP1A2, as observed in liver microsomes (K_i between 4 and 9 μM) [12, 68]. It was also found that some psychotropic drugs might directly inhibit rat CYP1A2 activity in liver microsomes [26–30]. Among the antidepressants examined, the tricyclic antidepressant drugs imipramine, clomipramine, and desipramine, and the SSRI sertraline are the most potent inhibitors of rat CYP1A2. However, the effect of antidepressants was approximetly ten times weaker in rats than in humans, indicating species-differences in CYP1A2 structure and function [27, 30]. Some phenotiazine neuroleptics added *in vitro* to control liver microsomes also directly inhibited rat CYP1A2 activity *via* competitive or mixed mechanisms, with a potency similar to that found for antidepressants in rat [26, 28, 30]. Promazine was the most potent inhibitor of the rat CYP1A2 among the phenothiazines studied (chlorpromazine, levomepromazine, thioriazine, and perazine). In contrast to promazine, haloperidol and atypical neuroleptics such as risperidone and sertindole had practically no effect on the oxidation of caffeine in rat liver microsomes. On the other hand, competitive inhibition of clozapine metabolism (a CYP1A2 substrate) by caffeine during concomitant intake of clozapine and caffeine was observed in patients [18].

In addition to the direct effect of psychotropic drugs on cytochrome P450 and caffeine metabolism (e.g. competitive inhibition), some psychotropics may influence caffeine metabolism indirectly *via* affecting cytochrome P450 regulation. Thus a two-week treatment with promazine, perazine, clozapine, or risperidone increased the level and activity of CYP1A2 in rat [26, 58]. Our recent study on the effect of antidepressants on the metabolism of caffeine showed that, when given chronically, fluoxetine increased the rate of 7-N-demethylation only, while sertraline and mirtazapine enhanced the rate of all caffeine oxidation pathways [52].The latter results indicated that chronic treatment with fluoxetine induces caffeine metabolism by enhancing the activity of CYP2C only, while administration of sertraline and mirtazapine acts mainly by elevating the activity of CYP2C and CYP1A2 [26, 44, 52]. In this way, these data appear to support the use of caffeine as a more universal "pharmacological tool", which may be applied for simultaneous estimation of not only CYP1A2, but also the CYP2C subfamily in pharmacological experiments in rat.

Finally, interactions of caffeine with other drugs such as allopurinol, antimycotics, cardiovascular drugs, histamine H_2 receptor antagonists, idrocilamide, methylxanthines (i.e. furafylline, theophylline), nonsteroidal anti-inflammatory drugs (paracetamol), oral contraceptives, phenylpropanolamine, proton pump inhibitors, psoralens, and quinolones have also been observed, as reviewed by Carillo and Benitez [18]. Moreover, a number of other factors, including gender, cigarette smoking (CYP1A2 induction by polycyclic aromatic hydrocarbons), dietary components of food, beverages, and fitness level also interact with caffeine metabolism [18, 33].

In conclusion, numerous drug interactions may occur between caffeine and neuroactive drugs or other pharmacological medications. Therefore, patients taking caffeine-containing medicine or coffee drinkers taking drugs that interact with CYP1A2 may require proper dosage adjustments upon caffeine ingestion and cessation.

Summary and conclusions

Due to its safety, favorable pharmacokinetic properties, and P450 isoform-selective metabolism, caffeine

has potential as a metabolic marker substance in both humans and rats, and as a more universal metabolic tool in the latter species. However, the qualitative and relative quantitative contribution of P450 isoforms to the metabolism of caffeine is species- and concentration-dependent. While 3-N-demethylation is quantitatively the main oxidation pathway in humans, 8-hydroxylation is the dominant metabolic pathway in rats. Both of these reactions in the two species are specifically catalyzed by CYP1A2.

The human 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; 2) 7-N-demethylation is non-specifically catalyzed, mainly by CYP1A2 and to a lesser extent by CYP2C8/9 and CYP3A4; 3) C-8-hydroxylation preferentially involves CYP1A2 and CYP3A4 and to a lesser degree CYP2C8/9 and CYP2E1 at a concentration of 100 μM, which corresponds to the maximum therapeutic concentration in human. At a higher caffeine concentration, the contribution of CYP1A2 to this reaction decreases in favor of CYP2C8/9.

The results obtained in rats show that 1) the 1-N- and 3-N-demethylation of caffeine is predominantly catalyzed by CYP1A2 and CYP2C; 2) 7-N-demethylation is governed by P450s of the CYP2C subfamily; 3) 8-hydroxylation, the main oxidation pathway of caffeine in the rat, is specifically mediated by CYP1A2 at a concentration of 100 μM. 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).

For the above reasons, caffeine may be applied as a marker substance for assessing the activity of CYP1A2 in human and rat liver, albeit by different reactions: 3-N-demethylation in human and C-8-hydroxylation in rat. In addition, caffeine can be used to preliminarily and simultaneously estimate CYP2C activity in rat liver using 7-N-demethylation as a marker reaction. Caffeine pharmacokinetics may be changed by drugs affecting the activity of CYP1A2 (human and rat) or CYP2C (rat), e.g. *via* autoinduction or treatment with certain antidepressants or neuroleptics. Therefore, patients taking caffeine-containing medicine or coffee drinkers taking drugs that interact with CYP1A2 may require proper dosage adjustments upon caffeine ingestion and cessation.

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