MASARYK UNIVERSITY

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THE ROLE OF CYTOCHROME P450 IN DRUG SAFETY AND EFFICACY

Habilitation thesis

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Brno 2018

Originality and Conflict of Interest Statement

The original data presented in this habilitation thesis were collected by the author together with his colleagues, students, and members of cooperating research groups who are co-authors of cited original manuscripts. The research was supported by following projects:

I hereby declare that I have no conflict of interest.

Acknowledgements

First of all, I would like to thank to all my colleagues, students, and supervisors. The research presented in this thesis would never have been done without their support, advice, and help. My special acknowledgement goes to Gabriela Dovrtělová, Kristýna Nosková, Michaela Sabová, and Jan Juřica, who are and always will be the core of our "pharmacokinetic research group". Thank you for the time spent in our lab as well as for the hundreds of hours consumed by the preparation of our manuscripts. Gábi, thank you for enthusiasm and patience with all the microsomal and HPLC methods. I would like to give many thanks to our laboratory technician Květoslava Sedlářová. You have always been the spirit of the lab and the one who gives a helping hand at the critical moments.

I would like to express my gratitude to my PhD supervisor Jiří Totušek and Professor Alexandra Šulcová who keep me motivated to continue with my research. Many thanks also go to the head of the Department Regina Demlová for her comprehension of the importance of our research. Many of the articles presented in this thesis would never have arisen without the co-operation of our colleagues from other research institutions. I have to give special mention to Pavel Anzenbacher and Anna W. Daniel, who are my cytochrome P450 step-parents. Thank you for your advice and methodological help. My great thanks also go to Nataša Hlaváčová from the Slovak Academy of Sciences and to Petr Pávek and his teammates from Charles University.

Finally, I would like to thank my family for their endless support and love. Thank you Lucia and Hugo.

Content

1   Annotation

This cumulative habilitation thesis consists of 8 original and 2 review articles published in the years 2008 – 2018. The work is thematically divided into four parts. My research work is focused on the preclinical and clinical research of cytochrome P450 enzymes (P450), which is probably the most important enzyme system involved in drug metabolism. Changes in its activity are therefore able to influence the levels of its substrates in plasma and target sites. Therefore P450 activity is related to the safety and efficacy of the administered drugs.

The P450 system is briefly reviewed in the introduction followed by a description of the mechanism of drug-drug interactions at the P450 level, and possible clinical consequences are explained. A study describing the co-administration of memantine and fluoxetine is given as an example of research in this field.

Interactions with P450 are a standard part of drug development. Another field of interest of my research is natural substances with promising potential for therapeutic use. The polyphenolic substances resveratrol, quercetin, and psychotropic substances crocin, safranal, and linalool were tested for their interactions with rat P450 in the second part of the thesis.

The activity of P450 is regulated by many endogenous factors including hormones, cytokines, and bile acids, but the role of the endocannabinoid system, a promising target of new therapeutic strategies, is unknown. Therefore the third part of the thesis summarises the state of the art of interactions between cannabinoids and P450 and postulates a hypothesis of how this system can be involved in the regulation of P450 activity. An experimental study with the endocannabinoid substance oleamide reveals some possible aspects of this regulation.

The methods for clinical phenotyping of P450 developed in cooperation with the Department of Psychiatry of University Hospital Brno are described in the final part of the work. The methods illustrate the clinical use of research that I have been involved in.

2   Introduction

The effectiveness of pharmacotherapy depends on many factors. Besides the essential requirement –
to correctly diagnose the disease and select a suitable drug, the proper dose, route of administration,
dosing schedule, and especially patient's adherence to the prescribed therapy determines the
outcomes of the therapy. Non-adherence is an age-old problem which is also reflected in a quote
from Hippocrates: "Keep a watch also on the faults of the patients, which often make them lie about
the taking of things prescribed (Hippocrates et al., 1923)." In fact, clinical studies document that the
number of patients with poor adherence to therapy is about 40% by the end of one year of therapy
(Blaschke et al., 2012). On the other hand we should ask the question: Why do patients not adhere to
their therapy? Why don't they follow our instructions? It is obvious that in some cases it can be
caused by a complicated dosing regimen. Simplification of the dosing scheme with a reduced
numbers of doses (Coleman et al., 2012) or single-pill combinations (Vrijens et al., 2017) could be a
suitable solution for this problem. Another reason for drug discontinuation or irregular use can be
inefficacy or side effects of a drug (Ascher-Svanum et al., 2010).

In most drugs used in clinical practice, the safety and efficacy of pharmacotherapy depend on the
interaction between the molecule of a drug and its target structure. But what determines the quality
and quantity of the final effect of a drug, and why is the effect not always the same when the same
drug, in the same dose, and in the same form is administered via the same route of administration to
different patients? The answer is simple. It is caused by pharmacodynamic and pharmacokinetic
variability. In other words, the final effect is determined not only by the presence of the target
structure and drug affinity to it, but also by the concentration of the drug at the target site. Today,
we are able to measure the density of different receptors and other target structures as well as the
binding affinity of a drug *in vivo*. Unfortunately, the methods for such an assessment are time-
consuming and too expensive to be used routinely for clinical practice (Ostad Haji et al., 2012). On
the other hand, pharmacokinetic variability can be easily measured by the assessment of drug
plasma levels. This could be used for the individualisation of drug dosing to limit adverse reactions
and to increase adherence. Therapeutic drug monitoring is used in drugs with a known relation
between their plasma level and effect (Ostad Haji et al., 2012) and especially for drugs with a narrow
therapeutic range (Kang and Lee, 2009). Pharmacokinetic variability seems to be easier to evaluate or
predict than pharmacodynamic variability.

One of the major causes of pharmacokinetic variability is the enzymatic system of cytochrome P450.
It consists of intracellular heme-binding enzymes found in all cellular living organisms, including
prokaryotes (Khmelevtsova et al., 2017). P450 enzymes are membrane-bound intracellular proteins
present mainly on the endoplasmic reticulum and to a minor extent also on plasma membranes and

mitochondria (Ahn and Yun, 2010). Their distribution in the human body varies in different tissues and organs with the predominant activity in the liver (Lin and Lu, 2001).

P450 has extremely low substrate specificity and it participates both in the biosynthesis and metabolism of various endogenous compounds (Gordeziani et al., 2016) and in the elimination of xenobiotic substrates, including a myriad of drugs (Zanger et al., 2008). Generally, it belongs to the Phase I enzymes of drug metabolism. The most common type of reaction catalysed by P450 is monooxydation (Anzenbacher and Anzenbacherová, 2003). Nevertheless, other types of oxidation are also catalysed by P450 (Hrycay and Bandiera, 2015). The catalytic apparatus of P450 is complex and requires other co-factors and enzymes for its reactions.

The system of P450 is classified into families and subfamilies with respect to their primary structure. There have been at least 18 human (Nelson, 2006), 62 plant (Xu et al., 2015), and 292 fungal (Moktali et al., 2012) families of P450, amounting to thousands of described unique P450 genes which can be found elsewhere (https://www.pharmvar.org/genes). The most clinically relevant P450 enzymes are CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP2B6. These five enzymes are involved in the metabolism of over 90% of P450 drug substrates (Pelkonen et al., 2008). Another important P450 in drug metabolism is also CYP1A2. Even though the number of drugs metabolized via this enzyme is not as high, it is involved in the activation of some procarcinogens (He and Feng, 2015) and its activity can be induced by tobacco smoke (Gunes et al., 2009).

3 Drug-drug interactions on the level of P450

The risk of drug-drug interactions rises with extensive drug metabolism via the P450 system and with the number of drugs co-administered. In fact, the induction and more frequent inhibition of P450 metabolic activity are probably the most frequent causes of documented drug-drug interactions (Lin and Lu, 1998). A review of drugs inhibiting and inducing P450 activity can be found elsewhere (http://medicine.iupui.edu/clinpharm/ddis/main-table/). The basic principle of this mechanism is the interaction of two drugs at the active site of the enzyme. Both of the drugs can be substrates of the enzyme or one can be a substrate and the other an inhibitor/inducer of the enzyme.

Cytochromes P450 are involved not only in the pharmacokinetic process of drug elimination, but in orally taken drugs also in their absorption in the intestine. Enterocytes together with the liver serve as the first line of defence of the human body against xenobiotics from the diet. Therefore, hepatocytes are rich in P450 enzymes and all substances absorbed from the gut enter the portal vein and are transported into the liver prior to entering systemic circulation. Drugs with extensive presystemic metabolism after oral use have increased bioavailability after P450 inhibition and conversely an increase in P450 activity decreases their bioavailability and plasma levels. With P450 inhibition, toxicity symptoms of P450 substrates can occur, while with induction the drug can become inefficient.

There are various mechanisms through which the drugs can interact at the P450 level. The induction of gene transcription and increase in protein levels is the most common mode of P450 induction (Waxman, 1999). A less frequently reported mechanism is positive cooperation, in which a substrate binds to a binding site and causes a conformational change leading to an increase in the affinity of other binding sites (Denisov et al., 2009). The dose of P450 substrates which induce their own metabolism has to be increased over time, as their elimination gradually increases (Lynch and Price, 2007).

Inhibition is a more frequent type of interaction with P450. The clinical impact of P450 inhibition depends mainly on the type of inhibition and the width of the therapeutic range of the substrate. The competitive inhibition between substrates is usually clinically insignificant in terms of pharmacokinetic variability in general. The seriousness of the interaction also exponentially declines with the number of alternative metabolic pathways. Signs of toxicity should be expected only when substrates of P450 with an extremely narrow therapeutic range are used. Such drugs are routinely a subject of therapeutic drug monitoring and their dosaging is individualized to reach the target plasma level and prevent adverse reactions and toxicity. Inhibitors of P450 enzymes can compete with the enzymes or act in a non-competitive manner and can bind to an enzyme molecule reversibly or

irreversibly. For clinical purposes, inhibitors are sometimes classified into weak, mild, and moderate or their strength of inhibition is compared with other known inhibitors. The same enzyme involved in the drug's metabolism can be inhibited by the drug or the drug can be metabolized by one P450 and inhibit another one. In rare cases, a P450 inhibitor is combined with its substrate to increase its plasma level (Lynch and Price, 2007).

Unlike inhibition, there is a delay between the beginning of inducer administration and the process of P450 induction. This is caused by the mechanism of induction itself, which requires the transcription of P450 genes to mRNA, subsequent synthesis of the protein structure, and time is also needed for translational processes and protein accumulation. The drug's half-life also plays a role in the speed of induction (Michalets, 1998). It should be also taken into account that the inhibition of P450 activity can persist even weeks after discontinuing administration of the inhibitor (Juřica and Žourková, 2013).

3.1 The interaction of memantine and fluoxetine in rats

Memantine is one the few antagonists of glutamatergic NMDA receptors used in pharmacotherapy. Besides acetylcholine esterase inhibitors, it is used in the treatment of dementia and it is believed to be a safe drug with a minimum of adverse effects in comparison to other NMDA blockers such as phencyclidine or MK-801.The most frequently is memantine prescribed in elderly as the incidence of cognitive impairment exponentially increases with age (Rocca, 2000). On the other hand co-morbidities, co-medications and risk of drug-drug interactions also rise in elderly patients. In our study, we researched the possible interaction between memantine and known P450 inhibitor fluoxetine in two different models in rats. Both drugs were administered intraperitoneally for 10 days at the following doses: fluoxetine 5 mg/kg/day (used only for *in vitro* part of the experiment) and 20 mg/kg/day, combination of both memantine and fluoxetine 5 mg/kg/day, and memantine 5 mg/kg/day. The activity of rat CYP2D2 which is orthologue to human CYP2D6 was assessed by dextromethorphan O-demethylation to dextrorphan (Yu, Dong, Lang, & Haining, 2001) in the model of isolated perfused rat liver or in the *in vivo* rat pharmacokinetic model. Memantine in our experiment significantly inhibited CYP2D2 activity as was documented by the decrease of dextrorphan formation. The strength of the inhibition of both memantine alone and combination of memantine and fluoxetine was comparable with the fluoxetine at the dose of 20 mg/kg/day. Interestingly, the effect of memantine, fluoxetine, and its combination differs in the *in vivo* model. Oppositely to perfused liver model the weakest inhibition of CYP2D6 activity was detected in animals with fluoxetine 20 mg/kg/day treatment. Memantine and its combination with fluoxetine inhibited CYP2D2 activity with similar potency.

Zendulka O., Juřica J., Zahradníková L., Sabová M., Šulcová A. Effects of combined treatment with cognitive enhancer memantine and antidepressant fluoxetine on CYP2D2 metabolic activity in rats. Neuroendocrinology Letters, 32, 727-732, 2011.

Impact factor: 1.296; Neurosciences Q4, Endocrinology & Metabolism Q4   Citations WoS: 0

Author's contribution: 35%

Effects of combined treatment with cognitive enhancer memantine and antidepressant fluoxetine on CYP2D2 metabolic activity in rats

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ORIGINAL ARTICLE

Abstract

OBJECTIVES: The drug-drug interactions can result in alterations of the therapeutical responses. The present study was designed to investigate possible pharmacokinetic interactions between the cognitive agent memantine and the antidepressant fluoxetine combined often in treatments of cognitive disorders including Alzheimer disease. The attention was focused on changes of the cytochrome P450 2D2 isoenzyme activity in two animal models.

METHODS AND DESIGN: The tested drugs were administered alone or in a combination to rat males and their effects on the 2D2 isoenzyme activity was determined after *in vivo* administration. The levels of marker dextromethorphan, its 2D2 specific metabolite dextrorphan were analyzed in plasma of rats and using the model of isolated perfused rat liver in the perfusion medium. The dextromethorphan/dextrorphan (DEM/DEX) metabolic ratios were determined as a sign of inhibitory influences on CYP2D2.

RESULTS: The analyses showed elevation of DEM/DEX metabolic ratio after all treatments: a) memantine, b) fluoxetine and c) memantine+fluoxetine, however the results were not completely identical. The intensity of inhibitory effects on the CYP2D2 activity were: memantine < memantine + fluoxetine < fluoxetine.

CONCLUSION: The results presented suggest that the clinical pharmacotherapeutical approach to combine memantine with fluoxetine is from the point of view of pharmacokinetic drug-drug interaction on the level of CYP2D2 isoenzyme safe and even of benefit as memantine could elicit a suppression of the inhibitory influence of fluoxetine.

Abbreviations:
AD   - Alzeimer's disease
DEM   - dextromethorphan
DEX   - dextrorphan
FLU   - fluoxetine
HPLC   - high-performance liquid chromatography
MEM   - memantine
MR   - metabolic ratio
NMDA   - N-methyl-D-aspartic acid

INTRODUCTION

Alzheimer disease and other neuropsychiatric disorders leading to dementia or cognitive deficits are supposed to be the major health concern of the coming decades (McDonald *et al.* 2010). The incidence of cognitive impairment is rapidly rising as the life expectancy is remarkably increasing over the last 50 years

and the exponential risk of age-related dementias is described (Rocca 2000). Although dementia is the problem of high importance, only few substances are approved and used in the therapy of this illness. Apart from the inhibitors of acetylcholinesterase, memantine (MEM) is still the only drug acting on glutamatergic NMDA receptors. It was originally synthesized in Eli Lilly Research Laboratories as an agent lowering hyperglycemia, but it was completely devoid of such effect and it was introduced into the therapy of cerebral ischemia and Alzheimer's dementia (AD) later on (Parsons *et al.* 1999). Memantine is believed to be a safe drug with a minimum of adverse effects in comparison to other NMDA blockers such as MK-801 or phencyclidine. The difference between these substances and MEM lays probably in the pathway of influencing the glutamate channels. Molecules with lower affinity, faster blocking/unblocking kinetics and weaker voltage-dependency (MEM, dextromethorphan, amantadine) are not burdened with negative psychotropic effects which are known to be associated with administration of high affinity NMDA receptor blockers with slow action (MK-801, phencyclidine) (Parsons *et al.* 1999). The most frequent side effect of memantine are psychomimetic responses, which appear in the case that a recommended starting dosing titration (5–20 mg over 3–4 weeks) is skipped or if MEM is co-administered with dopaminergic agents (Parsons *et al.* 1999). The same expectations were reviewed recently (Repantis *et al.* 2010) on the basis of some stimulant-like effect reports. Those reports on negative effects of MEM were not a cause for treatment discontinuation as described in the meta-analysis containing 6 trials in 2312 subjects (Doody *et al.* 2007).

The most frequent MEM prescription can be found in elderly with high probability of commorbidities requiring other pharmacotherapeutical intervention. Rather often with some other psychotropics, due to apathy (Wuwongse *et al.* 2010) or depression (the incidence from 3.2 up to 27% patients with AD) (Newman 1999, Castilla-Puentes & Habeych 2010). MEM is also reported to enhance effects of antipsychotics on negative symptoms (Krivoy *et al.* 2008), positively influence treatment of substance abuse (alcohol, heroin) (Zdanys & Tampi 2008) and it also showed the synergic effect with fluoxetine (FLU) in the combined treatment of obsessive compulsive disorder (Wald *et al.* 2009). The proposed combination either with psychotropics or with other drugs of other classes brings out the problem of drug-drug interaction. MEM is believed to be a safe drug. It is not metabolized via cytochrome P450 (CYP) system and it is believed to produce irrelevant changes in the activity of these enzymes, thus no pharmacokinetic interactions on that level are expected (Nameda). Nevertheless some discrepant reports can be found (Keltner & Williams 2004, Micuda *et al.* 2003). There was investigated in the present study the activity of rat CYP2D2, which is an orthologue of human CYP2D6

(Zahradnikova *et al.* 2007), one of the most clinically relevant CYP isoenzymes. The influences of MEM, CYP2D2 inhibitor FLU, and MEM+FLU were analysed in rats in vivo and in the isolated liver.

MATERIAL AND METHODS

Animals

The experiment was carried out on male Wistar albino rats weighing $200 \pm 20\,g$ (Biotest, Czech Republic) at the beginning of experiment. Animals were housed under controlled conditions (temperature: $22 \pm 2°C$; air humidity 50–60%; light regime 12 h light/12 h dark, lights on 6:00–18:00) in standard plastic cages with free access to water and pellet diet and underwent a 7 day long acclimatization before the start of the experiment. Rats were randomly subdivided into 5 groups per 10 animals for the isolated liver experiment. Tested substances were dissolved in saline and administered intraperitoneally, 1 bolus/day for 10 days according to the following design: M5 (MEM 5 mg/kg/day); FLU5 and FLU20 (FLU 5 or 20 mg/kg/day), MF5 (MEM+FLU 5 mg/kg/day both) and C (saline in adequate volume of 1 ml/kg/day as a control). Another 4 groups per 18 animals served for the pharmacokinetic experiment *in vivo*. These groups were administered identically as the M5, MF5, F20 and C. All procedures of animal care were approved by the Czech Central Commission for Animal Welfare.

The model of perfused rat liver

The activity of CYP2D2 was assessed in the model of isolated perfused rat liver as described elsewhere (Zendulka *et al.* 2008). The animal was anesthetized with the mixture of ketamine (100 mg/kg) and xylazine (16 mg/kg), *vena portae* was cannulated and the liver was isolated from the abdominal cavity. The liver was perfused with tempered saline during the isolation. Then, saline was changed for Williams medium E (120 ml) and liver was perfused in the modified recirculating apparatus described by Miller (Miller *et al.* 1951) for 120 minutes from the start time: the addition of DEM (1.2 mg) into the perfusion medium. The perfusion medium samples were collected in the 30th, 60th and 120th minute of perfusion.

The in vivo rat pharmacokinetic model

Animals were anesthetized with the same anesthetic mixture of ketamine and xylazine as described in the previous model. DEM was injected into the tail vein at the dose of 10 mg/kg. Experimental groups were subdivided into three subunits per 6 animals. Three samples were withdrawn from each animal to obtain nine sampling intervals for each group at all. Blood was withdrawn from *plexus retrobulbaris* by a heparinized glass capillary tube in the following intervals after the DEM injection: 10th; 20th; 40th; 60th; 90th; 120th; 180th; 240th

and 300th minute. Coagulated blood was then centrifuged to obtain serum samples.

Sample analyses

Analyses of plasmatic and perfusion medium samples were performed after the incubation with β-glucuronidase and a liquid/liquid extraction using HPLC methods described by Zimova (Zimova *et al.* 2001). Metabolic ratios (MRs) were calculated using equation MR = conc. DEM / conc. DEX.

Statistical analyses

Values of measured DEM and DEX concentration were tested for outliners with Dixon's Q-test and outliners were rejected (95% confidence level). Remaining values were statistically analyzed with the F-test and Student's t-test (Microsoft Excel 2000). The level $p \leq 0.05$ was considered to be a statistically significant difference.

Fig. 1. Levels of dextromethorphan in the perfusion medium in animals administered with memantine 5 mg/kg/day (M), fluoxetine 5 or 20 mg/kg/day (F5 and F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C). Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.
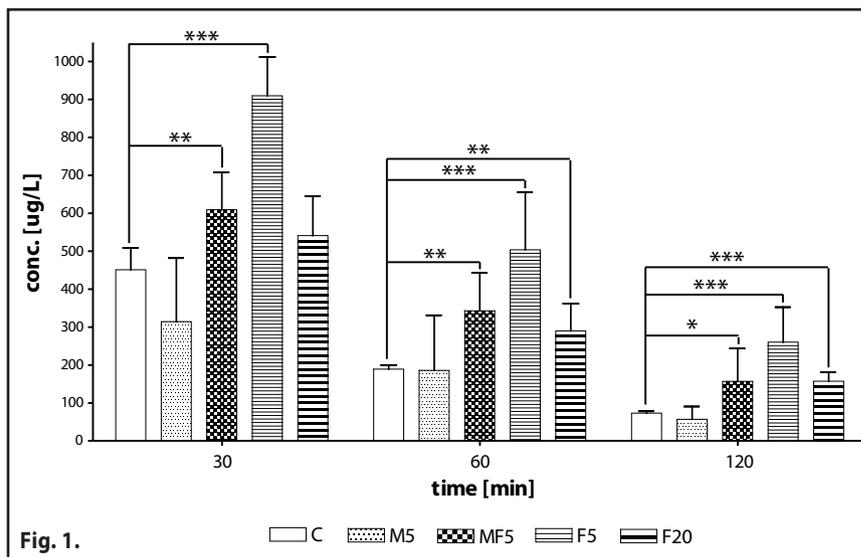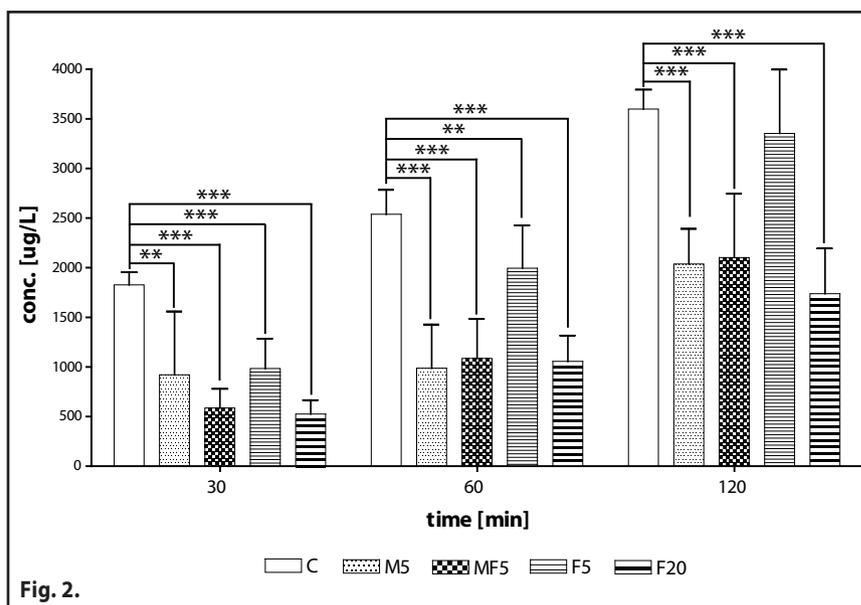
Fig. 2. Levels of dextrorphan in the perfusion medium in animals administered with memantine 5 mg/kg/day (M), fluoxetine 5 or 20 mg/kg/day (F5 and F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C). Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.

Fig. 3. Metabolic ratios of dextromethorphan/dextrorphan in animals administered with memantine 5 mg/kg/day (M), fluoxetine 5 or 20 mg/kg/day (F5 and F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C). Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.

Fig. 1.

Fig. 2.

Fig. 3.

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Tab. 1. Plasmatic levels of dextromethorphan [µg/L] in animals administered with memantine 5 mg/kg/day (M), fluoxetine 20 mg/kg/day (F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C), in the pharmacokinetic experiment *in vivo*. Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.

Time [min]	Group			
	C	M5	MF5	F20
10	2089.0 ± 424.2	530.0 ± 20.1***	285.9 ± 103.4***	1017.0 ±123.0**
20	1746.3 ± 194.7	333.1 ± 41.4***	176.6 ± 92.4***	822.2 ± 57.3***
40	273.0 ± 34.1	217.1 ± 110.8	110.9 ± 19.2***	535.5 ±96.6***
60	161.9 ± 27.2	130.4 ± 36.4	82.4 ± 20.6***	440.3 ± 39.9***
90	91.8 ± 6.8	88.7 ± 35.3	60.4 ± 6.6***	332.8 ± 72.7***
120	91.9 ± 41.3	66.1 ± 18.2	38.0 ± 7.3**	222.0 ± 83.3***
180	104.9 ± 45.0	25.4 ± 3.8**	31.7 ± 7.2**	191.4 ± 46.8***
240	69.5 ± 38.7	42.7 ± 24.2	12.8 ± 1.7**	122.6 ± 32.7**
300	25.2 ± 10.8	22.6 ± 7.3	20.5 ± 8.4	119.1 ± 46.2***

Tab. 2. Plasmatic levels of dextrorphan [µg/L] in animals administered with memantine 5 mg/kg/day (M), fluoxetine 20 mg/kg/day (F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C), in the pharmacokinetic experiment *in vivo*. Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.

Time [min]	Group			
	C	M5	MF5	F20
10	1281.5 ± 486.6	142.7 ± 24.7**	37.4 ± 9.7**	442.7 ± 262.7**
20	2100,8 ± 373.1	160.6 ± 40.7***	67.4 ± 41.2***	841.1 ± 366.1***
40	546.5 ± 47.0	198.3 ± 105.0***	87.8 ± 16.0***	920.8 ± 401.5*
60	514.6 ± 161.8	169.4 ± 70.7**	83.7 ± 31.4***	710.1 ±74.3*
90	328.0 ± 93.5	104.1 ± 32.0***	78.5 ± 15.2***	819.1 ± 466.9
120	424.1 ± 146.3	288.2 ± 216.6	59.7 ± 13.1***	611.5 ± 160.0
180	1405.0 ± 608.7	104.2 ± 60.8***	114.4 ± 68.7***	1110.5 ± 425.6*
240	1483.1 ± 434.6	272.6 ± 103.8***	389.4 ± 87.0***	1071.1 ± 313.3
300	1550.3 ± 408.2	657.4 ± 196.2***	327.1 ± 87.0	1241.3 ± 502.3

Tab. 3. Dextromethorphan/dextrorphan metabolic ratios in animals administered with memantine 5 mg/kg/day (M), fluoxetine 20 mg/kg/day (F20), memantine+fluoxetine both 5 mg/kg/day (MF5) and in control animals (C), in the pharmacokinetic experiment *in vivo*. Experimental conditions are described in Material and Methods. Data represent mean ± S.E.M. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ versus C group.

Time [min]	Group			
	C	M5	MF5	F20
10	1.80 ± 0.64	3.79 ± 0.54***	8.12 ± 3.78**	3.02 ± 1.64
20	0.85 ± 0.20	2.18 ± 0.54***	2.87 ± 0.72**	1.30 ± 0.61
40	0.51 ± 0.03	1.19 ± 0.37**	1.30 ± 0.30***	0.55 ± 0.15
60	0.34 ± 0.11	0.83 ± 0.19***	1.04 ± 0.22***	0.61 ± 0.08***
90	0.14 ± 0. 13	0.87 ± 0.06***	0.85 ± 0.11***	0.40 ± 0.14*
120	0.26 ± 0.13	0.38 ± 0.26	0.66 ± 0.16***	0.43 ± 0.24
180	0.09 ± 0.06	0.34 ± 0.22**	0.44 ± 0.29*	0.20 ± 0.07**
240	0.05 ± 0.03	0.22 ± 0.22	0.04 ± 0.01	0.12 ± 0. 04**
300	0.02 ± 0.01	0.03 ±0.01**	0.06 ± 0.01***	0.11 ± 0.05***

RESULTS

The model of isolated perfused rat liver

Levels of DEM measured in the perfusion medium were elevated during the whole perfusion in the F5 and MF5 and in the 60th and 120th min in F20 animals (Figure 1). The administration of MEM did not influence levels of DEM (M5) as they resembled those in the control (C) group. In opposite DEX concentrations were lower than in controls in all groups studied, independently on the treatment regimen (Figure 2). The only insignificant difference was measured in the F5 group in the 120th minute (Figure 2). MRs were elevated in all groups at all intervals with the exception of the 120th min in the group M5, but a trend close to the level of significance (p=0.077) was still present (Figure 3). The MRs increase in the MF5, F5 and F20 was similar with no significant differences between groups monitored.

The in vivo rat pharmacokinetic model

The influence of administered drugs on the levels of DEM varied. In the MF5 group the amounts of DEM were decreased. In opposite, FLU elicited in the F20 elevation of DEM and MEM (M5) showed practically no influence (Table 1). Concentrations of DEX were also altered, but did not reflected changes in DEM levels. DEX levels were lowered in the M5 animals and this effect was enhanced by FLU co-administration in the MF5 group (Table 2). Amounts of DEX similar to controls were found in the F20, where differences were present mainly in the first 90 minutes. In all experimental groups elevated values of MRs were found (Table 3). The significant difference between the M5, MF5 animals and controls lasted for the whole experiment with the exception of the MR in the 240th min interval. The F20 experimental group also showed elevation of MRs with later upset (after 180 minutes). All three experimental groups had elevated MRs in the end of experiment and the groups differ among each other. The highest MRs were calculated for the F20 and it was higher compared to the MF5 value, which was still more elevated than MRs of the M5.

DISCUSSION

The present study proved MEM as an inhibitor of the rat CYP2D2 enzyme. This correlates with human liver microsomes study (Micuda *et al.* 2004) which described inhibition of CYP 2B6 (K_i=76.7 μM) and 2D6 (K_i=94.9 μM). The other published data available report no drug-drug interactions on CYP level caused by MEM (Sonkusare *et al.* 2005; Nameda 2010).

The results obtained from both our experimental models are similar but not identical. On the contrary to *in vivo* testing there was registered a time dependent effect of MEM on CYP2D2 in the model of isolated perfused rat liver. The inhibition caused by MEM was detected only in the first two sampling intervals while

it was not present in the 120th min (the p-value (0.07) was close to the level of significance – p=0.05). This effect was more pronounced in the *in vivo* experiment when MEM inhibited CYP2D2 activity even longer (up to the 300th min). One could speculate that the higher DEM concentration available at the beginning of the *in vivo* experiment is the factor modulating the inhibition capacity of MEM. Thus the similar DEM levels during sampling in both models found could be a sign of higher inhibition caused by MEM *in vivo*. (The relatively high variability in DEM levels measured *in vitro* could probably result in non-significant differences calculated.)

There was documented in our study an interesting interaction between MEM and FLU changing the inhibitory effect on the CYP2D2 activity. Despite both drugs suppressed the enzyme activity with lower impact of MEM the combined treatment MEM+FLU showed milder effect than FLU alone. FLU is described as a moderate competitive inhibitor of human CYP2D6 with K_i value 0.6 μM (Pelkonen *et al.* 2008) and its major metabolite norfluoxetine showed the same activity (Otton *et al.* 1993, Crewe *et al.* 2004). The inhibition of CYP2D after FLU treatment is observed even after a single dose, is dose-dependent (Jeppesen *et al.* 1996) and can vary among different tissues and organs (Haduch *et al.* 2011). The mechanisms of the interaction between FLU and MEM as modifiers of CYP2D2 activity can be competitive, uncompetitive or mixed-type according to binding to the enzyme target. However the antagonism between enzyme modifiers MEM and FLU desribed in our study belongs to effects classified as rare and possible to be predicted only under very particular circumstances (Schenker & Baici 2009). Furthermore, the principles of interaction between two enzyme inhibitors can depend on the type of inhibition caused as well as on their concentrations (Schenker & Baici 2009). In our case, comparable amounts of MEM and FLU were administered (28 μM/kg/day and 16μ M/kg/day respectively) while K_i was different. Additional experiments with various other MEM and FLU doses applied can help to clarify this drug-drug interaction.

There are six CYP2D isoenzymes that have been genetically identified in rats compared to only one in humans (Hiroi *et al.* 2002). The sequential similarity between rat CYP2D2 and human CYP2D6 is over 70% (Soucek & Gut 1992). DEM is biotransformed into DEX exclusively via CYP2D2 enzyme in rats (Kobayashi *et al.* 2002) and there are two more metabolites present (Zendulka *et al.* 2009). The CYP2D2 catalyzes other reactions specific for the human 2D6 isoenzyme, too (Hiroi *et al.* 2002). Thus, the correlation between results obtained in CYP2D2 rat models and in CYP2D6 human studies is usually high. The dose of 5 mg/kg/day in our study was selected on the basis of MEM pharmacokinetic properties to simulate drug plasmatic levels in rats similar to those reached in humans treated with the therapeutic doses of the drug. The MEM dose used in

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our work is likely to be sufficient to reach peak serum concentrations at 20–30 min at the upper limit of doses seen in serum of healthy volunteers and patients with well tolerated doses of MEM (Parsons *et al.* 1999).

CONCLUSION

We conclude that the co-administration of therapeutically relevant doses of MEM with CYP2D6 drug substrates or even inhibitors of this enzyme would not be rated as a risk of possible clinical drug-drug interaction occurrence. The combination of MEM with CYP2D6 inhibitor could even be profitable, because MEM can moderate FLU inhibitory influence as was documented in the present study.

ACKNOWLEDGEMENTS

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4 The role of P450 in drug development and safety

The development of new drugs includes a number of preclinical tests in different models to describe the pharmacodynamics and basic pharmacokinetic characteristics of the tested compound prior to phase I of clinical tests. A description of the influence of the tested compound on the activity of P450 is part of standard testing. The U.S. Food and Drug Administration (FDA) provides lists of recommended P450 substrates, inducers, and inhibitors for both *in vitro* testing and clinical implications (FDA, 2017b). There is also a draft guidance for clinical drug interaction studies with a chapter on P450 interaction testing released by the FDA in 2017 (FDA, 2017a). A similar document applies for European countries. The European Medicines Agency (EMA) issued a guideline on the investigation of drug interactions, where P450 *in vitro* inhibition and induction testing are described (EMA, 2012).

Experimental drug testing for P450 interactions includes *in vitro* tests with human recombinant P450 enzymes. In such experiments, the tested molecule can be investigated for its ability to change the catalytic activity of P450 enzymes and compared with known inducers and inhibitors. Molecules causing a strong inhibition or induction of P450 activity can be disqualified from subsequent clinical testing because of high drug-drug interaction potential. The involvement of P450 in the metabolism of the investigated drug can be assessed as well.

On the other hand, the activity of P450 can be changed not only by direct inactivation of the enzyme molecule, but also via regulation of the amount of enzyme or its gene transcription, respectively (Zanger and Schwab, 2013). The transcription of P450 genes is regulated through specific nuclear receptors, namely constitutive androstane receptor (CAR), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), peroxisome proliferator activated receptor (PPAR), retinoic acid receptor (RXR), or vitamin D receptor (VDR) (Kawase et al., 2007; Terashima et al., 2018; Wang et al., 2008). MicroRNAs (miRNAs) are a mechanism of post-transcriptional regulation of the amount of P450. Different miRNAs are involved directly in the regulation of P450 levels (Dluzen and Lazarus, 2015) and indirectly via the regulation of nuclear receptors (Takagi et al., 2008). These types of interactions cannot be simply evaluated, as they require preserved intracellular P450 regulation mechanisms. Therefore primary screening with recombinant human P450 should be supplemented with experiments on primary human hepatocytes or with *in vivo* animal testing to obtain more detailed information about the effects of the investigated drug on P450 activity.

Although animal *in vivo* testing in preclinical pharmacology is common, the results are not simply transferable to humans. The problem of P450 activity testing is not in the sequential homology

between animal and human enzymes, which is as high as 60 to 80% for rats (Soucek and Gut, 1992), but lies in the substrate specificity, P450 distribution, and proportional representation (Hiroi et al., 2002; Nedelcheva and Gut, 1994).

4.1 Experimental testing of P450 interactions with natural substances

My experimental research in the field of natural substances and their interaction with P450 was focused on two main types of molecules. The first is natural substances with psychotropic activity. These are not a part of a common diet, but are sold as food supplements and nutraceuticals and marketed as an alternative to synthetic drugs. The second group are polyphenols – a common part of the human diet and known for many protective effects including antioxidative (Pandey and Rizvi, 2009), anti-inflammatory (Joseph et al., 2016), antiproliferative (Kawaii et al., 2001), antimutagenic (Valdez-Morales et al., 2014), and antiatherogenic activities (Rosenblat et al., 2015).

4.1.1 The influence of linalool and active compounds of saffron on P450 activity

There is a schizophrenic situation in the field of neuropsychopharmacology today. The use of psychotropics is increasing worldwide on one side (Curkovic et al., 2016; Steinhausen, 2015) while pharmaceutical companies have sidelined the research of new molecules on the other side (Hyman, 2013). The group of psychotropics is still stigmatized among patients and also some physicians as drugs with many side effects. This is partially a remnant of the past, because the first antipsychotics and antidepressant really evoked a variety of side effects. Despite new drugs and new treatment strategies including therapeutic drug monitoring and personalized/tailored medicine being available today, some "old school" measures leading to the adverse effects of psychotropics persist. A classic example is the use of benzodiazepines in anxiety and insomnia, especially in elderly people. Even the newest drugs are still not free of adverse effects. Therefore there is a significant interest in the search for new psychoactive drugs with better safety profiles. Using natural molecules isolated from herbs appears to be a promising approach. It seems that patients found natural substances to be safer in contrast to synthetic drugs in general. The demand for these molecules is met by a range of nutraceuticals and food supplements containing different herbs and herbal extracts. Such preparations are then frequently combined with pharmacotherapy. Some of these herbal psychoactive substances are known to cause drug-drug interactions at the P450 level (Zhou et al., 2004b). Therefore, knowledge of whether new constituents of these preparations influence P450 activity could improve the safety and efficacy of co-administered drugs.

Saffron, a spice prepared from Crocus sativus L., is a herb used in traditional medicine, besides others things, for its antidepressant properties. The effect of extract of saffron on mild to moderate depression is comparable with the effect of the synthetic antidepressant fluoxetine (Akhondzadeh Basti et al., 2007) or imipramine (Akhondzadeh et al., 2004). The chemical analysis of saffron extracts

reported more than 150 different chemical entities (Bathaie and Mousavi, 2010). Crocin is the water-soluble carotenoid that gives saffron its characteristic colour (Lage and Cantrell, 2009), while terpene safranal is the major constituent of the essential oil from saffron stigmas and is responsible for saffron's aroma (Tarantilis and Polissiou, 1997). Besides its antidepressant activity, safranal also has anxiolytic, hypnotic, and anticonvulsant activity, and a protective effect on the brain, skin, and respiratory tract were also proved (Rezaee and Hosseinzadeh, 2013). Crocin is reported to have a wide range of biological activities, especially in the central nervous and cardiovascular systems (Alavizadeh and Hosseinzadeh, 2014).

The monoterpenic alcohol linalool is present in the essential oils of flowers from the Lamiaceae (Nascimento et al., 2014), Lauraceae (Simić et al., 2004), and Rutaceae (Md Othman et al., 2016) families. Many herbs including essential oils with linalool are used in traditional medicine for various purposes. Besides its anxiolytic (Souto-Maior et al., 2011) and sedative (Linck et al., 2009) activities, linalool was proven to have other biological activities including analgesic (Peana et al., 2003), local anaesthetic (Zalachoras et al., 2010), antimicrobial (Herman and Tambor, 2016), anti-inflammatory (Peana et al., 2002), and antioxidant (Seol et al., 2016) activities. A clinical trial with linalool in the therapy of anxiety was conducted (Kasper et al., 2010), and a herbal medicinal product with linalool was licensed for this indication in Germany (Uehleke et al., 2012).

4.1.2   The influence of crocin and safranal on rat P450

We tested the effect of safranal and crocin on the activity of P450 in rat liver microsomes (RLM). The microsomes were both prepared from male Wistar albino rats after repeated doses of safranal given intragastrically and from animals administered repeated doses of crocin via intraperitoneal injection at doses of 4, 20 or 100 mg/kg/day. The different routes of administration for these two substances arose from knowledge of the poor bioavailability of crocin after oral use (Singla and Bhat, 2011). With safranal, we used intragastric injections as an alternative to oral use. This measure enables us to administer an accurate dose of the drug while the natural way in which the drug is taken up in the form of spice or dietary supplements remained preserved. The activity of CYP2C11, CYP3A, CYP2B, and CYP2A were assessed with testosterone as a probe substrate. Its conversion to 2β-, 2α-, 7α-, 6β-, 16α-, and 16β-hydroxytestosterone is selectively catalysed by distinct P450 enzymes. An overview of testosterone's metabolite and the involved P450 enzyme can be found in Table 1. The activities of P450 were compared with control animals treated with solvents alone.

To the best of our knowledge, our study is the first to describe the influence of safranal and crocin on P450 content and activity. Both tested substances increased the total protein content and total P450 content in RLM. The effect was significant at a dose of 100 mg/kg/day for safranal and 4 and 20

mg/kg/day for crocin. Such a change also indicates an increase in the metabolic activity of P450 which was measured for CYP 2B1 and CYP3A1 in animals with the highest dose of safranal and for CYP2C11 with a significant effect at the lowest dose. Surprisingly, the activity of all tested P450 after crocin treatment was decreased, despite increased protein and P450 levels. The actual metabolic activity, not the amount of total protein or P450, determines the risk of a drug-drug interaction, therefore the exact mechanism of this interesting effect was not further studied. Both tested substances were found to interact with P450 enzymes and were able to change the plasma levels of co-administered P450 drug substrates. Decreased plasma levels and efficacy of CYP3A1, CYP2B1, and 2C11 substrates are possible and also depend on the dose of the administered drug. In contrast, signs of toxicity and increased plasma levels should be expected when crocin is combined with substrates of the tested P450 enzymes.

Table 1: Overview of testosterone P450 probe reactions

P450	Reaction	Reference
2A1	7α-hydroxylation	(Yamazaki et al., 1994)
2B1	16β-hydroxylation	(Donato et al., 1994)
2C11	2α-hydroxylation 16 α-hydroxylation	(Wójcikowski et al., 2013)
3A1	2β-hydroxylation 6β-hydroxylation	(Eberhart et al., 1992)

Dovrtělová G., Nosková K., Juřica J., Turjap M**., Zendulka** O. Can bioactive compounds of Crocus sativus L. influence the metabolic activity of selected CYP enzymes in the rat? Physiological Research, 64 (Supp. 4), S453-S458, 2015.

Author's contribution: 30%, corresponding author

Can Bioactive Compounds of *Crocus sativus* L. Influence the Metabolic Activity of Selected CYP Enzymes in the Rat?

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Summary

Safranal and crocin are biologically active compounds isolated from *Crocus sativus* L., commonly known as saffron. Clinical trials confirm that saffron has antidepressant effect, thus being a potential valuable alternative in the treatment of depression. The aim of the present study was to determine, whether systemic administration of safranal and crocin can influence the metabolic activity of CYP3A, CYP2C11, CYP2B, and CYP2A in rat liver microsomes (RLM). The experiments were carried out on male Wistar albino rats intragastrically administered with safranal (4, 20, and 100 mg/kg/day) or with intraperitoneal injections of crocin (4, 20, and 100 mg/kg/day). Our results demonstrate the ability of safranal and crocin to increase the total protein content and to change the metabolic activity of several CYP enzymes assessed as CYP specific hydroxylations of testosterone in RLM. Crocin significantly decreased the metabolic activity of all selected CYP enzymes, while safranal significantly increased the metabolic activity of CYP2B, CYP2C11 and CYP3A enzymes. Therefore, both substances could increase the risk of interactions with co-administered substances metabolized by cytochrome P450 enzymes.

Key words

Crocin • Safranal • CYP • Rat liver microsomes

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Introduction

Crocus sativus L. is a perennial flowering plant and a member of the Iridaceae family. Saffron, the dried stigmas of *Crocus sativus* flowers, is a well-known spice. Chemical analysis of saffron extracts has revealed about 150 different compounds from which the most studied are crocin, safranal, and picrocrocin (Winterhalter and Straubinger 2000, Bathaie and Mousavi 2010). Crocin as a water-soluble carotenoid gives saffron its typical reddish or yellowish color. Safranal is a monoterpenic aldehyde responsible for saffron's characteristic odour, and the bitter taste of saffron is attributed to picrocrocin, which is also a precursor of safranal (Tarantilis *et al.* 1995, Melnyk *et al.* 2010). Saffron has not always been primarily a spice (for flavoring and coloring of food), but rather a medicinal plant, which was used in traditional medicine as e.g. sedative, anxiolytic, expectorant, aphrodisiac, antispasmodic, and for the treatment of premenstrual syndrome, asthma, or pain (Schmidt *et al.* 2007, Ríos *et al.* 1996).

Several food supplements containing saffron extracts are already available worldwide. The antidepressant effect of saffron extract was described in several double blind, randomized, placebo-controlled clinical trials (Moshiri *et al.* 2006, Akhondzadeh *et al.* 2005) and its activity was comparable to the effect of clinically used antidepressants, namely fluoxetine (Basti *et al.* 2007, Noorbala *et al.* 2005) and imipramine (Akhondzadeh *et al.* 2004). Thus, saffron may become a valuable alternative to classical antidepressants. Safranal and crocin have been intensively studied in the

last few years also for other biological effects, such as antiproliferative, antioxidative, hypnosedative, etc. (Rezaee and Hosseinzadeh 2013, Alavizadeh and Hosseinzadeh 2014, Hosseinzadeh and Noraei 2009, Moshiri *et al.* 2014).

It was described that the metabolic activity of cytochrome P450, which is one of the most important enzymatic systems for xenobiotic biotransformation, is influenced by a large number of natural substances, including carotenoids. The modulation of CYP metabolic activity could lead to clinically relevant changes in plasma concentrations of concurrently administered drugs, and thus also to changes in their pharmacological properties. The knowledge of the influence of saffron or its compounds on CYP enzymes is lacking. Therefore, it is important to reveal whether the activity of CYP enzymes could be affected. The aim of the present study is to determine the effect of systemic administration of safranal and crocin on the metabolic activity of CYP2C11, CYP3A, CYP2B, and CYP2A, and the total protein and total cytochrome P450 (CYP) content in rat liver microsomes (RLM).

Methods

Chemicals

Crocin and safranal were purchased form Sigma-Aldrich (St. Louis, MO, USA). Other compounds used in the study were following: NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $MgCl_2$, EDTA, prednisone, testosterone, KH_2PO_4, NA_2HPO_4, sucrose, and KCl, all of them provided by Sigma-Aldrich (St. Louis, MO, USA). The metabolites of testosterone, namely 2β-, 2α-, 7α-, 6β-, 16α-, and 16β-hydroxy-testosterone were purchased from Steraloids Inc. (Newport, RI, USA). Chemicals and organic solvents for HPLC analysis (acetonitrile, methanol, and dichloromethane) were provided by Lach-ner (Neratovice, CZ).

Animal procedures

The experiments were carried out on male Wistar albino rats (280±20 g), which were housed under standard laboratory conditions (22±2 °C room temperature; 55±5 % room humidity; 12:12 h light/dark cycle). Animals had free access to water and food and they were sacrificed 24 h after the last drug administration. All experiments were performed in accordance with the Czech act No. 246/1992 and with the

approval of both the local and national Czech Central Commission for Animal Welfare.

In the Experiment I, after 5 days of acclimatization, rats were randomly divided into four groups per 9-10 animals and were treated intragastrically with safranal dissolved in a mixture of propylene glycol and 5 % glucose (1:1) at the doses of 4, 20, and 100 mg/kg/day. The administrations were repeated for 10 consecutive days. The control group was administered with appropriate volume of vehicle (2 ml/kg). Safranal was administered intragastrically to simulate the same route of administration of food supplements and due to its poor solubility in aqueous solvents and thus inability to prepare appropriate parenteral solution.

In the Experiment II, after 5 days of acclimatization, rats were randomly divided into four groups per 9-10 animals and were treated intraperi-toneally with crocin dissolved in saline at the doses of 4, 20, and 100 mg/kg/day for 9 consecutive days. The control group was administered with appropriate volume of vehicle (1 ml/kg). Crocin was administered intraperitoneally due to its poor absorption through the gastrointestinal tract (Singla and Bhat 2011).

Preparation of rat liver microsomes

The RLM were isolated from 3 grams of liver tissue of individual animals by differential ultracentrifugation (19 000 × g and 105 000 × g) in 20 mM Tris/KCl buffer (pH=7.4) including washing with 0.15 M KCl and finally diluted in 0.25 M Tris/sucrose buffer (pH=7.4). The total protein content in the microsomal preparations was assessed according to Lowry *et al.* (1951) using bovine serum albumin as a standard. Determination of total CYP content was assessed using CO-difference spectroscopy method according to Omura *et al.* (1964).

Determination of cytochrome P450 activity in rat liver microsomes

Assessment of the activity of CYP2A, CYP2B, CYP2C11, and CYP3A enzymes was based on the rate of testosterone biotransformation in RLM with a NADPH generating system according to the previously described method of Wójcikowski *et al.* (2008) with a slight modification. The incubation mixture of final volume of 0.5 ml contained phosphate buffer (50 mM; pH=7.4), EDTA (1.1 mM), NADP (1.2 mM), glucose-6-phosphate (4.4 mM), $MgCl_2$ (3.2 mM), glucose-6-phosphate dehydrogenase (0.5 U in 0.5 ml), RLM (25 µl), and

testosterone, which was added to be in the final concentration of 400 μM. The reaction was stopped after 15 min of incubation at 37 °C by adding 50 μl of methanol and by cooling down in ice.

Table 1. CYP specific metabolites of testosterone (modified from Wójcikowski *et al.* 2008, Kot and Daniel 2008, Chovan *et al.* 2007, Kobayashi *et al.* 2002).

CYP	Metabolites of testosterone
2A	7α-hydroxytestosterone
2B	16β-hydroxytestosterone
2C11	2α-hydroxytestosterone
2C11	16α-hydroxytestosterone
3A	2β-hydroxytestosterone
3A	6β-hydroxytestosterone

The concentrations of testosterone and its specific metabolites were measured by a HPLC system (Shimadzu LC-10) with the UV detector (Shimadzu SPD-M10AVP) by the modified method of Haduch *et al.* (2006). After addition of the internal standard (prednisone), the analytes were extracted from the microsomal suspension with dichloromethane (4 ml), and the residue obtained after evaporation of extracts was dissolved in 200 μl of 50 % methanol. An aliquot (45 μl) was injected into the HPLC system, and the mobile phase was used in the following gradient mode: time 0 to 12 min 1:59:40 (v/v/v acetonitrile/water/methanol), from 12:00 to 17:50 min 1:48:51 and from 17:50 to the end of the analysis in the 25:00 min 1:59:40. The flow rate was 0.8 ml/min. Analytical column (Kinetex 2.6u PFP 100A, 150 × 4.60 mm) was purchased from Phenomenex (Torrance, CA, USA), and the absorbance was measured with DAD detector at the wavelength 245 nm. Metabolic

activities of all CYP enzymes were studied by measuring the rates of CYP specific reaction (Table 1) and expressed as the metabolite molar concentration/min/mg of total protein in RLM.

Statistical analysis

The results were statistically evaluated using the non-parametric Kruskal-Wallis test, and performed using the Statistica 12 software (StatSoft, Inc. 2013). Results were regarded as statistically significant when p≤0.05.

Results

Experiment I (safranal)

The analysis revealed that the systemic administration of the highest dose (100 mg/kg) of safranal significantly increased the total protein content (149 % of the control group value) and also the total CYP content (151 % of the control group value) (Fig. 1A). Other doses of safranal did not show any significant changes at the levels of the total protein and CYP content in RLM. However, both parameters were slightly elevated in comparison to the controls.

Changes in the metabolic activity were detected in all selected CYP enzymes except CYP2A (measured as a rate of 7α-hydroxylation of testosterone) (Fig. 2). The metabolic activity of CYP2B (measured as a rate of the 16β-hydroxylation of testosterone) was significantly increased only at the highest dose of safranal. The effect of safranal on CYP3A is not convincing because the rate of 6β-hydroxylation was significantly increased, while 2β-hydroxylation of testosterone was not changed (both believed to reflect CYP3A metabolic activity). The lowest dose of safranal significantly increased the rate of 2α- and 16α-hydroxylation of testosterone, which is considered as CYP2C11 specific reaction.
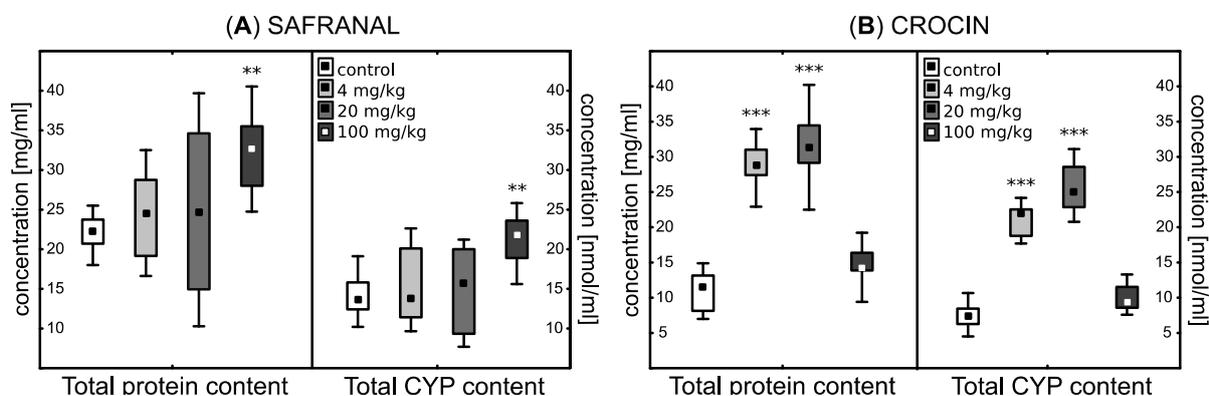

Fig. 1. The effect of systemic administration of safranal (**A**) and crocin (**B**) on the total protein and the total CYP content in RLM. All values are expressed as box plots with median (box 25 % − 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with **p≤0.01, ***p≤0.001.
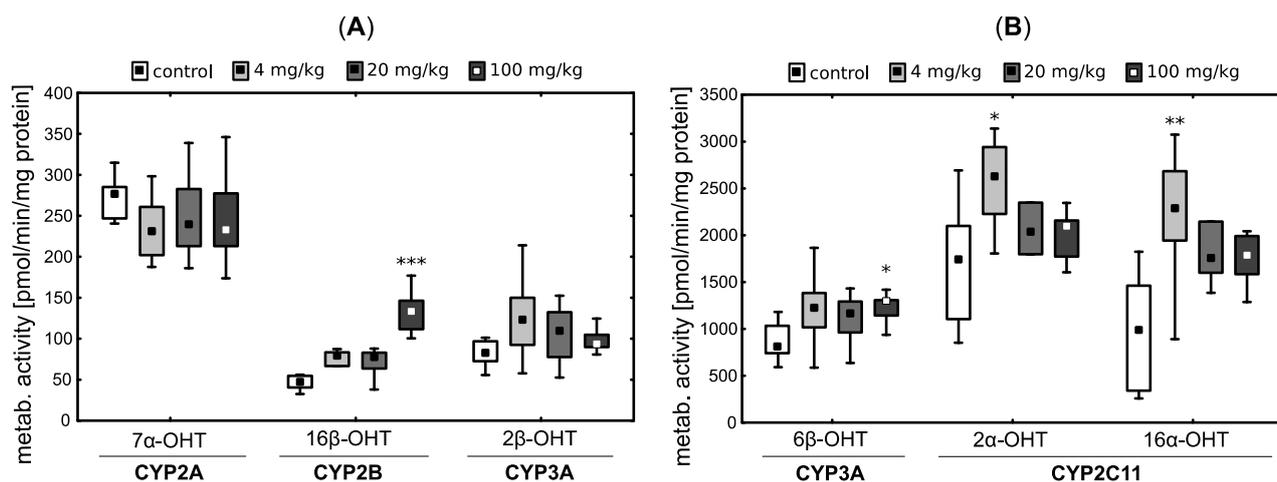
Fig. 2. The metabolic activity of selected CYP enzymes in RLM after safranal systemic administration, measured as the rate of testosterone hydroxylation. (**A**) The metabolic activity of CYP2A, CYP2B and CYP3A (2β-hydroxylation). (**B**) The metabolic activity of CYP3A (6β-hydroxylation) and CYP2C11. All values are expressed as box plots with median (box 25 % − 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with *p≤0.05, **p≤0.01, ***p≤0.001.
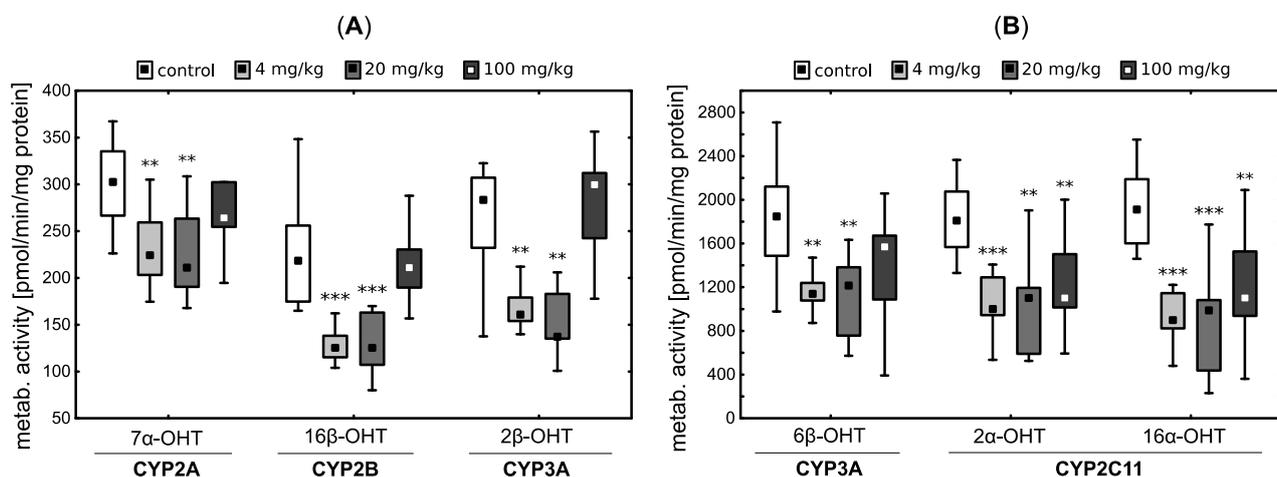
Fig. 3. The metabolic activity of selected CYP enzymes in RLM after crocin systemic administration, measured as a rate of testosterone hydroxylation. (**A**) The metabolic activity of CYP2A, CYP2B and CYP3A (2β-hydroxylation). (**B**) The metabolic activity of CYP3A (6β-hydroxylation) and CYP2C11. All values are expressed as box plots with median (box 25 % − 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with *p≤0.05, **p≤0.01, ***p≤0.001.

Experiment II (crocin)

The obtained results showed that the systemic administration of crocin significantly increased the level of both the total protein and the total CYP content, at the doses 4 mg/kg (up to 259 % and 280 % of the control, respectively) and at 20 mg/kg (up to 278 % and 343 % of the control, respectively) (Fig. 1B). The amount of total protein and the total CYP content remained unchanged at the highest dose of crocin.

The administration of crocin, unlike safranal, caused reduction of the metabolic activity of selected CYP enzymes (Fig. 3). The determination of CYP2B metabolic activity revealed significant decrease at the doses of 4 and 20 mg/kg and the same result was

observed in the metabolic activity of CYP2A. All doses of crocin significantly decreased the metabolic activity of CYP2C11. In the case of CYP3A, significant decrease was observed in the rate of both 6β-hydroxylation and 2β-hydroxylation of testosterone at the doses of 4 and 20 mg/kg.

Discussion

Our results demonstrate the ability of systemic administration of safranal and crocin to increase the total protein and the total CYP content in RLM and to change the metabolic activity of different CYP enzymes. Thereby, tested substances could raise the risk of

interactions with co-administered drugs metabolized by the same pathway. Several clinical trials confirmed that saffron may be of therapeutic benefit especially in the treatment of mild to moderate depression (Basti *et al.* 2007, Akhondzadeh *et al.* 2004, Moshiri *et al.* 2006). This is of interest because currently psychiatric patients with depression are treated by a wide range of synthetic antidepressants, but up to 30 % of them are pharmacoresistant without a significant clinical improvement (Micale *et al.* 2013). Food supplements containing saffron extract are relatively safe, and so they are a viable alternative to the conventional drugs. The daily dose of saffron extract is 30 mg according to clinical trials recommendations (Basti *et al.* 2007, Akhondzadeh *et al.* 2004, Moshiri *et al.* 2006).

To the best of our knowledge, no studies exist up to date dealing with the effects of safranal or crocin on CYP3A, CYP2C11, CYP2B, and CYP2A enzymes. However, it was described that metabolic activity of CYP enzymes is influenced by a large number of natural carotenoids, and the authors reported either decreased or increased CYP metabolic activity (Louisa *et al.* 2009, Jewell and O'Brien 1999, Satomi and Nishino 2013, Wang and Leung 2010). Our experiment showed that the metabolic activity of CYP2A, CYP2B, CYP2C11, and CYP3A was decreased after administration of carotenoid crocin. Safranal significantly increased the metabolic activity of CYP2B and CYP2C11, while the metabolic activity of CYP2A enzyme was without any significant changes. The effect of safranal on the testosterone hydroxylation *via* CYP3A was uncertain because 6β-hydroxylation was significantly increased, while 2β-hydroxylation of testosterone was not changed. Therefore, in our future experiments, we aim to use other probe substrates (nifedipine or midazolam) to determine the influence of safranal on CYP3A.

Interestingly, the total protein content was increased by safranal as well as by crocin. However, the metabolic activity of CYP2B, CYP2C11 and CYP3A enzymes was increased in the case of safranal and decreased in the case of crocin and CYP2A, CYP2B, CYP2C11, and CYP3A. The specific mechanism by which safranal or crocin influence CYP enzymes is unknown and we can therefore only hypothesize that this discrepancy could be explained by increase in CYP content including those CYP enzymes whose activity was not assessed in this study. Thus, we have observed apparent decrease in the CYP activity when the activity was calculated per mg of total protein or per nmol of total CYP. Other explanations could also exist, such as the negative feedback on reduced activity of main metabolic enzyme system. It is essential to carry out further experiments to confirm or reject these hypotheses.

To conclude, the obtained results showed that safranal and crocin influence the apparent metabolic activity of CYP enzymes in RLM. It would be necessary to take into account the risk of possible interactions with substances metabolized by CYP enzymes in the case of use of these substances in the clinical practice. This risk becomes more serious as food supplements containing saffron extracts are already available and their influence on the human CYP enzymes is not estimated yet. We report the ability of safranal and crocin to influence the activity at least of some rat liver CYP enzymes. Nevertheless, the daily intake of crocin and safranal from the food supplements is much lower in comparison to the doses administered to rats in our study and the substrate specificity of the rat CYP enzymes is not fully identical to human enzymes.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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4.1.3 The influence of linalool on rat P450

Two different enantiomers of linalool exist, and we investigated the effects of (-)-linalool on P450 activity in the model of RLM. Male Wistar albino rats were treated intragastrically with (-)-linalool at doses of 40, 120, and 360 mg/kg/day for 13 days. The total protein, total P450 content, and activities of CYP2A, CYP2B, CYP2C11, and CYP3A were assessed with testosterone as a probe substrate. For the measurement of CYP2C6 activity, the 4'-hydroxylation of diclofenac was used as a marker reaction. The interaction of (-)-linalool with CYP2C6 in naïve RLM was also tested. Neither total protein nor the total P450 content was changed in the RLM of (-)-linalool treated animals. The increase in activities of CYP2A, CYP2B, CYP3A, and CYP2C6 were insignificant except for the highest dose of (-)-linalool, which increased the activity of CYP2A significantly. The activity of CYP2C11 was in contrast inhibited, and this effect of (-)-linalool was again insignificant. (-)-linalool also exhibited a weak inhibition of rat CYP2C6 in direct *in vitro* inhibition studies. The IC$_{50}$ was calculated to be 84 µM. With regard to the obtained results, (-)-linalool probably does not significantly influence P450 metabolic activity, and its effect on human P450 should be tested to confirm our findings for clinical practice.

Nosková K, Dovrtělová K., **Zendulka** O., Řemínek R., Juřica J. The effect of (-)-linalool on the metabolic activity of liver CYP enzymes in rats. Physiological Research, 65 (Supp. 4), S427-S440, 2016.

Impact factor: 1.643; Physiology Q4   Citations WoS: 2

Author's contribution: 15%

The Effect of (-)-Linalool on the Metabolic Activity of Liver CYP Enzymes in Rats

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Summary

(-)-Linalool is the major floral scent occurring mainly in families *Lamiaceae, Lauraceae* and *Rutaceae* and is the main active compound of lavender oil. The purpose of this study was to reveal the influence of subchronic systemic treatment with (-)-linalool on the metabolic activity of CYP2A, 2B, 2C6, 2C11 and 3A in rat liver microsomes (RLM). The second aim was to reveal possible inhibitory effect of (-)-linalool on CYP2C6 *in vitro*. Wistar albino male rats were treated with (-)-linalool intragastrically at the doses of 40, 120, and 360 mg/kg/day for 13 days. Treatment with (-)-linalool at the dose of 360 mg/kg increased the metabolic activity of CYP2A assessed with testosterone as a probe substrate. (-)-Linalool showed weak competitive inhibition of CYP2C6 in rat liver microsomes, with IC_{50} of 84 µM with use of diclofenac as a probe substrate.

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Introduction

Linalool is a monoterpenic alcohol present either in monocotyledonous and dicotyledonous plants. There were identified two enantiomers in plants. (3S)-(+)-Linalool, also known as coriandrol, is the main component of essential oils of *Coriandrum sativum* seeds. (3R)-(-)-Linalool, known as licareol, is the main component of essential oil of *Lavandula officinalis* flowers. Except of the distribution, the enantiomers differ in a fragrance and biological activity. (+)-Linalool is described as sweet, floral and herbaceous while (-)-linalool as a woody, lavender-like aroma (Aprotosoaie *et al.* 2014). The subject of this study is (-)-linalool, the main active substance of *Lavandula spp.* which is used in traditional medicine to treat anxiety or mild depression (EMA/HPMC 2012). Many biological activities of (-)-linalool have been proven, for example anti-inflammatory (Peana *et al.* 2002), antimicrobial (Park *et al.* 2012) and antioxidant activity (Liu *et al.* 2012). Many studies have also shown diversity of other pharmacological properties such as sedative (Sugawara *et al.* 1998), anxiolytic (Souto-Maior *et al.* 2011), anticonvulsant (Elisabetsky *et al.* 1999), analgesic (Li *et al.* 2016) and local anesthetic activity (Zalachoras *et al.* 2010). Food supplements containing (-)-linalool whose efficacy in treatment of anxiety disorders was confirmed by clinical trial (Kasper *et al.* 2010) are available in Central Europe. Furthermore, lavender oil preparation has been licensed in Germany as a herbal medicinal product for the treatment of restlessness in anxiety disorders (Uehleke *et al.* 2012).

According to the increasing popularity of alternative medicine and rising use of herbal preparation, knowledge on pharmacodynamic and pharmacokinetic herbal-drug interactions are needed and required by state authorities in the registration procedure of herbal medication. The aim of this study was to evaluate the influence of subchronic administration of (-)-linalool on the metabolic activity of liver CYP2A, CYP2B, CYP2C6, CYP2C11 and

CYP3A enzymes in rats and to determine their IC_{50} for a purpose to predict possible herbal-drug interactions.

Methods

Animals

Male Wistar albino rats (280±20 g, 8 weeks old) were housed in groups of 5 under standard laboratory conditions (12/12 h light-dark regime at the temperature 22±2 °C and room humidity 55±5 %). Water and pelleted diet were provided *ad libitum*. All experiments were performed in accordance with the Czech act No. 246/1992 and with the approval of both the local and national Czech Central Commission for Animal Welfare.

Experimental design

After 5 days of acclimatization, rats were randomly divided into four groups per 10 animals. Animals were treated intragastrically with (-)-linalool dissolved in a 1 % Tween 20 at the doses of 40, 120 and 360 mg/kg/day. The control group was administered with appropriate volume of vehicle (5 % glucose + 1 % Tween 20, 1 ml/kg). The administration was carried out for 13 consecutive days. Animals were sacrificed by decapitation 24 h after the last drug administration and the liver was sampled and frozen until microsomes isolation.

Chemicals

(-)-Linalool, NADP, glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, $MgCl_2 . 6 H_2O$, EDTA, KH_2PO_4, Na_2HPO_4, HCl, prednisone, ibuprofen, testosterone, diclofenac, Tween 20, KCl, Tris, 4′-hydroxydiclofenac and sucrose were provided by Sigma-Aldrich (St. Louis, MO, USA). Metabolites of testosterone (2β-, 2α-, 7α-, 6β-, 16α-, 16β- hydroxyl-testosterone) were purchased from Steraloids Inc. (Newport, RI, USA). All the organic solvents were of HPLC purity (acetonitrile, methanol, dichloromethane and diethyl ether) and were supplied by Lach-Ner (Neratovice, CZ).

Preparation of RLM

Microsomes were isolated from 3 grams of liver tissue of individual animals by differential ultracentrifugation (19000 g for 20 min and 2×105000 g) in 20 mM Tris/KCl and finally diluted in 0.25 M Tris/sucrose buffer (pH=7.4). The total protein content in the microsomal preparations was assessed according to Lowry *et al.* (1951) method using the bovine serum albumin as a standard. Total CYP content was assessed by CO-difference spectroscopy according to Omura *et al.* (1964) method.

Determination of Cytochrome P450 activity in RLM

The activities of CYP2A, 2B, 2C11 and 3A were assessed by measuring the rate of testosterone hydroxylation in positions: 7α (CYP2A), 16β (CYP2B), 2α and 16α (CYP2C11), 2β and 6β (CYP3A) according to the modified method of Wójcikowsky *et al.* (2008) with slight modifications (Turjap *et al.* 2014) as was described previously (Dovrtělová *et al.* 2015). The activity of CYP2C6 was assessed by measuring the rate of 4′-hydroxylation of diclofenac. Incubation mixture of final volume 0.5 ml containing phosphate buffer (50 mM, pH=7.4), EDTA (1.1 mM), NADP (1.2 mM), glucose-6-phosphate (4.4 mM), $MgCl_2 . 6 H_2O$ (3.2 mM), glucose-6-phosphate-dehydrogenase (0.5 U in 0.5 ml), 50 µl of RLM (1 mg/ml of protein) and diclofenac (100 µM). Drugs' effects were evaluated in the range of linear dependence of the product formation on time and concentrations of substrates. The reaction was carried out at 37 °C on horizontal vortex at 180 rpm and stopped after 20 min by addition of 50 µl of ice cold methanol and cooling down on the ice. Metabolites formed during activity assays were measured by HPLC (Shimadzu LC-10) with the DAD detector (Shimadzu SPD-M10AVP). Internal standard (ibuprofen) was added to the analytes and 4′-hydroxydiclofenac was extracted by 10 min of vortexing with diethyl ether (4 ml). The residue obtained after evaporation of extracts was dissolved in 250 µl of mobile phase. An aliquot of 20 µl was injected into the HPLC system and the mobile phase was used in following isocratic mode: 57:43 v/v KH_2PO_4 (20 mM, adjusted to pH=2.8)/acetonitrile. The flow rate was 0.55 ml/min. Analytical column (Kinetex C18 2.6u, 150 x 4.6 mm) was purchased from Phenomenex (Torrance, CA, USA). The absorbance was measured with DAD detector at the wavelength 225 nm (ibuprofen) and 276 nm (diclofenac and 4′-hydroxydiclofenac). Metabolic activities of all CYP enzymes were expressed as the metabolite molar concentration/min/mg of total protein in RLM. Inhibitory potency of (-)-linalool on CYP2C6 metabolic activity was evaluated in RLM incubated with diclofenac after 10 min of pre-incubation with (-)-linalool (1 µM – 1 mM).

Data analysis

Data were statistically evaluated with the Statistica 12 software (StaSoft, Inc. 2013) using non-parametric Kruskal-Wallis test. Results were regarded as statistically significant when p≤0.05. IC$_{50}$ value was determined through nonlinear regression of relative reaction rate at single substrate concentration (25 µM) and 0.25 mg/ml of total protein in the presence of varying inhibitor concentrations (1 nM – 1 mM) and calculated by using SigmaPlot (SPSS, Inc., Chicago).

Results

Neither total protein content nor total CYP content in RLM were affected by any of treatments (Fig. 1). Treatment with (-)-linalool showed insignificant, dose dependent tendency with increase in the rate of testosterone 7α-(CYP2A), 6β-(CYP3A), and 16β-hydroxylation (CYP2C11) but only dose 360 mg/kg exhibited statistically significant increase in the rate of 7α-hydroxylation of testosterone (CYP2A) (Fig. 2). (-)-Linalool exhibited also dose-dependent trend on the rate of 4′-hydroxylation of diclofenac (CYP2C6) which did not reach statistical significance at any of treatment regimes (Fig. 2). Within 1 nM – 1 mM (-)-linalool exhibited weak competitive inhibition of CYP2C6, with IC$_{50}$ of 84 µM (Fig. 3).
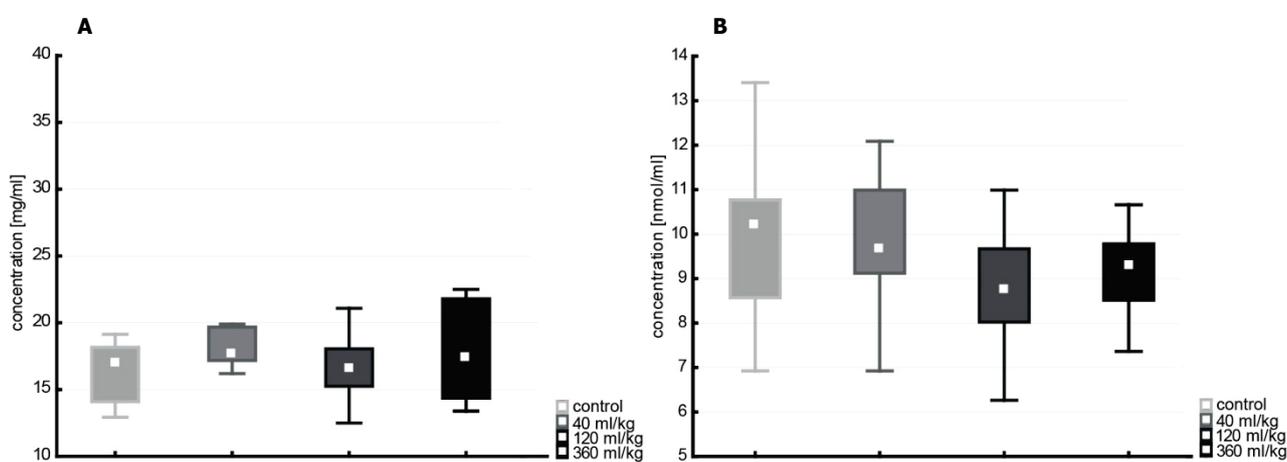
Fig. 1. The effect of subchronic administration of (-)-linalool on total protein (**A**) and total CYP content (**B**) in RLM. Values expressed as box plots with median (box 25 – 75 %, whiskers min – max outliers).
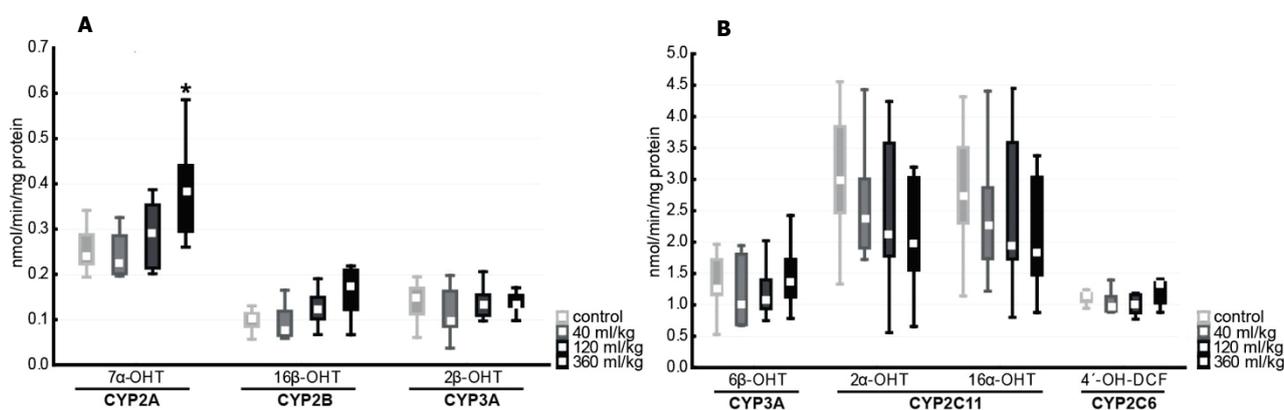
Fig. 2. The metabolic activity of selected CYP enzymes in RLM after systemic subchronic administration of (-)-linalool expressed as nmol/min/mg of total protein (OHT = hydroxytestosterone, 4′-OH-DCF = 4′-hydroxydiclofenac). All values are expressed as box plots with median (box 25 – 75 %, whiskers min-max without outliers). Statistical significance with respect to the control group is indicated with * p≤0.05.
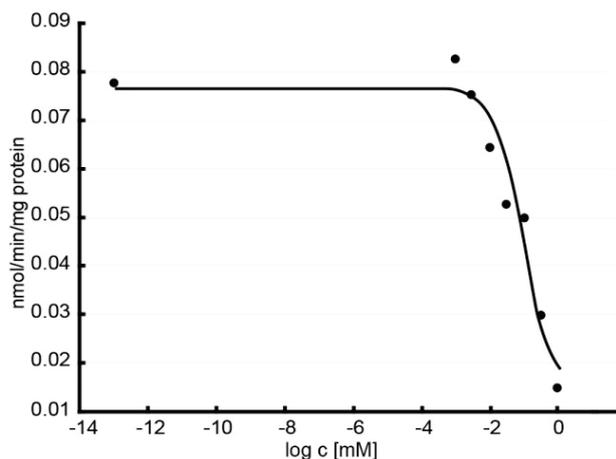
Fig. 3. Effect of (-)-linalool on the *in vitro* biotransformation of diclofenac to 4´-hydroxydiclofenac by RLM with NADPH generating system. A fixed concentration of diclofenac (25 µM) was incubated with various concentrations of (-)-linalool (1 nM – 1 mM). The IC$_{50}$ value was determined by nonlinear regression analysis.

Discussion

The objective of this study was to assess *in vitro* metabolic activity of selected CYP enzymes in RLM after subchronic administration of (-)-linalool to rats. The metabolic activity of CYP2A, CYP2B, CYP2C11 and CYP3A was assessed with the use of testosterone and the metabolic activity of CYP2C6 with diclofenac as a probe substrate. The results from the study demonstrate that dose 360 mg/kg of (-)-linalool significantly increased the rate of 7α-hydroxylation of testosterone, which correspond to increased metabolic activity of CYP2A. In rat, the CYP2A family includes CYP2A1, CYP2A2 occurring in hepatic tissue and CYP2A3 occurring in lungs. The rat CYP2A show about 60 % homology in amino acid sequence to human CYP2A6 (Martignoni *et al.* 2006). There are only few drugs which are predominantly metabolized *via* CYP2A (coumarin, nicotine), whereas for the most of xenobiotic substrates CYP2A represents only minor pathway of their biotransformation (halothane, methoxyflurane, valproic acid etc.). However, CYP2A displays restricted substrate specificity, it was found that can metabolically activate a number of carcinogens including nitrosamines and aflatoxins (Raunio *et al.* 2008).

To the best of our knowledge, up to date there is no study which tested the effect of (-)-linalool on CYP enzymes. Doroshyenko *et al.* (2013) evaluated effect of lavender oil preparation in 16 humans with result that 160 mg/day of lavender oil preparation had no clinically relevant effect on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes *in vivo*. Kasper *et al.* (2013) reviewed 7 clinical trials investigating the anxiolytic efficacy of lavender oil preparation, which generally confirmed that doses 80 – 160 mg/day have the anxiolytic effect in patients with subsyndromal anxiety and generalised anxiety disorder. The doses tested by ours in the experiment with subchronic administration of (-)-linalool to rats were much higher, and therefore we assume that possible influence of linalool on drug metabolizing enzymes is not clinically relevant.

Conclusions

In the light of our results, (-)-linalool could be considered as safe anxiolytic/antidepressant adjuvant to conventional treatment in the light of drug-drug interactions. For confirmation of our results, it is necessary to replicate our results in other experimental system, e.g. human liver microsomes or immobilized CYP microreactor system (Schejbal *et al.* 2016) which could enable repeated use of human CYP enzymes with series of CYP probe substrates.

Conflict of Interest
There is no conflict of interest.

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4.1.4. The influence of *trans*-resveratrol and quercetin on rat P450

Polyphenolic compounds seem to be promising molecules for the prevention of diseases because of their pleiotropic protective effects on the human body. There was a surge in their research after articles describing the "French paradox" were released in the 1980s. *Trans*-resveratrol was thought to be at least one of the most active if not the most important molecule of the described paradoxical situation between the high fat intake and low incidence of atherosclerosis in France. Despite the fact that the existence of the "French paradox" is disputable, the research of *trans*-resveratrol provided some interesting findings on its broad protective effects which have been reviewed elsewhere (Tsai et al., 2017).

Quercetin is another common polyhenolic compound with the flavonol structure, and is abundant in various types of fruits and vegetables (Nishimuro et al., 2015). Similarly to *trans*-resveratrol, it possesses a wide range of biological activities (Anand David et al., 2016). A controversy about its possible mutagenic and genotoxic effects detected in *in vitro* studies was discussed. Nevertheless, it seems that quercetin at estimated dietary intake levels is safe and produces only positive health effects *in vivo* (Harwood et al., 2007).

In one of my experiments, we tested the influence of *trans*-resveratrol and quercetin on the metabolic activity of CYP1A2 in Wistar albino rats. The activity of P450 was measured in the model of isolated perfused rat liver with phenacetin as a probe substrate. The principle of the assessment is phenacetin O-deethylation to paracetamol, a reaction characteristic for CYP1A2 (von Moltke et al., 1996). CYP1A2 was selected due to its role in the activation of procarcinogens (Guengerich and Shimada, 1998), to reveal one of the possible modes of the anticarcinogenic effects of the tested compounds. The effect of *trans*-resveratrol was tested in both male and female rats while quercetin was only administered to male rats. The difference in the experimental design was based on the knowledge that *trans*-resveratrol is able to interact with estrogenic receptors (Henry and Witt, 2002), which are known to also modulate CYP activity (Tsuchiya et al., 2005). Both substances were administered intraperitoneally for ten days because of poor bioavailability from the intestine (Biasutto et al., 2010).

The tested substances differed in their effects on the P450 activity. Our results documented that *trans*-resveratrol at a dose of 5 mg/kg/day administered intraperitoneally did not influence the activity of CYP1A2 regardless of sex. On the other hand, a significant difference in the activity of CYP1A2 between male and female rats was detected. Higher levels of paracetamol were found in female rats, indicating higher CYP1A2 metabolic activity than in males. Quercetin, on the other hand, inhibited the activity of the studied enzyme.

Zendulka O., Zahradníková L., Juřica J., Totušek J. The influence of *trans*-resveratrol and quercetin on the activity of CYP1A2 in rat. Czech Journal of Food Sciences, 26, S60-S64, 2008.

Impact factor: 0.472; Food Science & Technology Q3   Citations WoS: 6

Author's contribution: 60%

The Influence of *Trans*-resveratrol and Quercetin on the Activity of CYP1A2 in Rat

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Abstract: Polyphenolic compounds are widely distributed in plants and are a common part of human diet. Polyphenols are known to be potent bioactive molecules, predominantly with protective effects. Many of xenobiotics, including polyphenols, influence the activities of various enzymatic systems. Such interactions can modulate the activities of co-administered drugs. The identification of polyphenols' potential for the interactions based on metabolic changes is thus necessary. Cytochrome P450, which takes part in the metabolism of more than 90% of used drugs, is an important enzymatic system which can be influenced. We therefore determined the influence of quercetin and *trans*-resveratrol on the activity of cytochrome P450 1A2 in rats. A perfused rat liver model and phenacetin as a marker of 1A2 activity were used. Moreover, we studied the dependence of *trans*-resveratrol's activity on sex in both sexes. *Trans*-resveratrol did not influence the 1A2 activity, but it enhanced sexual differences in the metabolic activity. Our results also confirmed different metabolic activities between sexes. Female rats metabolised faster through 1A2. Based on our results, we suggest that quercetin is an inhibitor of cytochrome P450 1A2 isoenzyme.

Keywords: *trans*-resveratrol; quercetin; CYP 1A2; rat

Human health has been influenced by many factors. One of the most important ones, which cannot be excluded from our lives, is a diet. The amount and composition of food can prevent human body from many diseases. On the other hand, improper diet can be a risk factor or a promotor of many serious diseases including obesity (Terra *et al.* 2008), diabetes mellitus (Sabu *et al.* 2002), and illnesses of cardiovascular system (Sesso *et al.* 2003). From this point of view, the modulation of diet composition is desirable for the prevention of these illnesses.

Positive effects of diet on human health are caused by many biologically active substances. However, the characterisation of such molecules is problematic because of the variability in their chemical structures and differences in biological activity. Generally, these compounds are mainly of non-nutritive character and often act as anti-oxidants. Many such molecules belong to the group of polyphenolic compounds. Polyphenols are widely distributed in plants (Bravo 1998) and their protective effects were proved in many *in vitro* and *in vivo* studies (Rechner *et al.* 2002). Their good availability from natural sources as well as high biological activity determine them to be a perfect highly valuable diet component. High amounts of fruits and vegetables in diet guarantee a high polyphenolic intake. Recent lifestyle and people's reluctance to change their diet habits lead to the formulation of many dietary supplements containing polyphenols, and to the production of polyphenol-enriched foods. Such preparations contain either polyphenolic extracts from various

plants or single polyphenolic compounds. *Trans*-resveratrol (*t*-res) and quercetin (Q) are often used in dietary supplements.

Biological activities of polyphenolic compounds are presented at different levels of human organism including changes in metabolic activity of specific enzymatic systems (He *et al.* 2007). This action can be crucial for the metabolism of other substrates of the goal enzyme. These interactions can have either a positive or negative influence. The inhibition of the enzyme action can decrease the conversion of xenobiotics to their toxic forms (e.g. procarcinogens to carcinogens) or, vice versa, the induction can stimulate the same reaction. A strong probability also exists of combining polyphenols (dietary supplements, high diet intake) and medication. The interactions between polyphenols and drugs at the enzymatic level can play a crucial role in the drug effectiveness and toxicity.

One of the major metabolic systems in organism is cytochrome P450 (CYP450). It consists of many substrate and reaction specific isoenzymes, which are localised at different sites of the organism, but predominantly in liver (Seliskar & Rozman 2007). The majority of the intaken xenobiotics, as well as some endogenous molecules, are substrates for this enzyme and many of them are able to change its activity. Clinically the most important isoenzymes of CYP450 are: 1A2, 2C9, 2D6 and 3A4. More than 95% of drugs are metabolised by these four isoenzymes (Anzenbacher & Anzenbacherova 2001). Isoenzyme 1A2, which metabolic activity was determined in this study, plays a significant role in the conversion of promutagens and procarcinogens into their active forms (Hammons *et al.* 2001). Flavonoids are usually described as inhibitors of CYP450 (Piver *et al.* 2003) and inhibition of CYP1A2 is believed to be a part of their cancer preventive action. On the other hand, some authors showed data indicating the inductive activity of polyphenols on CYP450 (Rahden-Staron *et al.* 2001).

The aim of our recent work was to determine the influence of polyphenols *t*-res and Q on the activity of hepatic CYP450 1A2 (CYP1A2) isoenzyme in rats. *Trans*-resveratrol has a phytoestrogenic activity (Bowers *et al.* 2000). That is why we tested the activity of *t*-res in male and female rats together. The differences in the metabolic activity of CYP1A2 between sexes and the influence of sex on *t*-res activity have not been reported either until now.

MATERIAL AND METHODS

Animals. The experiments were carried out on male and female Wistar albino rats (Biotest, Czech Republic) weighing 200 ± 40 g. The rats were randomly divided into groups of 10 animals and were housed in a room with controlled standard conditions. The rats were acclimatised for 5 days before the start of the experiment. *Trans*-resveratrol and Q (both Sigma-Aldrich, USA) were dissolved in 30% DMSO/saline solution. The solutions were administered to the animals by intraperitoneal injections. One group of male and female rats was treated with *t*-res in the dose of 5 mg/kg/day. Quercetin in the same dose was administered to one group of male rats. To the control groups, the male and the female ones, 30% DMSO/saline solution in an adequate volume (1.0 ml/kg/day) was administered. Animals were used for liver perfusion after 10-day premedication. All experimental procedures were approved by the Czech Central Commission for Animal Welfare.

Model of isolated perfused rat liver. The rats were anesthetised with intraperitoneal injection of a mixture of ketamin – 2 ml/kg and xylazine – 0.8 ml/kg live weight (both Spofa, Czech Republic). The liver was isolated from the donors using a standard surgical technique. A plastic cannula was inserted into the portal vein after opening the abdominal cavity by wide laparotomy. The liver was shortly washed out by tempered (38°C) saline equilibrated with Carbogen – 95% O_2 and 5% CO_2 (Linde Technoplyn, Czech Republic). The saline solution was changed for tempered and Carbogen equilibrated William's medium (Sigma-Aldrich, USA) in a short time. A recirculating apparatus was constructed according to the principles described by Miller (1951). The marker substance phenacetin (Sigma-Aldrich, USA), in the final concentration of 10 mg/l was added as a bolus into the perfusion medium after 20 min of preperfusion. The samples of perfusate (1.0 ml) were collected in the 30th, 60th and 120th min of perfusion and were stored at –75°C until the analysis.

Extraction and determination of phenacetin and paracetamol. Quantitative analysis of phenacetin (PHE) and its CYP1A2 specific metabolite paracetamol (PAR) levels in the perfusate samples was performed after liquid-liquid extraction. Briefly, 500 µl of sample was mixed with 100 µl of internal standard – chlorpropamide (40 mg/l) (Sigma-Aldrich, USA). After the addition

of diethyl ether, the samples were shaken for 10 min and then centrifuged. The organic phase was separated, evaporated, and reconstituted in 250 µl of mobile phase for HPLC analysis. The levels of PHE and PAR were analysed using Shimadzu LC10 series (Japan) HPLC system. The mixture of 10mM KH_2PO_4 and acetonitrile in the 60:40 (v:v) ratio was used as the mobile phase. The separation was performed on Luna C18 (150 × 4.6 mm, 5 µm) column and the detection of the analysed molecules was performed on UV-VIS detector.

Statistical analysis. For statistical calculations, *F*-test and Student's *t*-test (Microsoft Excel 2000) were used, $P \leq 0.05$ was considered to be a statistically significant difference.

RESULTS AND DISCUSSION

The influence of sex on the activity of CYP1A2

The concentration curve courses of the markers and metabolites in the control groups did not differ from the expected ones. The levels of PHE decreased, while PAR levels increased with the ongoing perfusion time. The difference between the male and female groups was observed in the speed rate of PHE-PAR metabolic transformation. The concentrations of PHE were significantly increasing ($P \leq 0.01$) in females only in the 30th min (Figure 1), while PAR levels were increased ($P \leq 0.05$) during the whole perfusion as compared to the male control rats (Figure 2). The higher metabolic activity of CYP1A2 in the female rats

confirms the results obtained with humans (Scandlyn *et al.* 2008).

The clinical relevance of different metabolic activities between sexes is usually insignificant due to the safety of the majority of the administered drugs. Higher metabolic activity of CYP1A2 in females should be taken into account when drugs with narrow therapeutic index are used.

The influence of trans-resveratrol on the activity of CYP1A2

The speed of conversion between *t*-res treated male and female rats was different, as well as in the control animals. The concentrations of PHE did not differ between sexes (Figure 1), but PAR was elevated ($P \leq 0.001$) in females for the whole perfusion time again (Figure 2). The data obtained with *t*-res pre-treated animals resembled the data obtained with the controls and no statistical difference was found. In our opinion, *t*-res at the dose of 5 mg/kg l.w. for 10 days did not influenced the activity of CYP1A2 in rats. On the other hand, the literature sources describe its diverse influence on various CYP 450 isoenzymes. Chan and Delucchi (2000) and other authors (Piver *et al.* 2003; Regev-Shoshani *et al.* 2004) described the inhibition of CYP3A4 and 2E1, while Kluth *et al.* (2007) defined *t*-res as an inducer of hepatal CYP450. None of the experiments cited was carried out using the methods with whole animals or organs, so their conclusions may be incongruous with reality.

The comparison between the sexes of *t*-res administered animals was again similar to that in the
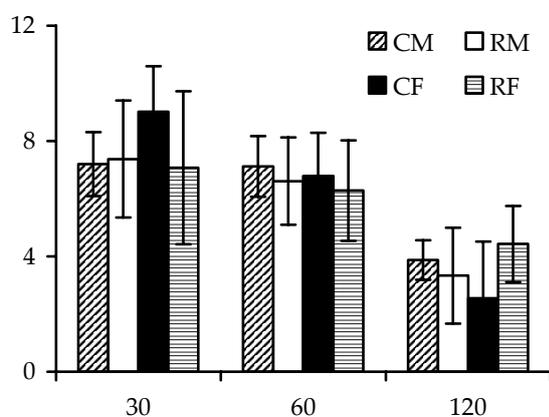

Figure 1. Levels of phenacetin in control male rats (CM), control female rats (CF), *trans*-resveratrol male rats (RM) and *trans*-resveratrol female rats (RF)
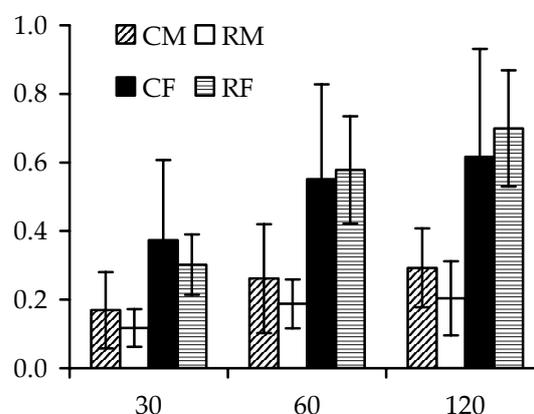
Figure 2. Levels of paracetamol in control male rats (CM), control female rats (CF), *trans*-resveratrol male rats (RM) and *trans*-resveratrol female rats (RF)
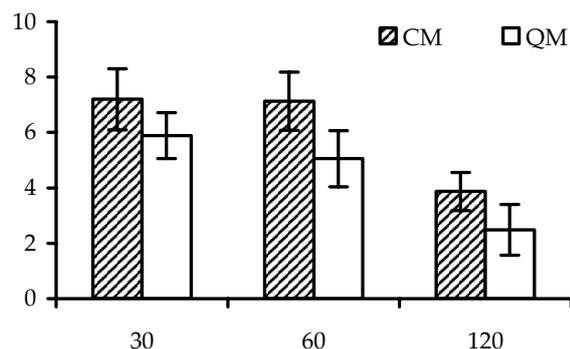
Figure 3. Levels of phenacetin in quercetin male rats (QM) and male control rats (CM)
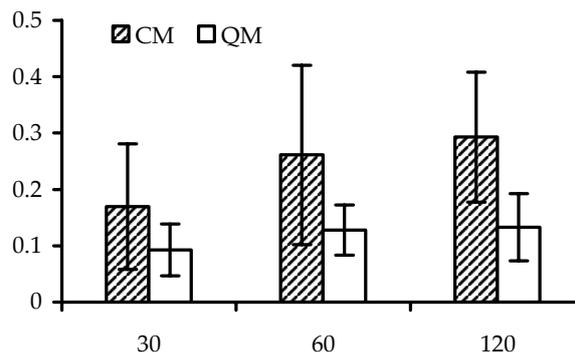
Figure 4. Levels of paracetamol in quercetin male rats (QM) and male control rats (CM)

controls. The females metabolised via CYP1A2 faster, which was confirmed by the elevation of PAR levels in them. This difference was statistically more significant ($P \leq 0.001$) than that in the controls ($P \leq 0.05$). In our opinion, *t*-res at the dose of 5 mg/kg l. w. enhances intersexual differences in rats. The possible explanation consists in the estrogenic effect of *t*-res which can bind to estrogenic receptors. Its agonistic effect on β-estrogen receptor was described (Bowers *et al.* 2000). Estrogens influence the activity of some CYP450 isoenzymes by various mechanisms (van Lipzig *et al.* 2005).

The influence of quercetin on the activity of CYP1A2

The administration of Q led to a decrease of both PHE and PAR levels. The concentrations of PHE as well as those of PAR in Q treated males were lower in the 60th ($P \leq 0.001$) and 120th min ($P \leq 0.01$) of perfusion as compared to the male control (Figures 3 and 4). The decrease of metabolite levels is a signal for CYP1A2 activity inhibition. Oppositely, the marker levels should be increased in the case of a slower biotransformation. Our explanation is that Q can change the activity of other pathways for the metabolic conversion of PHE, like CYP 2A enzymes (Devore *et al.* 2008). The induction of this enzymatic subfamily can cause a decrease in the levels of PHE in the perfusate, and consequently a lack of the substrate for CYP1A2, followed by the reduction of PAR production. We conclude that Q probably acts as an inhibitor of isoenzyme 1A2 and simultaneously induces other CYP isoenzymes, predominantly of 2A family. Ciolino *et al.* (1999) referred on the

bindings of Q to arylhydrocarbon receptor and the induction of CYP1A2. However, a majority of works (Tsyrlov *et al.* 1994; Obach 2000) resulted in the inhibition of CYP1A2 activity by Q. The inhibition of CYP1A2 by polyphenols is frequently mentioned in relation to their antimutagenic and tumour protective effects.

CONCLUSION

It can be concluded from our results that the metabolic activity of cytochrome P450 in rats is sex dependant. Female rats metabolise phenacetin faster than male rats do. This sexual difference is enhanced by the administration of *trans*-resveratrol. Resveratrol alone does not influence the activity of CYP1A2. Quercetin is probably an inhibitor of CYP1A2 and the induction of 2A subfamily is possible.

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4.1.5 Intersexual differences in the inhibitory effect of *trans*-resveratrol on rat P450

Sex-based differences in the pharmacokinetics of drugs are well known and can lead to individual differences in drug response. The variations in pharmacokinetics of drugs between males and females arises mainly from different body weight, proportion of body fat, organ perfusion, or plasma volume (Waxman and Holloway, 2009). Differences in hepatic metabolism between the sexes arise from the difference in hormonal levels between males and females. It is known that the expression of some P450 enzymes is sex-dependent in both humans (Scandlyn et al., 2008) and rats (Kato and Yamazoe, 1992). We demonstrated a difference in the metabolic activity of CYP1A2 between male and female rats (Zendulka et al., 2008). Nevertheless, the clinical impact of these differences seems to be irrelevant, as few drugs exhibited pharmacokinetic sex-related differences that resulted in a different pharmacodynamic response (Fadiran and Zhang, 2014). The pharmacokinetic variability between the sexes is instead dependent on the other factors mentioned above than on P450 sex-based differences.

In the following experiment we tested the sex-based differences in the metabolic activity of CYP2D2 in rats together with the effect of *trans*-resveratrol premedication (5 mg/kg/day i.p.). The activity of rat CYP2D2, which is an orthologue to human CYP2D6, was assessed by dextromethorphan O-demethylation to dextrorphan (Yu et al., 2001). The results of this study also documented sex-related differences in the activity of the tested enzyme. In contrast to the previous experiment, the metabolic activity of CYP2D2 was decreased. *Trans*-resveratrol inhibited the formation of dextromethorphan in both males and females, but with a greater impact in males. The data documents sex-based differences in the effect of *trans*-resveratrol. Extrapolation of the data to humans is difficult due to known interspecies differences in P450 between rats and humans.

Zendulka O., Totušek J., Šulcová A. Intersexual differences in inhibitory influence of *trans*-resveratrol on activity of cytochrome P450 2D2 in rats. Neuroendocrinology Letters, 30, 88-91, 2009.

Impact factor: 1.047; Neurosciences Q4, Endocrinology & Metabolism Q4   Citations WoS: 4

Author's contribution: 75%

Intersexual differences in inhibitory influence of trans-resveratrol on activity of cytochrome P450 2D2 in rats

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Abstract

OBJECTIVES: Differences in the metabolism between males and females have been seen over time. Hormonal regulation of cytochrome P450 activity is understood to be involved. *Trans*-resveratrol (RES) is an estrogenically active plant polyphenol with many protective biological activities including neuroprotection. The present report studied the influence of sex and RES on variances in rat's cytochrome P450 2D2 hepatic metabolic activity.

METHODS AND DESIGN: Isolated perfused rat liver was used for determination of cytochrome P450 2D2 activity. Wistar albino rats of both sexes were treated with RES at the dose of 5 mg/kg/day for 10 days prior to liver isolation. Levels of marker substance dextromethorphan (DEM) and its 2D2 specific metabolite dextrorphan (DEX) were measured during perfusion. The metabolic ratios (DEM/DEX) and the levels of DEM and DEX in perfusate were compared.

RESULTS: In the controls, the activity of CYP2D2 was found to be higher in male rats compared to females. RES produced inhibition of CYP2D2, expressed by significant changes of both DEM and DEX levels in males and significant increase of only DEM levels in females. There were no gender changes in DEX levels in RES treated animals whilst DEM levels were significantly increased during the whole perfusion in females.

CONCLUSION: The results confirmed gender differences in the metabolic activity of CYP450 2D2 with a higher rate in male rats. *RES* acted as an inhibitor, however again with greater impact in males than in females. This metabolic divergence could be a cause for different sensitivity or even toxicity of drugs metabolized by the CYP450 2D2.

INTRODUCTION

Intersexual metabolic differences in humans are known in general, but specific divergences are usually not described in details. Such variance can influence biotransformation of xenobiotics including drugs. Appropriate data describing particular differences can improve drug dosing optimization or prevent adverse effects. It can also clarify higher sensitivity to diseases caused by different xenobiotic polutants metabolized in human body. Enzymatic transformation of xenobiotics is usually involved in detoxification Phase I and II processes. One of the major systems of Phase I is cytochrome P450. Its activity is not rigid and can be regulated by many endogenous systems and

influenced by various exogenous factors. Beyond age, genetic polymorphism and xenobiotic influence, the hormonal regulation also takes a part, including sexual hormons what may be a reason for gender differences in CYP450 metabolism. Estrogens are proved to influence the amounts of some CYP450 isoenzymes in liver (Williams et al, 2004). Phytoestrogens are natural substances with estrogenic activity found in plants. Their ability to activate the estrogenic receptors can also be manifested in changes of CYP activity. *Trans*-resveratrol (RES) is a plant polyphenol with many protective effects on human organism including neuroprotection (Athar *et al.* 2007). RES is structurally similar to estrogens with phytoestrogenic activity. It binds to estrogen receptors probably in partial agonistic manner (Bowers *et al.* 2000). Influence of RES on some CYP450 isoenzymes has been also described (Piver *et al.* 2001). The use of RES in different food supplements declared as a neuroprotective agents can probably lead to changes in CYP450 activity in clinical practice. Among many of CYP450 isoenzymes CYP2D6 metabolizes a variety of drugs with psychotropic effects (antidepressant, antipsychotic). Some of them (fluoxetine or paroxetine) are also inhibitors of this isoenzyme and can even cause a switch from an extensive to poor metabolizer phenotype (Zourkova *et al.* 2008). There is a strong probability of changes in the metabolism of drugs which are CYP2D6 substrates when combined with RES treatment. In this study the activity of rat CYP2D2 (an orthologue of human CYP2D6) enzyme (Zahradnikova *et al.* 2007) was studied. The aims were to investigate possible intersexual differences in CYP2D2 activity and the influence of estrogenically active polyphenol RES in rats.

MATERIAL AND METHODS

Experiment was carried out on male and female Wistar albino rats weighing 200 ± 20 g (Biotest, Czech Republic). Animals were housed in standard plastic cages (540 x 320 x 200 mm) with wood shavings and with free access to water and commercial pelleted diet. Controlled conditions were kept in the animal room: temperature 21-22 °C; humid-

ity 50-60%; light regime – 12h light/12h dark (lights on from 6:00 to 18:00). Male and female rats were randomly subdivided into 2 groups per 8 animals and underwent a 7 day acclimatization before experiment. RES (Sigma-Aldrich, Czech Republic) was dissolved in 30% DMSO/saline solution and administered intraperitoneally at the dose of 5 mg/kg/day for 10 days prior to liver isolation. All experimental procedures were approved by the Czech Central Commission for Animal Welfare.

The model of isolated perfused rat liver was used for CYP450 2D2 activity assessment as described elsewhere (Zendulka *et al.* 2008). The animal was anesthetized (ketamine+xylazine), vena portae was cannulated and the liver was isolated from the abdominal cavity. Liver was perfused in modified recirculating apparatus described by Miller (Miller *et al.* 1951) with tempered and oxygenated William's medium E. Levels of 2D2 isoenzyme specific marker dextromethorphan (DEM) and its 2D2 specific metabolite dextrorphan (DEX) (*Fig.1*) were measured in withdrawn samples in the 30th, 60th and 120th minute of perfusion. Analyses of samples were performed after incubation with β-glucuronidase and liquid/liquid extraction using HPLC methods described by Zimova (Zimova *et al.* 2001). Metabolic ratios (MR) were calculated using the formula MR = conc. DEM / conc. DEX.

F-test and Student's t-test (Microsoft Excel 2000) were used for statistical calculations. p values lower than 0.05 were considered to be a statistically significant difference.
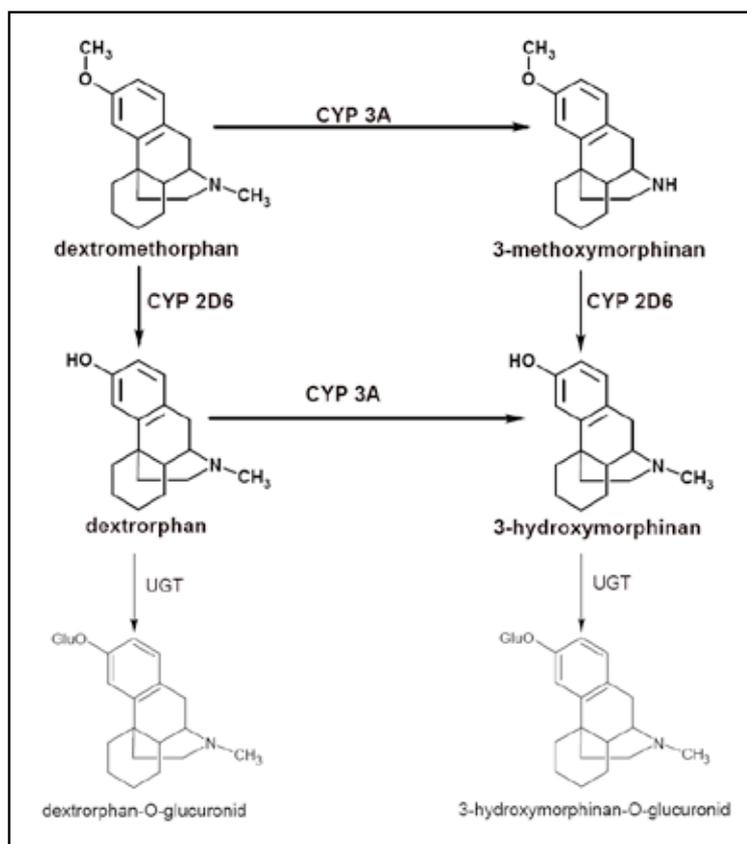
Figure 1. Dextromethorphan's metabolic pathways

Ondrej Zendulka, Jiří Totusek, Alexandra Sulcova
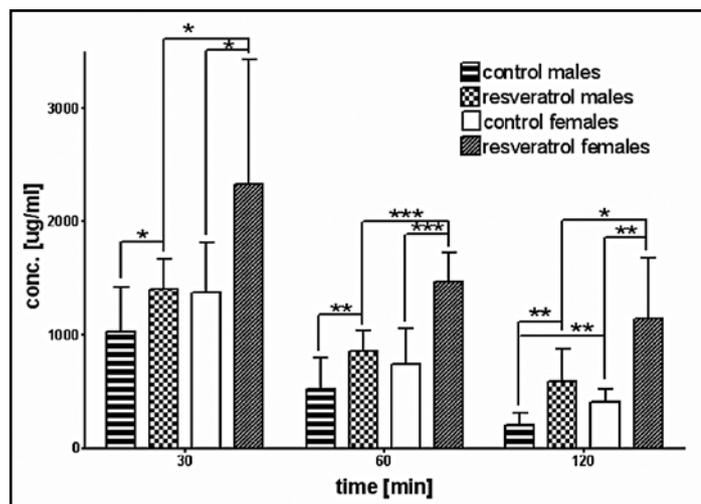
Figure 2. Levels of DEM in perfusion medium in male and female controls and trans-resveratrol pretreated animals (5 mg/kg/day, 10 days). Data represent mean ± S.E.M. * p≤0.05; ** p≤0.01; *** p≤0.001.
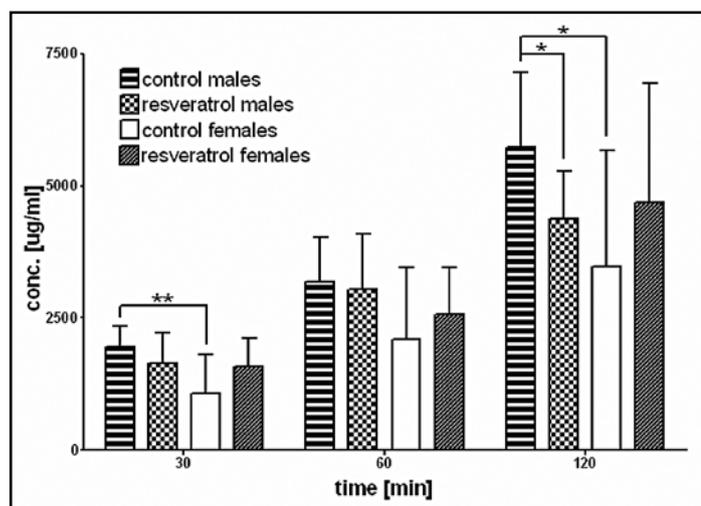
Figure 3. Levels of DEX in perfusion medium in male and female controls and trans-resveratrol pretreated animals (5 mg/kg/day, 10 days). Data represent mean ± S.E.M. * p≤0.05; ** p≤0.01.
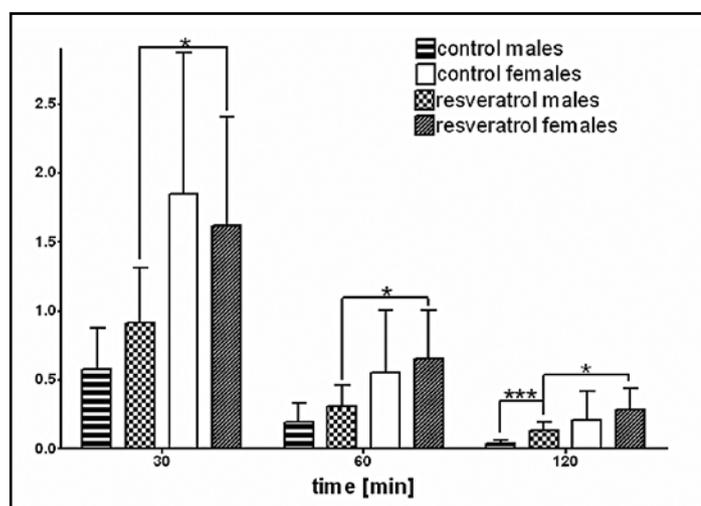
Figure 4. DEM/DEX metabolic ratios in male and female controls and trans-resveratrol pretreated animals (5 mg/kg/day, 10 days). Data represent mean ± S.E.M. * p≤0.05; ** p≤0.01.

RESULTS

The intersexual differences in CYP2D2 activity. Measured values of DEM in samples were different between male and female animals (**Fig. 2**), but statistical significance was found only in the 120th minute of perfusion ($p≤0.01$). The concentration of DEM was increased by 53% in females. On the other hand amounts of DEX were significantly increased in males (**Fig. 3**). Levels were changed in the 30th ($p≤0.01$) and 120th minute ($p≤0.05$). These results corresponded with DEM levels and declare a higher metabolic activity of CYP2D2 in male rats than in females and a faster conversion of DEM to DEX. Furthermore MR values confirmed this finding (**Fig. 4**). MR was significantly higher ($p≤0.01$) in females in the 30th minute and in other two intervals (60th and 120th min) the *p* values were close to significance ($p=0.06$ and 0.08 respectively).

The influence of RES on CYP2D2 activity The administration of RES caused an inhibition of CYP2D2 activity. This was demonstrated by an increase in levels of DEM and a decrease in amounts of DEX in both sexes. Compared to controls in RES treated males the concentrations of DEM were significantly raised (Fig. 2) by 26% in the 30th min up to 65% in the end of perfusion and associated with lower levels of DEX (Fig. 3), which were significantly changed only in the 120th min. These results correlate with lower values of MR in the control males (Fig. 4), specifically in the 120th min ($p≤0.001$). The data obtained from females administered with RES resemble DEM levels changes in males with significant due time increase from 41% to 62%.

The intersexual differences in influence of RES on CYP2D2. Differences between RES administered males and females correlate with controls only in the 120 min.

As shown in the Fig. 2, DEM levels were significantly elevated in females and there were registered no sex differences in changes of DEX levels (Fig. 3). Metabolic ratios were significantly higher in female rats (Fig. 4) during the whole perfusion ($p≤0.05$).

DISCUSSION

The working hypothesis that there could exist sex differences in the activity of CYP2D2 isoenzyme postulated in the view of our earlier results describing sex dependent activity of CYP1A2 in rats (Zendulka *et al.* 2008) was confirmed in the present study. Conversely to 1A2 isoenzyme, male rats metabolized substrates of 2D2 faster than females. Approximation of our results to humans is difficult, while there are some interspecies (Langsch *et al.* 2009) and even interstrain (Schulz-Untermoehl

et al. 1999) differences in CYP450 activities and data describing intersexual differences on CYP2D6 are inconsistent (Scandlyn *et al.* 2008; Zahradnikova *et al.* 2007). Some studies on humans resulted in no CYP2D6 sex specific difference (Aichhorn *et al.* 2005), however others found either higher metabolic rate in males (Gexfabry *et al.* 1990, Pritchard *et al.* 1992) or faster metabolization via CYP2D6 by females (Tamminga *et al.* 1999). We suggest that results of human studies focused on gender differences in CYP2D6 activity are probably highly dependent on the marker substance used and methodology of CYP450 activity assessment.

The second outcome of the present study is that the phytoestrogen RES produced an inhibition of CYP2D2 activity with at least some sex differences suggesting greater susceptibility of males. However, this effect was weaker comparing to some other CYP2D2 inhibitors we have studied previously, e.g. fluoxetine (Zendulka *et al.* 2009) as the significant changes were measured only at the end of perfusion.

Although the influence of polyphenolic compounds on the CYP450 metabolic system is extensively studied, the influence of RES on human 2D6 or rat 2D2 isoenzyme was not according to the literature available described yet. RES is reported to be an inhibitor of 1A2 and 3A4 isoenzymes (Piver *et al.* 2001). Mechanism of 1A subfamily inhibition by RES is believed to be present due to RES biotransformation via this metabolic pathway (Piver *et al.* 2004), which cannot be the case of 3A4 and 2D6 isoenzymes. RES estrogenic activity might be involved in sex differential regulation of 2D2 isoenzyme. RES perhaps can enhance intersexual difference by influencing the activity of 2D2 more in males than in females, similarly as described with 1A2 isoenzyme (Zendulka *et al.* 2008).

It can be concluded that metabolic activity of 2D2 isoenzyme is higher in male rats and is inhibited by RES administration. The effect of RES is sex dependent with greater impact on male rats.

Acknowledgements

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5. Endogenous regulation of P450 activity

The metabolism of xenobiotics is just one function of the P450 enzymes. They protect the human body against possible harm from the chemicals of the environment. On the other hand, the same importance should be attached to the role of P450 in the metabolism of endogenous substrates. Many products of reactions catalysed by P450 are involved in various physiological roles in the human body. Most of the P450 enzymes involved in drug elimination are different from those involved in the synthesis and metabolism of endogenous substances. However, some overlap between them can be found. Both CYP3A4 and CYP1A2 are extensively involved in the metabolism of drugs and also in the biotransformation of estrogens (Guengerich, 2017). A change in their activity can consequently lead not only to a change in the metabolism of drugs, but also to side effects due to changes in the level of their endogenous substrates.

Xenobiotics, namely drugs, are systematically tested to identify their potential to influence the activity of P450. The results of such research are obligatory and required by national authorities for the approval of the drug for clinical use. On the other hand, the research of endogenous regulators and regulatory pathways of P450 activity is mostly academic. It is not surprising that unlike xenobiotic regulators of P450 activity, some of the endogenous regulatory pathways are still not fully understood.

The mechanism of endogenous regulation of P450 activity is different from the simple substrate-enzyme interaction often found in drug inhibitors. The essential mode of regulation seems to be a change in the enzyme amount and in the regulation of gene expression. The mechanism of P450 gene expression via nuclear receptors and regulation of post-translational processes via microRNA is mentioned in chapter 4 of this thesis. Different steroid hormones serve as ligands of these receptors (Sever and Glass, 2013), while the effect of cytokines is probably mediated by another mechanism (Richardson and Morgan, 2005).

5.1 Bile acids and P450

Bile acids are essential for the digestion of lipids from the diet. Their amphiphilic character gives them the emulsifying activity necessary for the formation of micelles. Moreover, bile acids are also involved also in some physiological processes, including glucose and lipid metabolism, thermoregulation, or immune response (Claudel et al., 2011). The regulatory role of bile acids involves interaction with several nuclear receptors (Chiang, 2009). Because of their involvement in many important processes of homeostasis, some bile acids may be used as therapeutic agents for cholestatic liver disease, inflammatory bowel disease, diabetes mellitus, or other metabolic disorders

(Camilleri and Gores, 2015; Gadaleta et al., 2010; Mudaliar et al., 2013). Besides this, bile acids are involved in the regulation of P450 activity.

We have focused on different types of interactions between bile acids, P450, and nuclear receptors in our review article. In the first part of the article, bile acids are described as ligands of various nuclear receptors and the influence of stimulation of these receptors on the P450 is summarized. In general, the activation of nuclear receptors by bile acids is related to a restriction of their synthesis and increase in their elimination. Bile acids are ligands of the farnesoid X receptor (FXR), vitamin D receptor, liver X receptor (LXR), PXR, and CAR. The activity of both P450 involved in the biosynthesis of bile acids and P450 of drug metabolism can be regulated.

The second part of the article is dedicated to the regulation of P450 associated with bile acid synthesis. CYP7A1 is identified as the rate-limiting step of bile acid synthesis (Chiang, 2013), and bile acids are recognized as its negative regulators (Gupta et al., 2001). The role of single nuclear receptors in this process is also described. Finally, the regulation of P450 involved in the metabolism of xenobiotics by bile acids is reviewed in the final part. Specifically, CYP3A4 regulation by ursodeoxycholic acid and the role of PXR and FXR are described.

Bile Acids, Nuclear Receptors and Cytochrome P450

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Summary

This review summarizes the importance of bile acids (BA) as important regulators of various homeostatic mechanisms with detailed focus on cytochrome P450 (CYP) enzymes. In the first part, synthesis, metabolism and circulation of BA is summarized and BA are reviewed as physiological ligands of nuclear receptors which regulate transcription of genes involved in their metabolism, transport and excretion. Notably, PXR, FXR and VDR are the most important nuclear receptors through which BA regulate transcription of CYP genes involved in the metabolism of both BA and xenobiotics. Therapeutic use of BA and their derivatives is also briefly reviewed. The physiological role of BA interaction with nuclear receptors is basically to decrease production of toxic non-polar BA and increase their metabolic turnover towards polar BA and thus decrease their toxicity. By this, the activity of some drug-metabolizing CYPs is also influenced what could have clinically relevant consequences in cholestatic diseases or during the treatment with BA or their derivatives.

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Introduction

The essential physiological role of bile and bile acids (BA) in digestion is to neutralize chyme and serve as emulsifiers of fat in small intestine. Thanks to their amphiphilic nature, BA are emulsifiers which enable absorption of lipids and lipid soluble vitamins. The production and secretion of bile is regulated by intestinal paracrine hormones cholecystokinin and secretin and moreover autoregulation *via* negative feedback exists, too. BA and phospholipids stabilize micellar dispersion of cholesterol in the bile and facilitate cholesterol excretion as well as excretion of hydrophobic metabolites of xenobiotics, toxins and metals. In past decade, it has been postulated that bile acids may also regulate lipid and glucose homeostasis, thermoregulation, and immune response (Claudel *et al.* 2011). Especially the role of BA in immune response is undoubtedly involved in the therapeutic effects of some BA in cholestatic liver diseases (Roma *et al.* 2011, Buryova *et al.* 2013).

The most common human BA (Fig. 1) are cholic acid (CA), chenodeoxycholic acid (CDCA), in much less proportion also glycocholic acid, taurocholic acid (TCA), lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA). BA and oxysterols are natural ligands of several nuclear receptors (NRs), membrane receptors and regulators of metabolism of lipids and glucose (Chiang 2004, Chiang 2009). At least, some of BA regulate above mentioned pathways *via* their farnesoid X receptor (FXR) agonistic activity. In particular, FXR agonists probably *via* production of glucagon-like peptides 1 and 2 increase insulin sensitivity, glucose uptake, and adipogenesis in extrahepatic tissues. Meanwhile, they increase fatty acid oxidation, decrease triglyceride, fatty acid, and cholesterol synthesis in the liver and increase insulin production in beta cells (Camilleri and Gores 2015, Adorini *et al.* 2012). BA as FXR agonists also decrease

activity of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase and thus, together with all above mentioned effects increase glucose tolerance and insulin sensitivity (Chiang 2013). BA also promote gut motility through TGR5 activation (Camilleri and Gores 2015) which is in agreement with reported adverse effects of therapeutically used BA (American Society of Health System Pharmacists 2016a, American Society of Health System Pharmacists 2016b). Primary BA are deconjugated and dehydroxylated by microflora, and these BA metabolites exert antimicrobial properties (Begley *et al.* 2005). Another physiological consequence of BA binding on FXR is increased synthesis of fibroblast growth factor-19 (FGF-19), which may reduce glycolysis and lipogenesis, improved insulin sensitivity, and reduce bile acid synthesis (Camilleri and Gores 2015).
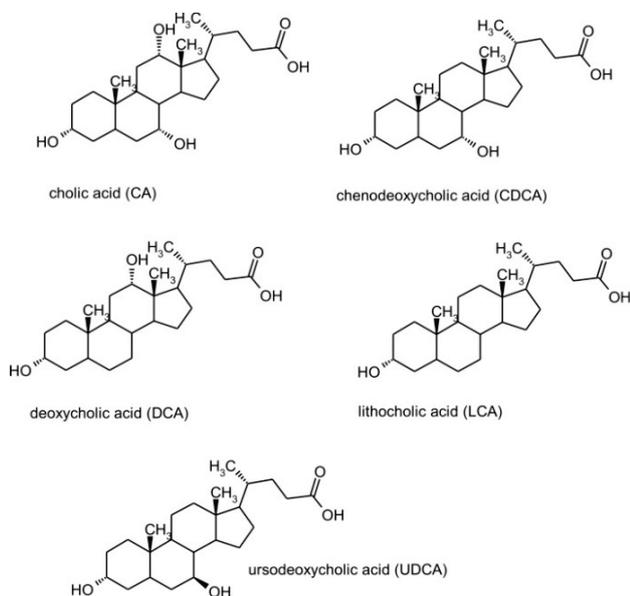
Fig. 1. Unconjugated primary and secondary bile acids in humans.

In agreement with above mentioned physiological roles of BA, some BA may be also exploited or tested as therapeutic agents, mostly in cholestatic liver diseases, inflammatory bowel disease (Gadaleta *et al.* 2010), diabetes mellitus and metabolic disorders (part Therapeutic use of BA).

This review summarizes the importance of BA as important regulators of various homeostatic mechanisms with detailed focus on cytochrome P450 (CYP) enzymes.

Synthesis, metabolism and circulation of bile acids

BA are synthetized in the liver from cholesterol ("classical pathway") in multiple steps catalyzed *via* CYPs, hydroxy-delta-5-steroid dehydrogenase (HSD3B7), Δ^{4-3}-oxosteroid-5β-reductase (AKR1D1) and 3α-hydroxysteroid dehydrogenase (AKR1C4) to form 5β-cholestan-3α,7α,12α-triol. Biosynthetic pathway of CDCA leads directly to 5β-cholestan-3α, 7α-diol *via* AKR1D1 and AKR1C4. Subsequently, those by products are hydroxylated in the position 27 and the hydroxylic group is then oxidized to aldehydic and carboxylic group. The products are further ligated to coenzyme A (CoA) and the side chain is shortened by β-oxidation to release – propionyl-CoA and cholyl-CoA or chenodeoxycholyl-CoA. Under physiological conditions, CA and CDCA occurs as Na^+ salts („bile salts") (Chiang 2004). Then, primary BA are conjugated with glycine and taurine. UDCA is 7-epimer of chenodeoxycholic acid and therefore is more hydrophilic than its structural analogue CDCA (Fig. 1) and forms about 4 % of BA pool (Roma *et al.* 2011). Hydroxylation in the positions 6α/β or 7β increases water solubility and decrease toxicity of BA (Chiang 2013).

Apart from this "classic pathway", alternative ("acidic") biosynthetic pathway exists in humans, which forms less than 10 % of total BA. In this pathway, which is believed to occur also in extrahepatic tissues, cholesterol is hydroxylated by series of steps (e.g. CYP27A1, CYP46A1, HSD3B7, 3βHSD), but finally has to be transported into the liver to complete synthesis of CA and CDCA (Chiang 2004, 2009; Russell 2000).

Conjugated BA are stored in gall bladder and secreted into the duodenum in response to ingestion of meal. Then, about 95 % of BA is reabsorbed in ileum, mostly by apical Na^+-dependant bile salt transporter (SLC10A2 or ASBT) (Camilleri and Gores 2015b). Resorbed BA are transported to the liver in blood through *vena portae* (Chiang 2013). BA which are not reabsorbed undergo deconjugation and dehydroxylation by intestinal flora to form secondary BA, LCA, and DCA, of which the most is excreted in faeces (Chiang 2009, Chiang 2013).

Bile acids – ligands of nuclear receptors

For many years, BA were thought to be fat emulsifiers and digestive surfactants as the only function of

the BA in the human body. However, recent observations in the last decade documented that BA are involved in the regulation of more complex processes including bile production, glucose and lipid metabolism, and in the modulation of immune response (Renga *et al.* 2013, Trauner *et al.* 2010, Claudel *et al.* 2005). The regulatory role of BA is a result of their interaction with various types of receptors including both intracellular nuclear receptors (NR) and cell surface membrane receptors (MR). BA acids vary in their ability to bind and activate different types of receptors. CDCA together with DCA are the most potent activators of FXR (Parks *et al.* 1999, Makishima *et al.* 1999), while LCA is the most potent activator of membrane G-protein coupled receptor TGR5 (Sato *et al.* 2008). Except of regulation of energy metabolism and immune system reactions, stimulation of different NRs and MRs by BA helps to maintain bile acid homeostasis *via* targeting the processes of their synthesis, release, reabsorption or metabolism (Copple and Li 2016).

BA are activators/ligands of various NRs, at least their ability to influence FXR, pregnane X receptor (PXR), vitamin D receptor (VDR), and liver X receptor (LXR) is well documented (Table 1, Chiang 2013, Song and Liao 2000). On the other hand some NRs which do not interact with BA, such as constitutive androstane receptor (CAR), can significantly influence the metabolism of BA. Most of these NRs are highly expressed in tissues exposed to high levels of BA like are liver or intestine (Kliewer *et al.* 1998, Forman *et al.* 1995, Gascon-Barré *et al.* 2003, Qatanani and Moore 2005).

NRs are ligand activated transcription factors consisting of N-terminal DNA binding domain (DBD) necessary to recognize the DNA response element in the target genes and C-terminal ligand-binding domain (LBD) interacting usually with small hydrophobic molecules. Activation of NRs by bile acids results in restriction of BA synthesis and increase of their degradation and excretion.

Table 1. Bile acids, their derivatives and metabolites as ligands of nuclear receptors.

Receptor	Ligands	Reference
FXR	CDCA>DCA≈LCA>CA>UDCA	Makishima *et al.* 1999, Parks *et al.* 1999)
	Bile alcohols, 6α-ethylCDCA	Pellicciari *et al.* 2002
	5β-cholanoic acid, 5β-norcholanoic acid, and 5α-cholanoic acid	Sepe *et al.* 2016
PXR	3-keto-LCA>LCA>CDCA>DCA>CA	Taudinger *et al.* 2001
	7α-OH-4-cholesten-3-one	Goodwin *et al.* 2003
VDR	LCA, 3-keto LCA	Makishima *et al.* 2002
LXR	Cholestenoic acid	Song and Liao 2000
	6α-hydroxylated BA	Song *et al.* 2000
	Oxysterols	Janowski *et al.* 1996, Lehmann *et al.* 1997

Farnesoid X receptor (FXR; NR1H4)

There are two genes (*FXRα* and *FXRβ*) coding the FXR. FXRβ represents a functional NR in mammalian species except of primates and humans, where the gene encodes the non-functional protein (Otte *et al.* 2003). FRXα encodes four isoforms of FXR different either in the use of promoters or in alternative splicing (Zhang *et al.* 2003). The structure of FXR corresponds with the general structure of NR with the LBD allowing receptor heterodimerization with retinoid X receptor (RXR) and interactions with co-regulators. After interaction with agonist, FXR binds with FXR response elements as heterodimer FXR/RXR or as

a monomer and regulates the gene expression (Ding *et al.* 2015).

FXR is mainly found in hepatocytes, enterocytes (Forman *et al.* 1995) and acts as a sensor in the enterohepatic system regulating BA homeostasis. BA are potentially toxic and their levels have to be strictly controlled. In the hepatocytes, FXR controls BA synthesis (*via CYP7A1*, *CYP8B1*), sinusoidal uptake, and canalicular secretion of BA. In the intestine, FXR regulates the absorption, trafficking from the apical to the basolateral membrane and basolateral efflux (Modica and Moschetta 2006). FXR plays a protective role against BA toxicity by feedback inhibition of *CYP7A1*, *CYP8B1,* and

CYP27A1 expression (Copple and Li 2016). The regulation of expression of above mentioned genes is not direct, but mediated *via* small heterodimer partner (SHP) interacting with liver-related homolog-1 (LRH-1) or hepatocyte nuclear factor 4α in the liver (Kwong *et al.* 2015) and fibroblast growth factor 19 (FGF19) binding to FGF receptor 4 in the intestine (Kir *et al.* 2011).

BA can activate the FXR in their free and conjugated forms. The most powerful ligand of FXR is CDCA with the EC_{50} approximately 10 µmol/l (Ding *et al.* 2015). Other endogenous BA bind to FRX with lower affinity as follows: CDCA>DCA≈LCA>CA>UDCA (Makishima *et al.* 1999, Parks *et al.* 1999).

Pregnane X receptor (PXR; NR1I2)

PXR acts mainly as a xenobiotic sensor. It is activated by steroidal substances including glucocorticoids and its main role is to form a barrier protecting inner environment from xenobiotics. Therefore, its highest expression was found within liver and intestine (Kliewer *et al.* 1998).

The PXR LBC is substantially larger than in other NRs and is poor in the number of polar groups. Such characteristic explains the high promiscuity and variability of its ligands (Handschin and Meyer 2005). PXR is activated for example by glucocorticoids, steroids, macrolide antibiotics, antifungals, and some herbal extracts (Jones *et al.* 2000, Lehmann *et al.* 1998, Ihunnah *et al.* 2011). From bile acids the most potent ligand of PXR is LCA with the $EC_{50} \sim 10$ µmol/l (Staudinger *et al.* 2001). It is questionable whether BA can activate the receptor under physiologic conditions when their plasma levels are below 100 nmol/l. It is hypothesized, that sufficient concentration can be reached after rupture of intrahepatic bile duct in cholestasis, when PXR can increase BA clearance by *CYP3A* induction and decrease its biosynthesis by suppression of *CYP7A1* expression (Copple and Li 2016). After binding the ligand it is translocated into nucleus and associated with RXR to form a heterodimer activating gene transcription.

The range of regulated genes is wide including many of phase I (Table 2) and II enzymes, or uptake and efflux transporters. The role of PXR in the expression of drug metabolizing enzymes is well documented by the study of Kandel *et al.* (2016), who treated human hepatocytes from 6 donors with rifampicin, a well proved human PXR agonist. Results confirmed the protective role of PXR against BA toxicity as expression of most of the CYP biotransformation enzymes genes were

increased (Table 2) while the *CYP7A1*, involved in BA synthesis, was decreased to 0.32-fold in comparison to control (Kandel *et al.* 2016).

Table 2. The role of NRs in the regulation of CYP genes expression.

CYP	Receptor					Reference
	FXR	**PXR**	**VDR**	**CAR**	**LXR**	
1A1				↑		1
1A2				↑		1
2A6			↑	↑		1, 2
2A7				↑		1
2A9			↑			2
2A13				↑		1
3A1		↑				3
3A2		↑				3
3A4[*]	↑	↑	↑	↑		1, 4, 5, 6, 7, 8, 9
3A7		↑		↑		1
7A1	↓	↓	↓		↑	1, 10, 11, 12, 13
27A1	↓					10
2B6		↑		↑		1, 4, 9, 14
2B7		↑		↑		1
8B1	↓			↑		10
2C8		↑		↑		1
2C9		↑		↑		1, 4
2E1				↓		1

↑ – expression increased, ↓ – expression decreased, [*] – human *CYP3A4* is orthologue of mice *CYP 3A11*, therefore results from animal models are represented as change in *CYP3A4* expression. Ref.: 1 – Kandel *et al.* 2016, 2 – Drocourt *et al.* 2002, 3 – Kliewer *et al.* 1998, 4 – Drocourt *et al.* 2001, 5 – Gnerre *et al.* 2004, 6 – Goodwin *et al.* 2002, 7 – Goodwin *et al.* 2000, 8 – Makishima *et al.* 2002, 9 – Sberna *et al.* 2011, 10 – Eloranta and Kullak-Ublick 2005, 11 – Han *et al.* 2010, 12 – Lehmann *et al.* 1997, 13 – Staudinger *et al.* 2001, 14 – Xie *et al.* 2000.

Vitamin D receptor (VDR; NR1I1)

In spite of the fact that hepatocytes do not express VDR, its significant levels are found in non-parenchymal liver cells such as Kupffer cells or sinusoidal endothelial cells (Gascon-Barré *et al.* 2003). The typical ligand of VDR is cholecalciferol, while most of the BA including CDCA, CA, DCA, or muricholic acid does not activate VDR. Similarly to ligands of PXR, VDR can be activated by LCA and its metabolite 3-keto-LCA with the $EC_{50} \approx 8$ µM and 3 µM, respectively (Makishima *et al.* 2002). It could be of clinical importance that therapeutically used UDCA can be converted to LCA (VDR and PXR agonist) by intestinal microflora (Xie *et al.* 2001, Staudinger *et al.* 2001).

The role of VDR to promote calcium and phosphate absorption is well known. Its protective role against BA toxicity similar to PXR or against infection of bile duct was documented in the past decade. Stimulation of VDR by both vitamin D and LCA induces production of antimicrobial peptide cathelicidin in the bile duct epithelial cells (D'Aldebert *et al.* 2009). Both ligands can also increase the expression of *CYP3A4* leading to elevated BA clearance (Makishima *et al.* 2002) while the expression of *CYP7A1* can be reduced through VDR activation (Han *et al.* 2010). Moreover, there is documented interaction between VDR and LXRα with antagonizing effects on the *CYP7A* gene, too (Jiang *et al.* 2006).

Liver X receptor alpha (LXRα; NR1H3)

This NR can be found in the tissues with high metabolic activity such as liver, small intestine, kidney or adipocytes (Sato and Kamada 2011), in comparison to LXRβ, which is ubiquitously expressed in all tissues (Teboul *et al.* 1995). LXR binds to its responsive elements as heterodimer associated with RXR and its influence on gene expression is tissue specific (Moschetta 2015).

Its known endogenous ligands are oxysterols and its physiologic role is the regulation of cholesterol, fatty acid, and glucose homeostasis (Zelcer and Tontonoz 2006). Oppositely to other mentioned NR, LXR activation leads to increased activity of *CYP7A1*, thus the formation of BA is increased. Together with increased cholesterol transport to bile *via* specific transporters and restriction of its absorption from intestine, the level of total plasma cholesterol is decreased. However, reduction in cholesterol levels *via* activation of LXR is associated with fatty liver and hypertriglyceridemia development (Moschetta 2015).

LXR is activated by cholestenoic acid with ED_{50} of 200 nmol/l (Song and Liao 2000) and also by different oxysterols (Lehmann *et al.* 1997, Janowski *et al.* 1996) and 6α-hydroxylated BA (Song *et al.* 2000) within the range of their physiologic levels.

Constitutive androstane receptor (CAR; NR1I3)

CAR is closely related to PXR, this NR acts similarly as xenobiotic sensors which regulate expression of genes significant for biotransformation and excretion of exogenous compounds. Both receptors are activated by toxic derivatives of endobiotic metabolism, too. However, CAR seems to be more sensitive to endogenous stimuli (Bing *et al.* 2014). Typical exogenous ligands of CAR represent phenobarbital, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCOBOP) (Timsit and Negishi 2007). When activated, it is translocated into nucleus *via* protein phosphate PP2A and association with RXR precede to binding to DNA (Timsit and Negishi 2007). BA do not seem to be direct ligands of CAR, nevertheless activation of this receptor increases activity of enzymes producing more hydrophilic and thus less hepatotoxic metabolites of BA (Beilke *et al.* 2009) and activates their excretion from hepatocytes (Wagner *et al.* 2005).

The genome-wide screening in the liver cells of human donors treated by CAR prototype ligand CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbal-dehyde O-(3,4-dichlorobenzyl)oxime) revealed 11 CYP genes among top 25 influenced genes. In all cases CITCO increased gene expression in the range between 1.28- and 2.19-fold in ascending order as follows: *2C9<1A2<3A7<3A4<2C8<2A6<2A13<1A1<2A7<2B6<2B7* (Kandel *et al.* 2016). On the other hand, *CYP2E1* was downregulated with 0.86-fold decrease of its expression (Kandel *et al.* 2016).

Besides binding to nuclear receptors, BA are also ligands of other types of receptors, including membrane G protein-coupled receptors. The first G-protein coupled receptor known to interact with BA was TGR5, GP-BAR1, or M-BAR. This receptor is widely distributed in different tissues and stimulation leads to different effects depending on the tissue and signalling cascade mediating the signal (Duboc *et al.* 2014). TGR5 is involved in energy metabolism; it protects liver and intestine from inflammation and steatosis, and improves insulin sensitivity (Chiang 2013, Li and Chiang 2015). The influence of some of BA on the immune response is also essential in their therapeutic effect in cholestatic liver diseases (Poupon 2012, Poupon 2014). This effect is in case of UDCA probably mediated by interaction with toll-like receptors TLR4 and TLR9 and glucocorticoid receptors (Poupon 2012).

Regulation of CYP enzymes involved in the metabolism of bile acids

The synthesis of BA is limited mainly through activity of cholesterol 7α-hydroxylase (CYP7A1), which is believed to be the only rate-limiting step in BA synthesis (Chiang 2013). The protein content and metabolic activity of CYP7A1 is regulated by variety of

Table 3. Bile acids as regulators of CYP expression.

Bile acid	CYP											
	1A1	1A2	3A1	3A2	3A4[+]	3A7	3A9	7A1	27A1	2B6[+]	2C8	2E1
CDCA	↓[7]	↓[7]	↑[5,6]↓[6*]	↑[5]↓[6*]	↑[3,6]↓[7]		↓[6*]	↓[1,7]			↓[7]	↓[7]
DCA					↑[3]			↓[1]				
UDCA					↑[2,3]			↓[1,2]		↑[2]		
LCA			↑[5]	↑[5]				↓[1]				
CA					↑[2,3]			↓[2]		↑[2]		
TUDCA					↑[3]							
TDCA									↓[4]			

↑ – expression increased, ↓ – expression decreased, [1] – Roma et al. 2011, [2] – Zollner *et al.* 2006, [3] – Schuetz *et al.* 2001, [4] – Rao *et al.* 1999, [5] – Khan *et al.* 2010, [6] – Khan *et al.* 2009, [7] – Krattinger *et al.* 2016. * – only in rat liver, + – human *CYP3A4* and *CYP2B6* are orthologue of mice *CYP3A11* and *CYP2B10,* respectively. Therefore results from animal models are represented as change in *CYP3A4* and *CYP2B6* expression.

factors. CYP7A1 metabolic activity is limited mainly by availability of its substrate cholesterol. Feeding the experimental animals with cholesterol lead to increase expression of CYP7A1, suggesting the stimulatory effect of substrate ("Km effect") on CYP7A1 (Chiang 2013). On the other hand, negative feedback exists and most of BA are negative regulators of CYP7A1 (Gupta *et al.* 2001). CDCA, DCA and with much lesser potency also other BA, such as UDCA and LTA, inhibit transcription of *CYP7A1* gene. Minor BA, UDCA, which is used therapeutically, increases expression of murine *CYP3A11, CYP2B10* and human *CYP3A4* – these enzymes catalyze hydroxylation of primary BA towards less toxic (hydrophilic) BA (Roma *et al.* 2011, Schuetz *et al.* 2001).

As mentioned above, BA are ligands of several NR, including FXR and it seems that BA downregulate *CY7A1* probably through activation of RXRα/FXR (Chiang 2013). FXR decreases *CYP7A1* expression by several indirect mechanisms, by induction of SHP, which in turn inhibits transactivation of *CYP7A1* and *CYP8B1* by hepatocyte nuclear factor 4αHNF4α) and liver-related homolog-1 (LRH-1). This is in line with finding of Peng *et al.* (2016), who reported that CYP7A1 is upregulated in young FXR nullizygous mice.

Moreover, activation of FXR further decreases intracellular BA content by increase of expression of canalicular BSEP (bile salt efflux pump, ABCB11). These feedback mechanisms seem to protect inner environment of hepatocytes from BA toxicity and liver damage. Taurodeoxycholic acid also downregulates *CYP27A1 via* activation of HNF1α (Rao *et al.* 1999).

The regulation of BA synthesis and metabolism seems to be very complex and some pathways are anticipatory, since activation of VDR may trigger decrease of SHP and this leads to induction of CYP7A1 (Chow *et al.* 2014). The expression of VDR itself in rat ileum and liver is regulated (among others) by some of BA (at least by CDCA), but not LCA. The VDR activation by 1,25-dihydroxycholecalciferol or LCA then leads to increase in transcription of *CYP3A1* and *CYP3A2* genes as well as CDCA treatment. However, when combined together with LCA the expression of *CYP3A1* and *CYP3A2* is reduced (Khan *et al.* 2010). It was also reported by the same team of authors that CDCA decreases expression of CYP3A1, CYP3A2, and CYP3A9 mRNA in rat liver, whereas the expression of *CYP3A1* in the rat ileum is increased and *CYP3A2* and *CYP3A9* are not influenced within the whole rat intestine (Khan *et al.* 2009). This means that unlike UDCA, CDCA could decrease the metabolism and increase the liver toxicity of hydrophobic BA (Khan *et al.* 2010). Moreover, the same team of authors reported that CDCA induced CYP3A4 in human liver (Khan *et al.* 2009). In another study with the primary culture of human hepatocytes, CDCA decreased expression of *CYP3A4, CYP7A1, CYP2C8, CYP1A1, CYP2E1* and *CYP1A2* genes after 48 hours of incubation, as well as it decreased expression of the *AHR* and *PPARγ* gene (Krattinger *et al.* 2016). The overview of BA influence on CYP enzymes is summarized in the Table 3. The results of experiments focused on the regulatory role of BA in the CYP expression seem to be highly variable and dependent on the model used. Moreover, the effects of hydrophobic and hydrophilic BA may differ, as there are differences in

their selectivity and affinity to nuclear receptors (Table 1). This is also resembled by different clinical effects of DCA and CA on one hand and UDCA, TUDCA and nor-UDCA on the other hand. While CA and CDA appear to be hepatotoxic, UDCA prevents from liver damage. Surprisingly, the genetic polymorphisms of *CYP7A1* do not seem to influence the production of BA (Xiang *et al.* 2012).

Other endogenous regulators seem to play a significant role in BA synthesis, such as blood glucose levels, insulin, thyroid hormone, glucocorticoids and glucagon (Siljevik Ellis 2006, Twisk *et al.* 1995, Chiang 2009, Xiao *et al.* 2016). Glucose increases both *CYP7A1* transcription (Li *et al.* 2010) and CYP7A1 metabolic activity by histone acetylation of gene promoter. The latter mechanism is also mediated by insulin (Li *et al.* 2012). Glucagon inhibits BA synthesis *via* block of *CYP7A1* expression (Song and Chiang 2006). In overall, fasting state seem to downregulate CYP7A1, while elevated glucose and insulin seems to increase expression or metabolic activity of CYP7A1 (Chiang 2009, Chiang 2013).

Bile acids as regulators of CYP enzymes involved in the metabolism of xenobiotics

Through activation of FXR and PXR, BA are regulating rate-limiting step in their biosynthetic pathway (CYP7A1), as well as several transporters involved in BA elimination from hepatocytes, as mentioned above.

BA may also influence CYP enzymes, which are primarily involved in the drug metabolism, such as CYP3A4, CYP2C8, CYP2E1 or CYP1A2.

Although PXR and CAR are primary regulators of CYP enzymes, other nuclear receptors of different types are also known to be involved in their regulation.

With respect to the proposed binding ability to the nuclear receptors, namely FXR, it is not surprising that BA may induce expression of *CYP3A4* (Schuetz *et al.* 2001), *SULT2A1* and *UGT2B4* genes (Poupon 2012) and increase CYP3A4 metabolic activity (Schuetz *et al.* 2001).

In particular, FXR nullizygous mice exhibit downregulation of bile salt export pump and thus increase hepatocellular BA content, which in turn, may activate PXR and upregulate *CYP3A* (involved in their metabolism), but also *CYP2B* and some ABC transporters (Schuetz *et al.* 2001). It is also note of worth, that BA are not equipotent ligands of FXR (Table 1). Hydrophilic BA are not considered to be agonists of FXR (Chiang, 2013)

and sometimes are reported to be partial agonists, and sometimes also partial antagonists of FXR, such as UDCA (Modica *et al.* 2010).

It was reported that UDCA may induce the enzymes of CYP3A subfamily (Schuetz *et al.* 2001) and thus decrease concentrations of substrates of CYP3A4 (such as cyclosporine A (CsA)) after UDCA treatment (Yan *et al.* 2008, Becquemont *et al.* 2006, Kurosawa *et al.* 2009, Uchida *et al.* 2014).

Interestingly, the rate of CsA elimination was not changed (Caroli-Bose *et al.* 2000), what implies for decreased bioavailability through induction of intestinal CYP3A4 and P-glycoprotein by activation of PXR (Schuetz *et al.* 2001). PXR is known as potent inducer of phase I metabolic enzymes, phase II conjugation enzymes and phase III drug transporters (Chiang 2013). Among BA, both taurine-conjugate of UDCA (TUDCA) and UDCA were reported to be the most effective inducers of CYP3A4 in primary human hepatocytes (Schuetz *et al.* 2001) which corresponds with their ability to decrease toxicity of more hydrophobic BA.

Information on the influence of UDCA on bioavailability of CsA are inconsistent and controversial, since there were published studies documenting decreased dosing of CsA when combined with UDCA, when AUC of CsA were increased twice (Gutzler *et al.* 1992). Similarly, the bioavailability of CsA was increased with either combination with TUDCA or CsA-TUDCA micellar solution (Balandraud-Pieri *et al.* 1997). This report may be explained in part by effect of excipient-based increase ion CsA bioavailability, since other micellar dispersions of CsA may also increase the bioavailability and AUC of CsA (Balandraud-Pieri *et al.* 1997).

Therapeutic use of BA

Dried bile of Chinese black bear was used as a remedy already during the dynasty of Tang in China (approximately 600-900 A.D.) (Guarino *et al.* 2013). Evidence-based use of bile acids as drugs dates about 30 years back (Beuers *et al.* 2015). In particular, CDCA was utilized as a treatment for gallstone dissolution, which was later displaced by UDCA due to the better safety profile and efficacy. CDCA caused diarrhea, increased serum total cholesterol and LDL-cholesterol, among other adverse effects (American Society of Health System Pharmacists 2016a). UDCA is currently the only drug approved by FDA for the treatment of primary

biliary cirrhosis (PBC, stage I. and II.) (Roma *et al.* 2011). UDCA delays progression and need for liver transplantation, increases survival and is well tolerated in PBC at the doses of 10-20 mg/kg/day (American Society of Health System Pharmacists 2016b, Poupon 2014, Roma *et al.* 2011). In some countries, UDCA is also recommended in primary sclerosing cholangitis (PSC) but evidence on benefit from treatment with UDCA is not as convincing as in PBC. Nevertheless, UDCA is reported to improve biochemical characteristics of disease, ameliorate inflammatory component of the disease, but it probably does not influence overall survival, liver histology nor time to the transplantation at the doses of 10-15 mg/kg/day (EASL 2009). UDCA is also registered for the dissolution of radiolucent, noncalcified gallbladder stones smaller than 20 mm in diameter at the doses of 10-114 mg/kg/day (American Society of Health System Pharmacists 2016b). UDCA is also recommended in the treatment of intrahepatal cholestasis in pregnancy (10-20 mg/kg/day). It alleviates pruritus and improves liver biochemical parameters in up to 80 % of patients (European Assoc Study 2009, Gabzdyl and Schlaeger 2015). Another use of UDCA is cystic fibrosis liver disease (CFLD, syn. CFALD – Cystic Fibrosis Associated Liver Disease, syn. CFAHD – Cystic Fibrosis Associated Hepatobiliary Disorders). UDCA improves biochemical parameters in cystic fibrosis patients, but not overall survival (Staufer *et al.* 2014). Concerning off-label use, UDCA is recommended by European Society for Blood and Marrow Transplantation (ESBMT) as protective agent in Hepatic Veno-Occlusive Disease after hematopoietic stem cell transplantation (RR=0.34) (Dalle and Giralt 2016). Due to the hepatoprotective effect of UDCA, there are some reports that UDCA improves biochemical parameters in non-alcoholic steatohepatitis (NASH), notably in higher doses (Xiang *et al.* 2013), but in overall there is not enough evidence for routine use of UDCA in NASH (Ratziu 2012, Georgescu and Georgescu 2007). UDCA in low doses may also prevent from colorectal cancer in patients with concomitant inflammatory bowel disease and PSC (Singh *et al.* 2013).

Some other derivatives of natural BA – "bile mimetics" – in particular FXR and TGR5 agonists, such as nor-UDCA and obeticholic acid, have been suggested to treat cholestatic liver disease (Poupon 2012, Adorini *et al.* 2012). Obeticholic acid (6α-ethyl-chenodeoxycholic acid) is a semisynthetic derivative of CDCA and potent FXR agonist (Neuschwander-Tetri *et al.* 2015). It was used in the treatment of PBC and NASH in patients non responding to UDCA (Camilleri and Gores 2015). Obeticholic acid also improved liver histology in NASH patients, but 23 % of the patients developed pruritus compared to 6 % in placebo group (Neuschwander-Tetri *et al.* 2015). Moreover, obeticholic acid (25 mg/day) improved liver enzymes, increased low density lipoproteins and decreased markers of fibrosis in patients with type II diabetes mellitus and non-alcoholic fatty liver disease (NAFLD) (Mudaliar *et al.* 2013). In another study, increase of total cholesterol with simultaneous decrease of HDL cholesterol was reported upon obeticholic acid treatment (Neuschwander-Tetri *et al.* 2015).

BA and BA sequestrants are currently investigated as possible adjustment to the treatment of diabetes mellitus (notably Type II) due to their ability to increase insulin sensitivity, and decrease gluconeogenesis (in particular through increase of glucagon like peptide 1) (Camilleri and Gores 2015).

Conclusions

BAs are essential physiological factors preserving homeostasis through their influence on nutrition, metabolism and excretion of both endo- and xenobiotics and their metabolites. BAs have also an important role in pathogenesis of cholestatic diseases and drug induced liver injury. Some of BA, their analogues and so called bile mimetics seem to be promising drugs. Apart of the effects of lipid absorption and xenobiotic excretion based on physico-chemical properties of BA, most of their regulatory and signalling properties are mediated through the nuclear receptors FXR, PXR CAR, LXR and VDR, as well as membrane bound receptors TGR5 and S1PR2 (Copple and Li 2016). The physiological role of interaction of BA with NRs is probably to decrease production of toxic hydrophobic BA and to increase their metabolic turnover towards polar and hydrophilic BA, to increase their excretion and thus decrease their toxicity in the hepatocytes. By this, the activity of some drug-metabolizing CYPs is also influenced since these are regulated by the same NRs, what could have clinically relevant consequences in cholestatic diseases or during the treatment with BA or their derivatives.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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5.2   Endocannabinoid system and P450

The therapeutic use of cannabinoids from cannabis in traditional medicine is known from antiquity, as well as its misuse for psychotropic effects. Nevertheless, the pharmacodynamics of cannabinoids was described as late as in the second half of the 20th century when the Δ^9- tetrahydrocannabinol (THC) was isolated (Gaoni and Mechoulam, 1964) and the CB1 receptor was first described (Devane et al., 1988). Later on, the basic constituents of the endocannabinoid system (ECS) were described: cannabinoid CB1 and CB2 receptors, their endogenous ligands called endocannabinoids, and enzymes of their synthesis and degradation.

The effect of the ECS in the human body can be described as pleiotropic. Components of the ECS are widely distributed throughout the whole body. It is involved in learning and memory, brain plasticity, nociception, inflammation, energy balance, and many others (Aizpurua-Olaizola et al., 2017). Its dysfunction is related to diseases such as depression (Huang et al., 2016), schizophrenia (Zamberletti et al., 2012), obesity (Engeli, 2008), and diabetes (Gruden et al., 2016).

There is great potential for cannabinoids to be used therapeutically for a wide range of illnesses. Today, herbal cannabis, standardized extracts of cannabis, and synthetic analogues of THC are used. Besides THC, cannabidiol (CBD) is another important constituent of cannabis preparations. It counteracts some unwanted psychotropic effects of THC (Niesink and van Laar, 2013). Besides this, it also has anti-inflammatory, neuroprotective, anxiolytic, and antipsychotic properties (Devinsky et al., 2014). There are a variety of synthetic ligands of cannabinoid receptors with various activities on cannabinoid receptors. Some of them, called "spice" or "K2", are misused for their psychotropic effects. The first synthetic selective cannabinoid antagonist/inverse agonist of CB1, rimonabant, was approved for the treatment of obesity and metabolic syndrome. Nevertheless, it was withdrawn from the market because of serious unwanted psychiatric effects (Gong et al., 2007). The role of ECS in the regulation of P450 activity as well as the influence of endocannabinoids is unclear.

5.2.1   The role of ECS in the regulation of P450 activity

In the brain, ECS serves as a retrograde modulator of other neuronal systems including the GABA, dopaminergic, adrenergic, and serotonergic pathways (Carvalho and Van Bockstaele, 2012; Haj-Dahmane and Shen, 2011; Ohno-Shosaku and Kano, 2014). Recently, it was reported that some of these neuronal circuits could be involved in the regulation of liver P450 (Bromek et al., 2013; Kot et al., 2015; Rysz et al., 2016; Wójcikowski et al., 2008). The mechanism of how the signal is transduced from the brain to the liver was studied, and hormones were identified to be involved (Wójcikowski et al., 2007).

Figure 1. Possible involvement of endocannabinoid system in regulation of P450 activity (Zendulka et al., 2016).
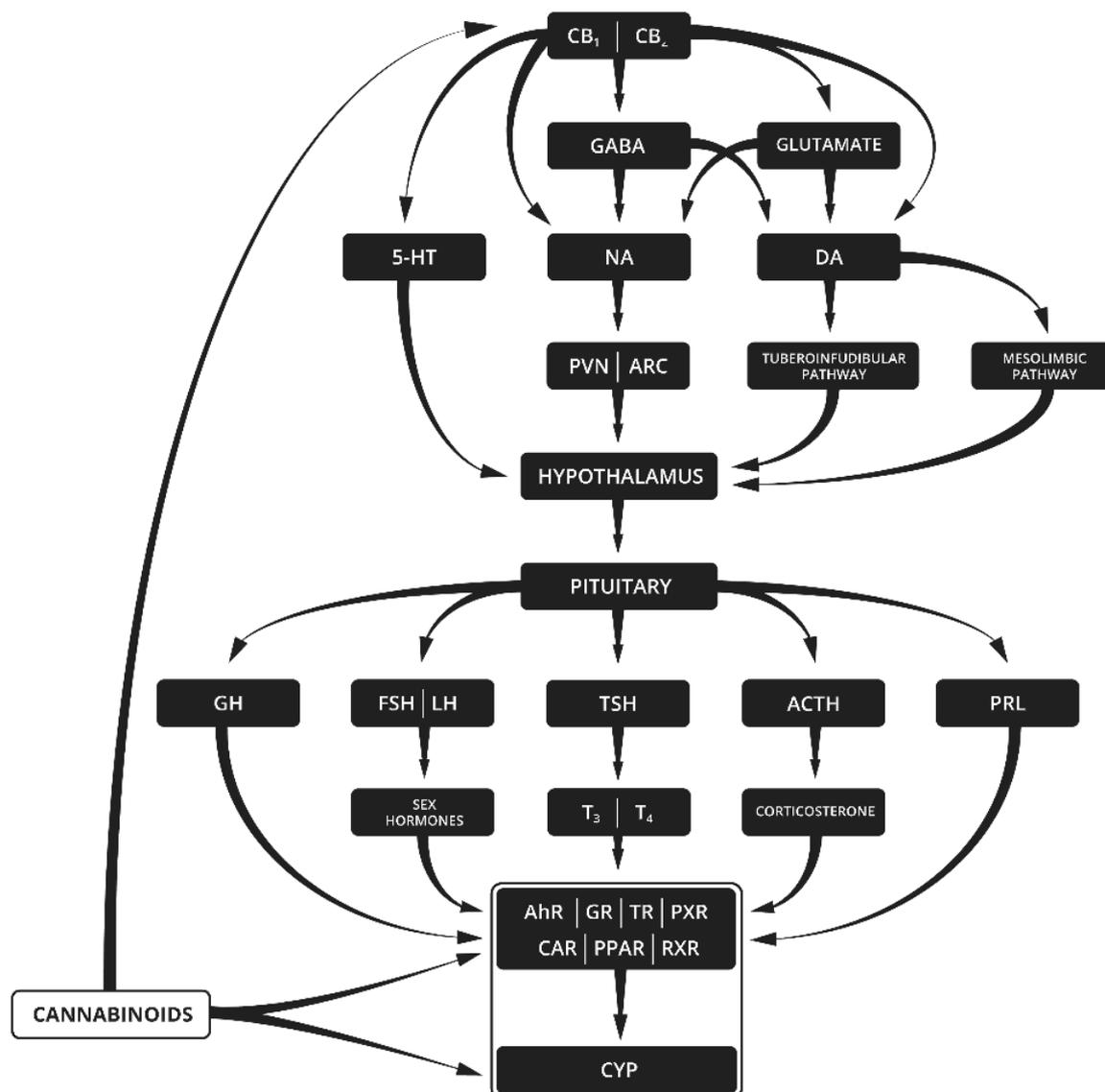
The stimulation or inhibition of particular neuronal pathways modulates the level of hormones released from the pituitary and subsequently from the peripheral tissues and organs. Nuclear receptors regulating the expression of genes of P450 involved in drug metabolism are under the control of glucocorticoids (Dvorak et al., 2003; Ferguson et al., 2005), gonadal steroids (Mwinyi et al., 2011), thyroid hormones (Liddle et al., 1998), or growth hormone (Dhir et al., 2006; Levitsky et al., 1989).

The role of the retrograde modulator of neuronal systems in the brain indicates that ECS could be indirectly involved in the regulation of P450 activity. This hypothesis is supported by the fact that cannabinoid receptors can form heterodimers with dopaminergic receptors (Kearn et al., 2005). We

described this theory in our article "Cannabinoid and cytochrome P450 interactions" (Zendulka et al., 2016), and a brief overview of this can be found in Figure 1. Besides the modulation of neuronal circuits, cannabinoids could also influence P450 activity via nuclear receptors or directly by interacting with the enzyme molecule.

Apart from the hypothesis, the article describes also the P450 enzymes as an alternative metabolic pathway for the degradation of endocannabinoids, herbal cannabinoids, and synthetic cannabinoids as well. Known drug-drug interactions of therapeutically used cannabinoids are also overviewed in the manuscript. A summary of results from preclinical and clinical research on the influence of cannabinoids on P450 amounts and activity is given too. Data from the *in vivo* models are diverse and indicates that ECS could regulate the activity of P450 by other mechanism than by simple enzyme inactivation.

Zendulka O., Dovrtělová G., Nosková K., Turjap M., Šulcová A., Hanuš L., Juřica J. Cannabinoids and cytochrome P450 interactions, Current Drug Metabolism, 17 (3), 206-226, 2016.

Impact factor: 2.659; Pharmacology & Pharmacy Q2, Biochemistry & Molecular Biology Q3

Citations WoS: 22 Author's contribution: 35%

Cannabinoids and Cytochrome P450 Interactions

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Abstract: ***Objective:*** This review consists of three parts, representing three different possibilities of interactions between cannabinoid receptor ligands of both exogenous and endogenous origin and cytochrome P450 enzymes (CYPs). The first part deals with cannabinoids as CYP substrates, the second summarizes current knowledge on the influence of various cannabinoids on the metabolic activity of CYP, and the third outline a possible involvement of the endocannabinoid system and cannabinoid ligands in the regulation of CYP liver activity.

Methods: We performed a structured search of bibliographic and drug databases for peer-reviewed literature using focused review questions.

Results: Biotransformation via a hydrolytic pathway is the major route of endocannabinoid metabolism and the deactivation of substrates is characteristic, in contrast to the minor oxidative pathway via CYP involved in the bioactivation reactions. Phytocannabinoids are extensively metabolized by CYPs. The enzymes CYP2C9, CYP2C19, and CYP3A4 catalyze most of their hydroxylations. Similarly, CYP represents a major metabolic pathway for both synthetic cannabinoids used therapeutically and drugs that are abused. In vitro experiments document the mostly CYP inhibitory activity of the major phytocannabinoids, with cannabidiol as the most potent inhibitor of many CYPs. The drug-drug interactions between cannabinoids and various drugs at the CYP level are reported, but their clinical relevance remains unclear. The direct activation/inhibition of nuclear receptors in the liver cells by cannabinoids may result in a change of CYP expression and activity. Finally, we hypothesize the interplay of central cannabinoid receptors with numerous nervous systems, resulting in a hormone-mediated signal towards nuclear receptors in hepatocytes.

Keywords: Cannabinoids, cytochrome P450, endocannabinoid system, interaction, metabolism, regulation.

1. INTRODUCTION

Cytochrome P450 (CYP) enzymes are haem-containing monooxygenases (EC 1.14.14.1) bound to the membranes of the endoplasmic reticulum or mitochondria in the liver, intestine, kidney, lung, brain, skin, and heart, with the highest level of expression in the liver and intestine [1, 2]. CYPs are functionally coupled with cytochrome P450 reductase, which enables the transfer of electrons from NADPH, the reduced form of NADP (nicotinamide adenine dinucleotide phosphate), to CYP. Microsomal enzymes from subfamilies CYP3A, CYP2C, CYP2D, CYP1A, and CYP2B play a pivotal role in the metabolism of xenobiotics [2]. Variability in the drug plasma levels may diverge depending on different factors, and according to some authors may reach up to 40-fold differences [3]. The most important factors influencing drug plasma levels include the activities of the CYPs with their genetic polymorphisms, epigenetic changes such as DNA methylation and histone deacetylation, together with exogenous factors. These factors substantially influencing CYP metabolic activity are the major source of variability in the pharmacokinetics of drugs and thus in drug responses [3]. CYPs are therefore of particular relevance in clinical pharmacokinetics. On the other hand, the importance of CYP in the metabolism of endogenous substances is also crucial. CYPs are involved in the metabolism of steroid hormones, cholesterol, vitamin D, bile acids and eicosanoids [1], and also most endocannabinoids [4].

Cannabinoids are a group of substances originally isolated from the cannabis plant (*Cannabis sativa*). Today over 100 different molecules with similar structure, most of them with a C21 terpenophenolic moiety, have been isolated and described [5, 6]. They are known to have a wide range of pharmacologic effects [7, 8], for which the hemp plant has been used for over 6000 years in herbal medicine and as a recreational drug.

The first cannabinoid isolated from the cannabis oil was cannabinol (CBN) in 1898 [9, 10], followed by cannabidiol (CBD) in 1940 [11]. Nevertheless, the major psychoactive compound of cannabis remained unknown until 1964, when Δ^9-tetrahydrocannabinol (Δ^9-THC) was isolated in its pure form [12, 13] and its structure was described [14]. The second breakthrough in cannabinoid research was the finding that Δ^9-THC elicits its activity by binding to specific receptors. The first two G-protein coupled receptors (GPCRs) to be discovered, which when activated inhibit adenylyl cyclase, were called the CB1 and CB2 receptors. The CB1 receptor was identified in the brain in 1988 [15], and the CB2 receptor in immune cells in 1993 [16]. These were the first pieces of direct evidence for the possible existence of the endocannabinoid system [17]. The cloning of both of these receptors [16, 18] opened the door to the identification of their endogenous ligands (endocannabinoids), and to the description of their distribution and transduction signal pathways. Anandamide (N-arachidonoylethanolamine) [19] and 2-arachidonoylglycerol (2-AG) [20, 21] are among the first detected and most studied endocannabinoids so far. More recent studies indicate that endocannabinoids, besides the cannabinoid receptor, can also activate multiple receptor targets, including nuclear peroxisome proliferator-activated receptors (PPARs) [22, 23], the transient receptor potential vanilloid type 1 receptor (TRPV1) [24, 25], and orphan G protein-coupled receptors, such as GPR55, GPR119, and GPR18 [26-29]. Other works indicate that cannabinoids have the ability to modulate the activity of additional receptors and their signal transduction pathways, for example

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opioid, serotonin, NMDA, and nicotinic acetylcholine receptors [29, 30].

Nowadays, the terminology concerning cannabinoids is not unified. Some authors describe cannabinoids as ligands of cannabinoid CB1 or CB2 receptors of herbal (phytocannabinoids), endogenous (endocannabinoids) or xenogenic origin (synthetic cannabinoids). Some others differentiate between a) true cannabinoids with the structure derived from endogenous arachidonic acid or natural herbal cannabis-derived compounds, b) synthetic cannabinoid-like compounds of different structures with either direct or indirect cannabimimetic effects, or compounds inhibiting the cannabinoid receptor activities. Moreover, some authors consider endogenous molecules with a similar structure, but without the ability to bind to CB receptors to also be cannabinoids. These endocannabinoid-like compounds can interfere with the activity of true endocannabinoids, as they are in several cases synthetized and biotransformed via the same pathways [31]. For the above reasons and to maintain the clarity the authors of this review decided to use the name cannabinoids for all of the substances described. An overview of endocannabinoids and endocannabinoid-like substances as well as the most common phytocannabinoids, synthetic ligands of cannabinoid receptors used in preclinical studies, and cannabinoid derived drugs is shown in Table **1**.

The aim of this work is to provide a comprehensive review of the interactions between CYPs and the endocannabinoid system and its ligands. Here, we describe the role of CYP in the metabolism of cannabinoids and vice versa the role of cannabinoids in the regulation of CYP activity.

2. CANNABINOIDS AS SUBSTRATES OF CYTOCHROME P450 MONOOXYGENASES

The endogenous and exogenous cannabinoids are substrates of various CYPs. Due to the possibility of interaction between endocannabinoids, phytocannabinoids, or synthetic cannabinoids and other drugs at the CYP site, there is a risk of treatment failure or drug toxicity. It is therefore important to identify possible sites of such interactions for the successful prevention of pharmacokinetic drug-drug interactions.

2.1. Endocannabinoids and Endocannabinoid-Like Compounds

Numerous amides of fatty acids, notably amides of arachidonic acid, its derivatives, and their metabolites, are potent ligands of cannabinoid receptors. To date, anandamide (AEA), 2-AG and its isomer 1-arachidonoylglycerol, oleamide (oleic acid amide), virodhamine (O-arachidonoylethanolamine), di-homo-γ-linolenoyl-ethanolamide, N-arachidonoyldopamine, noladin ether (2-arachidonylglyceryl ether), and N-arachidonoylserine were identified and proved to be endogenous ligands of at least some cannabinoid receptors. Other endogenous N-acylethanolamines, N-acylethanolamides, and N-acyl-aminoacids such as palmitoylethanolamide, N-arachidonoyltaurine, N-arachidonoylglycine [32-35], N-docosatetraenoyl-ethanolamine, N-docosahexaenoylethanolamine, or N-eico-sapentaenoylethanolamine were found in mammalian tissues over the last decade and exhibit varying affinity to cannabinoid receptors CB1 and CB2. It is also possible that they potentiate the effects of „classical" endocannabinoids such as anandamide and 2-AG independently of binding to CB receptors. Therefore, they are sometimes called „endocannabinoid-like compounds" [4, 35-37].

The metabolism of AEA and 2-AG, being the first investigated and most studied endocannabinoids, was recently reviewed by Snider *et al*. [38] and Zelasko *et al*. [4].

The biological effects of most endocannabinoids are terminated by transport to the cells and enzymatic inactivation. It was hypothesized that the transport of endocannabinoids to the cells may also regulate their biological effects. Mechanisms such as simple diffusion, facilitated diffusion or endocytosis are thought to uptake AEA to the cells [38]. A major degradation pathway is catalyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [4, 38]. The oxidative degradation of endocannabinoids is only a minor pathway and involves the enzymes cyclooxygenase-2 (COX-2), 12- and 15-lipoxygenase (12-LOX, 15-LOX, respectively), and CYPs [38-41]. Since some of the metabolites of endocannabinoids originating via CYP enzymes are active ligands of CB receptors, the role of this oxidative pathway remains unclear [4, 35, 37, 38]. Due to the focus of this review, CYP-mediated pathways are described in detail.

Table 1.   **Overview of cannabinoids.**

Endocannabinoids	Endocannabinoid-like compounds	Phytocannabinoids	Synthetic cannabinoids
anandamide	palmitoylethanolamide	Δ^9-tetrahydrocannabinol	dronabinol[a]
2-arachidonoylglycerol	N-docosatetraenoylethanolamine	Δ^8-tetrahydrocannabinol	nabilone[a]
noladin ether	di-homo-γ-linolenoyl-ethanolamide	cannabidiol	rimonabant
virodhamine	2-oleoylglycerol	cannabinol	methanandamide
arachidonoyldopamine	N-oleoylethanolamine	cannabigerol	JWH-0133
N-arachidonoylserine	N-eicosapentaenoylethanolamine	cannabichromen	AM-251
homo-γ-linolenoylethanolamide	N-docosahexaenoylethanolamine	cannabivarin	ACEA
			ACPA
7,10,13,16-docosatetraenoylethanolamide	oleamide	cannabielsoin	WIN 55,212-2
	N-arachidonoylglycine	cannabitriol	CP 55,940
	1- arachidonoylglycerol		HU-210
	N-arachidonoyltaurine		

[a] synthetic analogues of Δ^9-THC

2.1.1. Arachidonic Acid

Since the CYP-mediated metabolic pathways of endocannabinoids are closely similar to the metabolism of arachidonic acid (AA), this CYP-mediated metabolism of AA is reviewed briefly so as to elucidate theoretical possibilities of the oxidations at the "fatty acid" site of endocannabinoid molecules.

CYPs are known to metabolize arachidonic acid by epoxidation, ω/ω-1 hydroxylation, bis allylic oxidations, and hydroxylation to conjugated dienols (Fig. **1**) [42-44]. As a result, a wide variety of metabolites with biological activities are produced.

Arachidonic acid has four double bonds and epoxidation may occur on any of them. The products of epoxidation, epoxyeicosatrienoic acids (EET), may be further hydrolyzed to dihydroxyeicosatrienoic acids (diHETE). EET are produced by several hepatic and extrahepatic CYPs - CYP2C8, CYP2C9, CYP1A2, and CYP2B6, with the latter playing only a minor role (Table **2**). The ω/ω-1 hydroxylations of arachidonic acid to hydroxyeicosatrienoic acid (HETE) are catalyzed by the CYP4A, CYP2E1, and CYP4F families (Table **3**). Finally, bis-allylic oxidations and hydroxylations with double bond migration are catalyzed by CYP families 1A, 3A, 2C, and 4F (Table **4**).

2.1.2. Anandamide

Anandamide, the first known endocannabinoid, was isolated from the porcine brain by L. O. Hanuš and W. A. Devane from the team of prof. R. Mechoulam at Hebrew University, Jerusalem in 1992 [19]. AEA is hydrolyzed by the membrane-bound enzyme FAAH, with the highest level of expression in the liver. This degradative pathway is the most important in the regulation of AEA cellular and tissue concentrations. FAAH hydrolyses AEA towards arachidonic acid and ethanolamine. Thus the inhibition of FAAH may become a useful alternative in cannabinergic treatment options [38]. COX-2, an enzyme expressed in an inducible manner in inflammation, converts anandamide to several prostaglandin ethanolamides [46, 47]. Oxidation of the aliphatic chain by 12-LOX and 15-LOX yields 12- and 15-hydroxyanandamide. 12-hydroxyanandamide in particular may play a significant role in the modulation of neuronal functions via its influence on neurotransmitter levels [48].
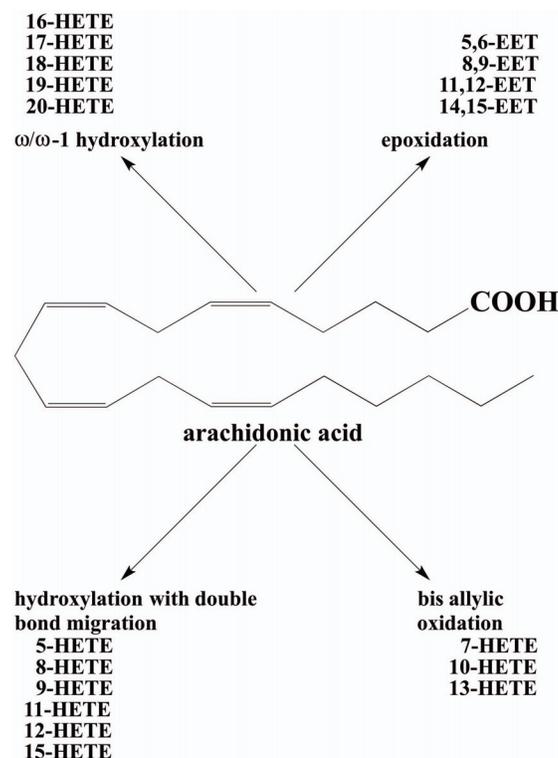
Fig. (1). CYP-mediated metabolism of AA [42-44].

CYPs involved in the degradation of AEA belong to the CYP3A and CYP4F families. The biodegradation of anandamide by CYPs was reported for the first time by Bornheim *et al*. in 1995 [49], who described its conversion by mouse liver microsomal fraction to approximately 20 products, whose structures were not identified. Furthermore, pretreatment with common CYP inducers such

Table 2.   Epoxidation of AA catalyzed by cytochrome P450 enzymes [42-44].

CYP	Product	Tissue
2B6	14,15-EET, 11,12-EET	liver
2C8	14,15-EET, 11,12- EET, 8,9-EET	liver, lung, vascular endothelium
2C9	14,15- EET, 11,12- EET, 8,9-EET	liver, lung, vascular endothelium
2C19	14,15- EET, 8,9-EET	liver
2J2	5,6- EET, 8,9- EET, 11,12- EET, 14,15-EET	kidney, GIT, pancreas
1A2	8,9- EET, 11,12-EET	liver, lung

Table 3.   ω/ω-1 hydroxylations of AA catalyzed by cytochrome P450 enzymes [44, 45].

CYP	Product	Tissue
4A11	19-, 20-HETE	liver, kidney
4F2	20-HETE	liver, kidney
4F3	hydroxy-LTB$_4$	polymorphonuclears
4F11	?	liver, kidney
4F12	18-HETE	liver, kidney
2E1	19(*S*)-HETE, 19(*R*)-HETE, 18(*R*)- HETE	liver, kidney

Table 4.　**Bis-allylic oxidations and hydroxylations of AA with double bond migration which are catalyzed by cytochrome P450 enzymes [42].**

CYP	Product	Tissue
1A2	7-, 10-, 13-HETE	liver
3A4	7-, 10-, 13-HETE	liver
2C8	11-, 13-, 15-HETE	liver
2C9	12-, 13-HETE	liver
2C19	13-, 19-HETE	liver
4F8	13-HETE	liver, ovary/testes
4F12	18-HETE	liver, kidney, GIT

as dexamethasone increased the formation of metabolites 5-15 fold, and pre-treatment with a CYP3A antibody diminished the production of anandamide metabolites. Bornheim *et al.* also suggested that the CYP3A, CYP2B, and CYP1A subfamilies are involved in the metabolism of AEA in mouse liver microsomes. Similarly, Costa *et al.* suggested that CYP3A and CYP2B subfamilies are involved in the metabolism of anandamide in rat liver microsomes [50].

To the best of our knowledge, Snider *et al.* was the first to investigate the biotransformation sites of AEA by human liver and kidney microsomal CYPs, and identified the metabolites [51]. The biotransformation routes are fundamentally similar to those of AA. Anandamide may be epoxygenated by several CYPs at positions 5-6, 8-9, 11-12, and 14-15 to form four epoxyeicosatrienoic acid ethanolamides (EET-EAs). At least in some of them, this oxidative pathway is more bioactivation than degradation, since 5,6-EET-EA seems to be a more stable CB ligand than AEA itself [38]. Nevertheless, all EET-EAs may be further hydroxylated in the ω positions (again, similarly to AA metabolism) predominantly by CYP2D6 and thus 20-hydroxy-epoxyeicosatrienyl ethanolamides (HEET-EAs) are produced [51]. With 5,6-EET-EA and 14,15-EET-EA, hydroxylations at positions 16, 17, 18, and 19 were also described. EET-EA may be hydrolyzed by epoxyhydrolase to form dihydroxylated EET-EA [52].

Besides these reactions, ω- and ω-1-hydroxylations of AEA were also described [4, 38, 51, 53]. Details on the oxidative metabolism of AEA are shown in Figure **2**.

2.1.3. 2-arachidonoylglycerol

The main metabolic degradation of 2-AG is catalyzed by MAGL, FAAH, and α,β-hydrolase domains (ABHD) 6 and 12 [4, 38, 54]. The structure of 2-AG suggests that they are subject to the same oxidative metabolism as AA and AEA, which would lead to four regioisomeric 2-epoxyeicosatrienylglycerol derivatives (EET-G) (Fig. **3**). In contrast to this assumption, only 2 EET-G were identified to date – 2-(11,12-epoxyeicosatrienyl)-glycerol and 2-(14, 15-epoxyeicosatrienyl) glycerol, which are produced by CYP2J2 in rat kidney and spleen [55] and in bovine and porcine myocardium [56, 57]. These metabolites demonstrate regulatory effects on blood pressure, as was shown in the study of Awumey *et al.* [58].

As well as in EET-EA, the CYP-mediated epoxygenation of 2-AG to EET-G seems to be a kind of bioactivation, since these metabolites exhibit a tighter binding to CB receptors than 2-AG [56]. EET-G may be oxidatively decomposed by CYP2J2 to AA and glycerol.

2.1.4. N-arachidonoyldopamine

N-arachidonoyldopamine (NADA) is another endocannabinoid known to exert significant biological activity, e.g. in the immune system and pain perception [59, 60]. Besides hydrolysis to AA and

dopamine by FAAH [35], NADA may be hydroxylated by rat microsomal protein in the presence of NADPH in the ω and ω-1 positions to form 19- and 20-hydroxyeicosatetraenyl dopamine (19-HETE-DA and 20-HETE-DA) [60] (Fig. **4**).

The question of epoxygenase reactions analogous to the CYP-mediated metabolism of AA and anandamide remains to be further elucidated.

2.1.5. Other Endocannabinoids and Cannabinoid-Like Compounds

The metabolic fate of the other endocannabinoids and cannabinoid-like compounds, such as virodhamine, oleamide, N-arachidonoylglycine, N-arachidonoylserine, or N-arachidonoyltaurine is not well understood, but hydrolysis with esterases or amide hydrolases is likely. On the other hand, hydrolysis of the ether group (e.g. noladin ether) by these enzymes is not likely, in contrast to oxidative metabolism, which may be an alternative degradative pathway for ethers, but there is still no direct evidence for this.

2.1.6. Concluding Remarks Concerning Endocannabinoid Metabolism

The metabolism of endocannabinoids via the hydrolytic pathway (namely FAAH) usually produces inactive metabolites, in terms of their affinity to bind to CB receptors. On the other hand, the products of the oxidative pathway may be both metabolites with a lower affinity to CB receptors (20-HETE-EA and 14,15-EET-EA) and products with a higher affinity to the CB (or PPAR) receptor than the parent compound, as shown with some 2-11,12-EET-Gs and 2-14,15-EET-Gs [38]. Moreover, a molecule with higher stability (5,6-EET-EA) can be produced. From this point of view, the inhibition of endocannabinoid degradation may be a valuable pharmacological target, and has been shown to produce anxiolytic-like and antidepressant-like effects in animal models [62]. Despite promising results from animal studies, there are no reliable data on efficacy from clinical studies. The clinical trials were focused mostly on safety; in general, the inhibitors were well tolerated and lacked typical "cannabinoid-like effects" [62]. There has also been a reported lack of efficacy in a clinical trial of an FAAH inhibitor in the treatment of osteoarthritic pain [63]. Modulation of the oxidative metabolic pathway was not studied in terms of a possible therapeutic approach; modulating the oxidative pathway would be problematic due to the involvement of CYPs (CYP2D6, CYP2C8, CYP3A) in the metabolism of other endogenous substances and possibly also co-administered drugs.

2.2. The Metabolism of Phytocannabinoids *via* Cytochrome P450 Monooxygenases

The term phytocannabinoids covers naturally occurring phytochemicals from *Cannabis sativa*, *Cannabis indica,* or *Cannabis*
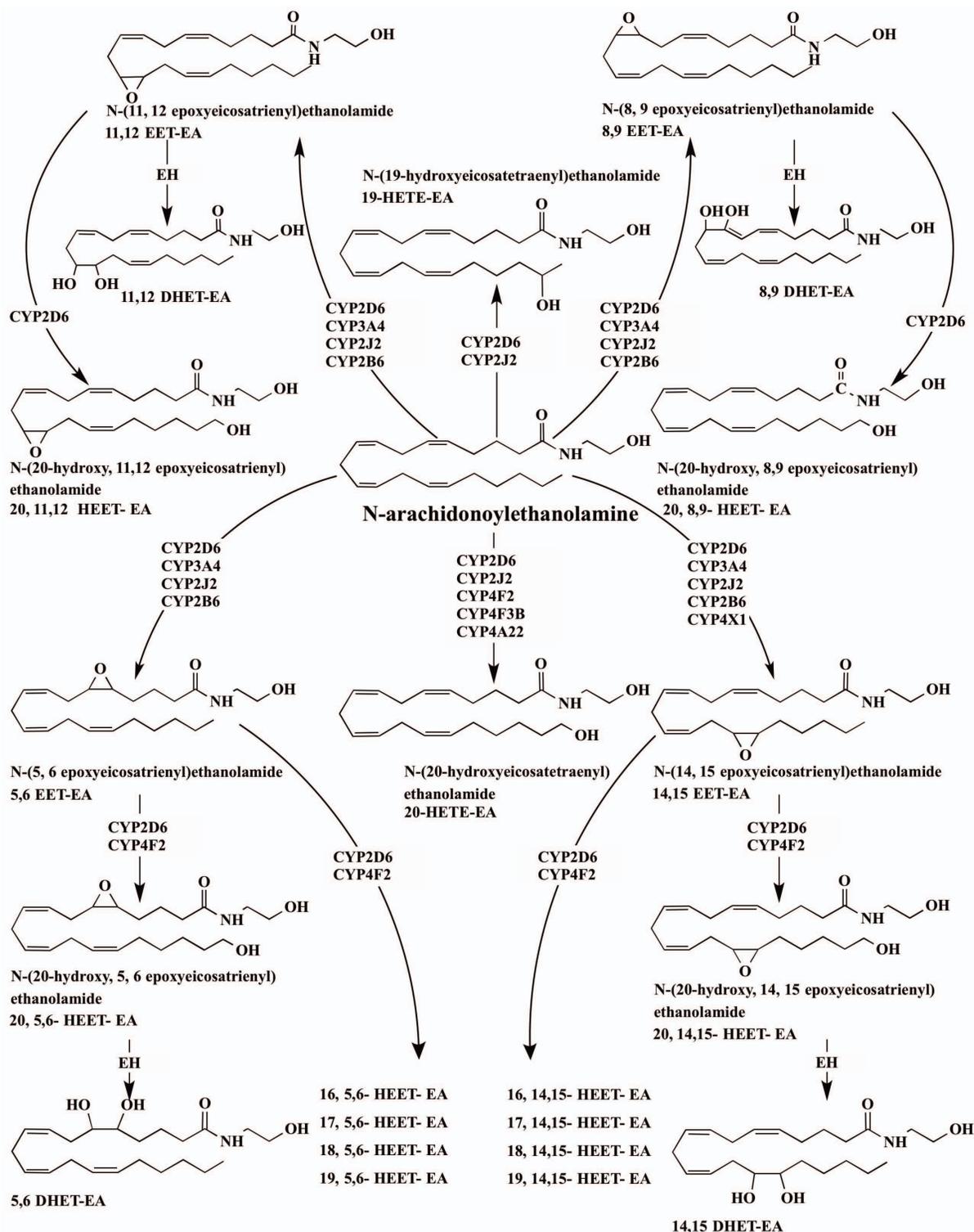
Fig. (2). CYP-mediated metabolism of anandamide [4, 38, 61].

ruderalis which are able to interact with cannabinoid receptors [64, 65]. Nearly 500 chemical entities were identified in *Cannabis* herbage, of which about 70 are phytocannabinoids. These compounds are present in the highest amounts in the viscous resin produced by the glandules of female cannabis inflorescence [64, 66]. As a result, several chemical classes of phytocannabinoids were defined by ElSohly *et al.* [66]: 1) cannabigerol type, 2) cannabichromene type, 3) cannabidiol type, 4) (-)-Δ^9-trans-tetrahydrocannabinol type,

5) (-)-Δ^8-trans-tetrahydrocanna-binol type, 6) cannabicyclol type, 7) cannabielsoin type, 8) cannabinol type, 9) cannabinodiol type, 10) cannabitriol type, and 11) miscellaneous type. In terms of this classification, the (-)-Δ^9-trans-tetrahydrocannabinol type, cannabinol type, and cannabidiol type are the most abundant and best known and studied. Out of 70 known phytocannabinoids, only Δ^9-THC, CBN, and CBD are reviewed in terms of oxidative metabolism by CYPs. No data were found for the other phytocannabinoids.
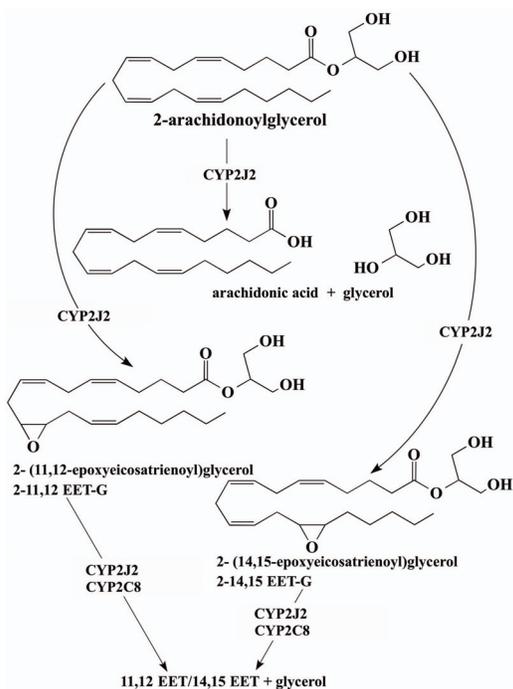
Fig. (3). CYP-mediated metabolism of 2-arachidonoylglycerol [4, 38].
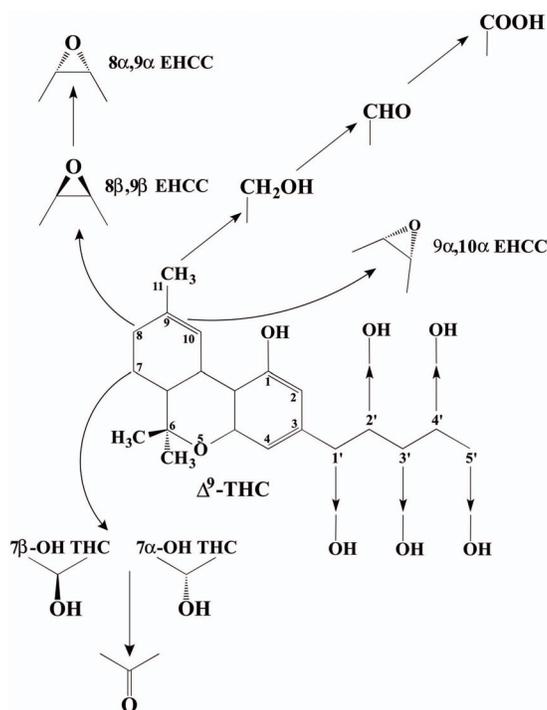
Fig. (5). Structure and CYP-mediated oxidative metabolism of Δ^9-THC [69].
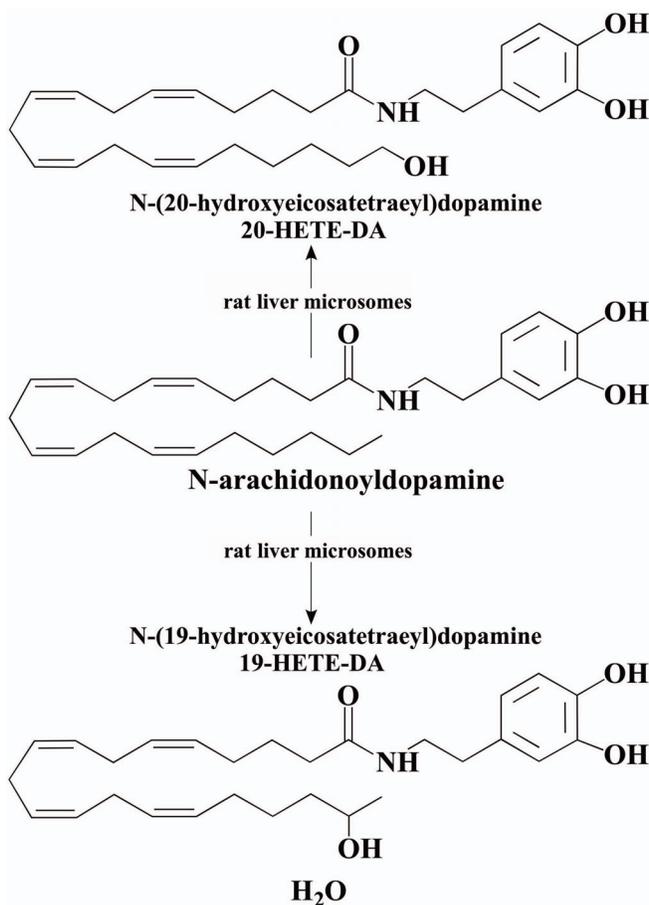
Fig. (4). CYP-mediated metabolism of N-arachidonoyldopamine [60].
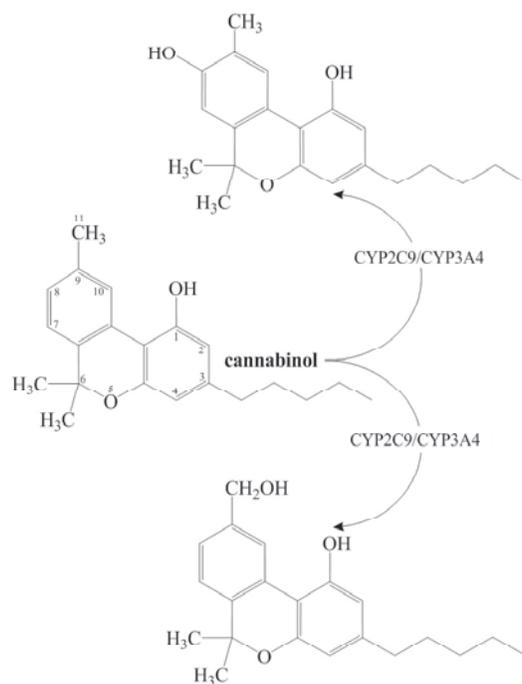
Fig. (6). Hydroxylation of CBN by CYP enzymes [69, 71].

2.2.1. Δ^9-tetrahydrocannabinol

The oxidative biotransformation of Δ^9-THC is quite complicated – approximately 80 metabolites were identified in humans [67]. The majority of the biotransformation processes of Δ^9-THC are catalyzed by CYPs (Fig. **5**). The first metabolite of Δ^9-THC was described back in 1970 by Nilsson *et al.*, who used NMR to identify 11-hydroxy-Δ^9- THC in an extract from the incubation of a crude
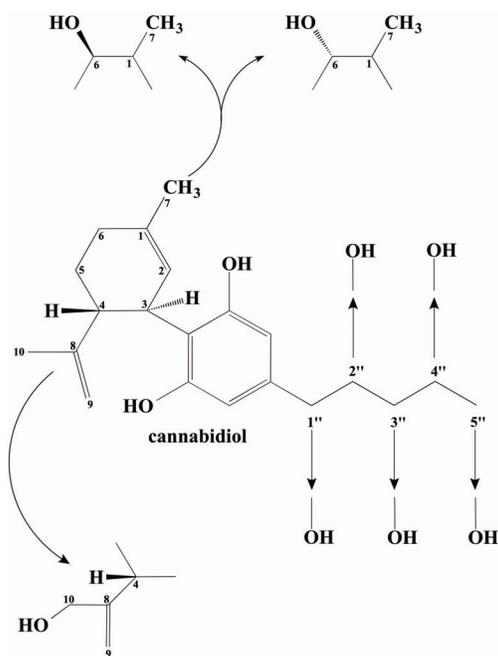
Fig. (7). Hydroxylation of CBD by CYP enzymes [72].

microsomal fraction of rabbit liver with Δ^9-THC [68]. This metabolite was formerly named 7-hydroxy-Δ^1-THC, because of the different numbering of the terpenophenolic ring in the past. The second most abundant hydroxy-derivative of Δ^9-THC is 8β-hydroxy-Δ^9-THC [69]. Later on, many other metabolites were identified, mostly in experiments with liver microsomes of different species, including humans, and the relative importance of CYPs was also examined, containing epoxygenated metabolites of THC [70]. The authors suggest that CYP2C9 and CYP3A4 probably play the most important roles in the oxidative metabolism of Δ^9-THC. Recently, Stout *et al.* [65] published a unique systematic review on the metabolism of cannabinoids.

Some of the metabolites of Δ^9-THC seem to be active (e.g. 11-hydroxy-Δ^9-THC) and therefore some authors think that the oxidative metabolism of Δ^9-THC may be necessary for the effects of cannabis [69].

2.2.2. Cannabinol

Cannabinol metabolism was studied by Kuzuoka *et al.* [71] and Watanabe *et al.* [69]. The chemical structure of CBN, being similar to Δ^9-THC, leads us to expect similar metabolic pathways mediated by microsomal monooxygenases. The hydroxylations occur at positions 8 and 11, and CYP2C9 and CYP3A4 are involved in their formation as reported in [65, 69, 71] (Fig. **6**).

2.2.3. Cannabidiol

The metabolism of cannabidiol was investigated both *in vivo* and *in vitro*. 33 different metabolites were found in human urine from a patient treated with CBD, 600 mg/day [72].

CBD is metabolized primarily by the enzymes CYP2C19 and CYP3A4 [65, 73]. The hydroxylation reactions occur at positions 6, 7, and positions 1″- 5″ of the aliphatic pentyl- and position 10 on the propenyl- substituent (Fig. **7**). Moreover, these metabolites may be further oxidized to form dihydroxylated metabolites and CBD-oic acid derivatives [72]. In an experiment with recombinant human liver microsomes, Jiang *et al.* proved that 7 out of 14 recombinant human CYP enzymes may be involved in CBD metabolism [73]. These include CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5.

Among monohydroxylated metabolites, the most abundant were 6α-OH-, 6β-OH-, 7-OH-, 1″-OH-, 2″-OH-, 3″-OH-, 4″-OH-, and 5″-OH-CBD [73]. The authors also confirmed the importance of CYP3A4 and CYP2C19 in the overall metabolism of CBD, namely in the 6α-, 6β-, 7-, and 4″-hydroxylations of CBD with the use of selective isoform-specific inhibitors and anti-CYP3A4 antibodies.

2.2.4. Other Phytocannabinoids

The metabolism of other phytocannabinoids has not been studied in humans, but the hydroxylation of several cannabinoids, including THC, CBD, CBN, cannabichromene (CBC), and cannabigerol (CBG) was studied *in vitro* in the liver microsomal fraction in several animal species [74]. In general, similar hydroxylation reactions are catalyzed by microsomal fractions, but particular CYPs responsible for the reactions were not identified. Hydroxylation occurs most abundantly at the allylic part of the molecule at positions C5' and C6'. Apart from C5' and C6' hydroxylations, hydroxylation also occurs at positions C2' and C1" to C5", and epoxidation at the double bond of the methylpentenyl group [74] (Fig. **8**).

Cannabigerol metabolism appeared to be similar to the metabolism of CBC. Hydroxylations at the terminal allylic group of the side chain were the most abundant reactions in the liver microsomes of all species except for mouse, where C6' or C7' epoxide was the most abundant [74] (Fig. **8**).
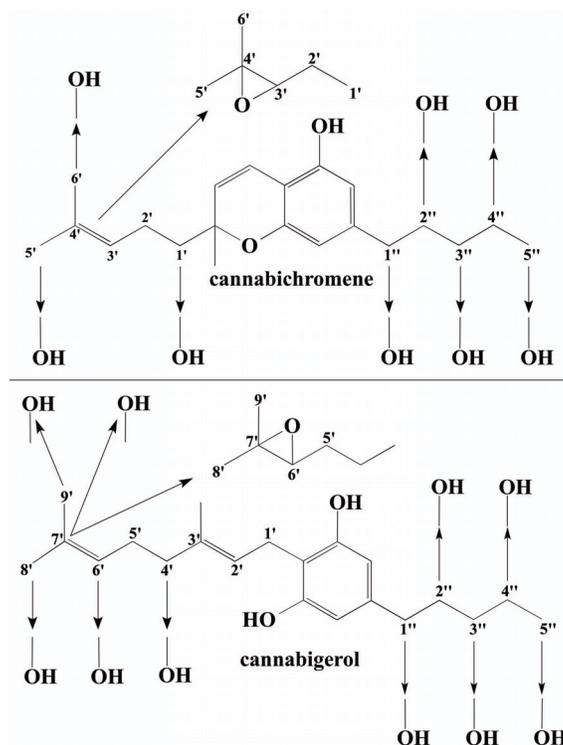
Fig. (8). Structure of CBC (**A**) and CBG (**B**) and positions of oxidative metabolism mediated by microsomal enzymes in mouse, rat, guinea pig, rabbit, hamster, gerbil, cat [74].

In summary, phytocannabinoids are extensively metabolized by CYP enzymes. For the most studied THC, CBN, and CBD, the enzymes CYP2C9, CYP2C19, and CYP3A4 catalyze the majority of hydroxylations. Most cannabinoids exhibit a similar pattern of oxidative metabolism [74]. At first, tricyclic cannabinoids (THC, CBD, and CBN) are the most effectively hydroxylated at the C-11 position and to a lesser extent also at the C-8 position. Various degrees of hydroxylation and epoxidation also occur at the carbons of the side chain in all cannabinoids, with the exception of CBN.

2.3. Synthetic Cannabinoids

Besides the substances isolated from natural materials, many other ligands of CB receptors were synthetized *in vitro*. Synthetic cannabinoids cover the whole spectrum of receptor ligand types from full agonists to inverse agonists, and their biological effects are therefore miscellaneous [75]. For the purposes of this review, synthetic cannabinoids are classified into groups of drugs for therapeutic purposes, molecules used as research tools, and abused drugs.

2.3.1. Synthetic Cannabinoids as Medicinal Products

Dronabinol (Marinol®) is a synthetic Δ^9-THC for oral use. It is approved for medical use in the United States and several other countries. Dronabinol is indicated for the treatment of anorexia associated with weight loss in patients suffering from AIDS and for the treatment of nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetic therapy [76]. It undergoes extensive first-pass hepatic metabolism, primarily by microsomal hydroxylation via multiple CYPs, yielding both active and inactive metabolites. Its principal active metabolite is 11-OH-Δ^9-THC [76].

Nabilone (Cesamet®) is a synthetic THC analogue for oral administration. It is registered in Canada, the USA, and several other countries for the treatment of nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetic treatments [77]. It has been suggested that the antiemetic effect of nabilone is mediated by its interaction with the cannabinoid CB1 receptor within the central nervous system. The metabolism of nabilone is extensive, and several metabolites have been identified. According to the data from Cesamet® SPC [77], there are at least two metabolic pathways involved in the biotransformation of nabilone. A minor pathway is initiated by the stereospecific enzymatic reduction of the 9-keto moiety of nabilone to produce the isomeric carbinol metabolite. Secondly, a metabolite of nabilone in faeces has been identified as a diol formed by reduction of the 9-keto group plus oxidation at the penultimate carbon of the dimethylheptyl side chain. In addition, there is evidence of extensive metabolism of nabilone by multiple CYPs. *In vitro* CYP inhibition studies using human liver microsomes showed that nabilone did not significantly inhibit the metabolic activity of CYP1A2, 2A6, 2C19, 2D6, and 3A4. In clinical use, nabilone is unlikely to alter the CYP-mediated metabolism of co-administered drugs [77].

Rimonabant (Acomplia®) was the first CB1 antagonist/inverse agonist to be approved for therapeutic use in metabolic syndrome and obesity [78]. Because of the significant risk of serious psychiatric adverse effects, it was withdrawn from the market [79]. *In vitro* experiments revealed CYP3A4 and amidohydrolase to be the major metabolic pathways involved in the biotransformation of rimonabant into inactive metabolites [80].

A buccal spray preparation containing *Cannabis* extracts, whose main active ingredients are Δ^9-THC and CBD (Sativex®), is now available in many countries including the UK, Spain, Italy, and Germany (not available in the US). It is used for the symptomatic relief of spasticity or neuropathic pain in multiple sclerosis and in cancer pain [81]. The active substances have the same structures as natural Δ^9-THC and CBD, therefore they undergo the same metabolic pathways.

2.3.2. Synthetic Cannabinoids as Experimental Tools

Compounds that are known to activate CB1 and CB2 receptors with approximately equal potency and that are most commonly used in the laboratory as CB1/CB2 receptor agonists fall essentially into one of four chemical groups: classical cannabinoids, nonclassical cannabinoids, amino-alkylindoles, and eicosanoids [29].

The classical group consists of dibenzopyran derivatives. The prototypic synthetic member of this group is HU-210, a synthetic analogue of (-)-Δ^8-THC. HU-210 displays a high affinity for CB1 and CB2 receptors, and also a high potency and relative intrinsic activity as a cannabinoid receptor agonist [29]. In the study of Kim *et al*. [82], the *in vitro* metabolism of HU-210 was investigated using human liver microsomes to characterize associated phase I metabolites. HU-210 was metabolized to yield a total of 24 metabolites, characterized as mono-oxygenated, mono-hydroxylated, di-oxygenated, or di-hydroxylated metabolites. The specific enzymes involved in the formation of the metabolites were not investigated.

The nonclassical group contains bicyclic and tricyclic analogues of Δ^9-THC that lack the pyran ring. The most widely used member of this group is CP 55,940. The oxidative metabolism of CP 55,940 was studied in mouse liver microsomes by Thomas *et al*. [83]. The mass spectral data indicated that five monohydroxylated metabolites had been formed differing in their position of hydroxylation. Two additional compounds were detected whose mass spectral data suggested that these metabolites were hydroxylated at two positions on the side chain. Side chain hydroxylation is consistent with the metabolic profile of Δ^9-THC [83].

The prototype of the aminoalkylindole group widely used in cannabinoid research is WIN 55,212-2. WIN 55,212-2 exhibits a relatively high efficacy at the CB1 and CB2 receptors and possesses CB1 and CB2 affinities in the low nanomolar range. The structure of WIN 55,212-2 bears no structural similarity to classical, nonclassical, or eicosanoid cannabinoids [84]. The metabolism of WIN 55,212-2 in rat liver microsomes was investigated in the study of Zhang *et al*. [85]. The HPLC chromatogram revealed two major and at least six minor metabolites derived from the parent compound. The two major metabolites (representing 60 to 75 % of the total metabolites) were each identified as dihydrodiol metabolites resulting from the arene oxide pathway. Three of the minor metabolites corresponded to structural isomers of the trihydroxylated parent compound, the other two represent monohydroxylated isomers and another was determined to be a dehydrogenation product. Specific enzymes involved in the formation of metabolites were not investigated.

Members of the eicosanoid group of cannabinoid CB1/CB2 receptor agonists have structures quite unlike those of classical, nonclassical, or aminoalkylindole cannabinoids. Two prominent members of this group are the endocannabinoids AEA and 2-AG.

The starting point for the development of the first CB1 selective agonists was the AEA molecule [29]. A number of agonists with significant selectivity for CB1 or CB2 receptors have been developed. Important CB1 selective agonists include the AEA analogues R-(+)-methanandamide, arachidonyl-2′-chloroethylamide (ACEA), and arachidonyl-cyclopropylamide (ACPA). Of these, both ACEA and ACPA share the susceptibility of AEA to enzymatic hydrolysis by FAAH [29, 86]. In contrast, methanandamide is less susceptible to enzymatic hydrolysis, probably because it is protected from this by the presence of a methyl substituent at the 1′ carbon [87].

The CB2 selective agonists most widely used as experimental tools have been the classical cannabinoid JWH-133, and the less selective aminoalkylindole JWH-015 [29]. The *in vitro* phase I metabolism of JWH-015 using human liver microsomes was studied by Mazarino *et al*. [88]. A total of 18 metabolites were formed. The biotransformation pathways detected consist of mono-hydroxylation, di-hydroxylation, tri-hydroxylation, carboxylation, N-dealkylation, dehydratation, and combinations of them, confirming data from the study with rat liver microsomes [89]. Specific enzymes involved in the oxidative metabolism were not studied.

2.3.3. Synthetic Cannabinoids as Abused Drugs

Synthetic cannabinoids recently became the largest group of compounds to be monitored in Europe by the EU Early Warning System on new psychoactive substances [90]. "Legal high" products containing synthetic cannabinoids (SCs) have probably been sold as herbal smoking mixtures since 2006. In 2008, a synthetic

cannabinoid JWH-018 was detected for the first time in a herbal mixture. In 2014, a further 30 new synthetic cannabinoids were reported for the first time, bringing the total number reported by the EU Early Warning System to 137 in February 2015 [91]. New drugs are synthesized by slight modifications of the known psychoactive "parent" compound, to obtain similar - or even stronger - psychoactive effects and to circumvent the law, being not yet included in the lists of controlled substances [92]. The common property of all SCs is that they interact with the CB1 and CB2 cannabinoid receptors and elicit cannabimimetic effects similar to Δ^9-THC. They are synthesized in clandestine laboratories and illegally added to commercial products such as herbal blends (these are sold under brand names such as "Spice" and "K2"), which are claimed to be air fresheners or herbal incenses. The most common way of administration is smoking.

The majority of compounds are chemically unrelated to Δ^9-THC. To date hundreds of SCs were categorized into the following structural groups: adamantoylindoles, aminoalkylindoles, benzoylindoles, cyclohexylphenols, dibenzopyrans, naphthoylindoles, naphthylmethylindoles, naphthylmethylindenes, naphthoylpyrroles, phenylacetyl-indoles, tetramethylcyclopropylketone indoles, quinolinyl ester indoles, and indazole carboxamide compounds [93].

Only limited data are available on the metabolism of the huge variety of synthetic cannabinoids. Due to the insufficient toxicity data, controlled human drug administration studies are not feasible. Therefore, *in vitro* experiments are alternative approaches for metabolite profiling and structure elucidation. Most of the recent *in vitro* metabolite-profiling studies utilize human liver microsomes or human hepatocytes. So far, we have identified such studies for the following synthetic cannabinoids: AB-CHIMINACA [94], AB-FUBINACA [95-97], AB-PINACA [95, 96, 98], 5F-AB-PINACA [98], ADB-FUBINACA [95], AKB-48 [99, 100], 5F-AKB-48 [100], AM-2201 [101, 102], APICA [103], CP 47,497 [104], HU-210 [82], JWH-015 [88], JWH-018 [105-108], JWH-073 [108], JWH-073 4-methylnaphthoyl analogue [108], JWH-122 [108-110], JWH-200 [109], JWH-210 [88], MAM-2201 [110], PB-22 [95, 111], 5F-PB-22 [95, 111], RCS-4 [112], RCS-8 [113], STS-135 (5F-APICA) [103, 114], UR-144 [102], and XLR-11 [115].

In vitro metabolite-profiling studies with subsequent confirmation in authentic specimens provide critically important information for the identification of suitable *in vivo* biomarkers to document the intake of SCs in clinical and forensic settings.

Two of the above-cited investigations also focused on the identification of specific CYP enzymes involved in oxidative metabolism. Chimalakonda *et al.* [101] studied the oxidative metabolism of [1-naphthalenyl-(1-pentyl-1H-indol-3-yl]-methanone (JWH-018) and its fluorinated counterpart AM-2201 [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone. Kinetic analysis using human liver microsomes and six human recombinant CYPs (CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4) identified CYP2C9 and CYP1A2 as the major CYPs responsible for the generation of hydroxylated and carboxylated metabolites of JWH-018 and AM-2201. The contribution of CYP2C19, 2D6, 2E1, and 3A4 in the hepatic metabolic clearance of these synthetic cannabinoids was minimal. These findings are further supported by the results of another investigation that observed a concentration-dependent inhibition of JWH-018 and AM-2201 oxidation in human liver microsomes by the CYP2C9- and 1A2-selective chemical inhibitors sulfaphenazole and α-naphthoflavone, respectively [116]. The study of Holm *et al*. [100] was focused on the elucidation of CYP enzymes involved in the oxidative metabolism of N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (AKB-48, also known as APINACA). Metabolite formation was screened using a panel of nine recombinant CYPs (CYP1A2, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4) and compared the metabolites formed to human liver microsomal incubations with specific inhibitors against CYP2D6, 2C19, and 3A4, respectively. The results demonstrate that CYP3A4 is the major

CYP responsible for the oxidative metabolism of AKB-48, preferentially performing the oxidation on the adamantyl moiety of the compound. Such detailed data are not available for other SCs. However, SCs are structurally diverse and the involvement of other CYPs and non-CYP enzymes in phase I biotransformations is likely. This was recently demonstrated for the quinolineindole synthetic cannabinoids PB-22, 5F-PB-22, and BB-22, where carboxylesterase 1 hydrolyzes an ester bond [96]. In addition, carboxylesterase 1 also hydrolyzes the primary amide group of two other synthetic cannabinoids, AB-PINACA and AB-FUBINACA [96].

Based on the recent evidence, synthetic cannabinoids are extensively metabolized in phase I and phase II biotransformation reactions. CYPs are involved in phase I metabolism. Oxidative metabolism forms preferably mono-, di-, and tri- hydroxylated, carboxylated, and N-dealkylated metabolites. Unlike Δ^9-THC metabolism, several SC metabolites retain a high affinity for the CB1 and CB2 receptors and exhibit a range of intrinsic activities. The majority of phase II SC metabolites are glucuronides.

3. CANNABINOIDS AS REGULATORS OF CYP METABOLIC ACTIVITY

The therapeutic use of cannabis, its extracts and synthetic cannabinoids together with the pleiotropic regulatory activity of the endocannabinoid system and the role of CYP in the metabolism of cannabinoids raises the question of drug-drug interaction with co-administered medicines. The decrease in the metabolic activity of individual CYPs can increase the plasma levels of their substrates, and symptoms of toxicity could appear. In the opposite direction, increased CYP activity will decrease the efficacy of its substrates, and can lead to the failure of a therapy.

The interactions between cannabinoids and CYPs could be simply caused by the competition of two substrates at the same CYP protein. Nevertheless, the huge metabolic capacity of CYP and involvement of alternative metabolic pathways decreases the clinical importance of such drug-drug interplay. The second possibility involves the direct interaction of cannabinoids with the CYP protein in a non-competitive (allosteric) manner, which usually leads to enzyme inactivation or a slowdown of the metabolic reaction. On the other hand, the possibility of allosteric induction for some substances was also described [117]. The final possibility for how cannabinoids can influence the CYP-mediated metabolism of co-administered drugs is by targeting the expression of CYP genes. This possibility is likely, due to the involvement of the endocannabinoid system in many physiological functions, including some metabolic pathways [118, 119], and its interaction with many other neuronal systems and circuits which might also be involved. Therefore, the results of research focused on the direct interaction of cannabinoids with CYP enzymes and the influence of cannabinoids on the metabolic activity assessed after systemic administration or in models using living cells with intact signal pathways could give different results and are described separately.

3.1. Direct Interactions of Cannabinoids and CYP Proteins

Rimonabant was tested for the ability to bind to CYPs in the model of human liver microsomes (HLM). Approximately 19 % of the metabolites produced were covalently bound to CYPs [120]. Therefore it is not surprising that a mechanism-based inhibition of CYP3A4 and decrease in the metabolic activity over 70 % was described. A search for other CB1 antagonists for possible therapeutic use led to the synthesis of aminopyrazine CB1 inverse agonists. The chlorine in the para position of the 5-phenyl ring was found to be responsible for the inhibition of CYP3A4 and its substitution with a trifluoromethyl moiety did not change the potency at the CB1 receptor, increased aqueous solubility, and decreased potential for CYP3A4 inhibition [121]. The negative psychotropic effects of rimonabant could be eliminated with analogues not crossing the blood-brain barrier but with the effect on peripheral CB

receptors. LH-21, a CB1 antagonist with poor brain penetration, is similar to rimonabant in both its anorectic affect and also CYP inhibiting activity [122]. It inhibits the metabolic activity of CYP3A4, 2C9, and 2D6 with the IC_{50} of 1.62, 8.14, and >105 μM, respectively. The inhibition was weaker in comparison to the control inhibitors ketoconazole, sulfaphenazole, and quinidine. LH-21 is reported to be a moderate inhibitor of CYP3A4 and CYP2C9 and weak inhibitor of CYP2D6.

Ashino *et al*. described the inhibition of the CYP1A2 metabolic activity of different synthetic cannabinoids with the indole structure moiety in the model of mouse liver microsomes [123]. MAM-2201 and JWH-019 with the naphtoylindole structure were the most potent inhibitors, and decreased the activity to 47.7 % and 64.3 % of the control values at a concentration of 100 μM. Most of the adamantoylindole derivatives inhibited the activity weakly (up to a 10 % decrease) except for STS-135, which exhibited an inhibition comparable to naphtoylindole ligands. The last molecule tested with the tetramethylcyclopropylindole core exhibited moderate inhibitory activity with a decrease to 73.4 % of the control values at a 100 μM concentration.

$Δ^9$-THC, CBN, and CBD are the most studied substances of the phytocannabinoids group in terms of CYP interactions. All of them competitively inhibit the CYP1A enzyme family, but with different strengths [124]. The most potent inhibitor of CYP1A1 is CBD followed by CBN, while the inhibition of CYP1A2 and CYP1B1 was stronger after CBN treatment. All the enzymes were inhibited by $Δ^9$-THC less potently, with a low selectivity for individual CYP1 enzymes. The subsequent studies revealed the pentylresorcinol [125] or methylresorcinol [126] structures to be important for the direct inhibition of CYP1A1. The same moiety is probably involved in the potent CYP2B6 inhibition by CBD [127], while $Δ^9$-THC and CBN have a weaker effect. All of the substances decreased the activity in a mixed fashion in comparison to the inhibition of CYP2A6, which is non-competitive, and the inhibition potency of all three phytocannabinoids was weaker than the inhibition of CYP2B6. Similar results were obtained with CYP2C9, which was also inhibited by all three substances [128]. The strongest inhibition was reported for CBN, followed by $Δ^9$-THC and CBD. The same substances were incubated with rat liver microsomes, and the 16α- and 2α–hydroxylation of testosterone was assessed [129]. The reaction is mediated by CYP2C11, which is considered to be the counterpart of human CYP2C9 [130]. However, the results are different from those obtained in the human studies. The inhibition was only detected in CBD-treated samples, while $Δ^9$-THC and CBN did not influenced the enzyme activity. Both CBN and $Δ^9$-THC decreased the omeprazole 5-hydroxylase activity, indicating their inhibitory effect on CYP2C19 [131], and at least one of the free phenolic groups and pentyl side chain are the structural determinants of this effect. The activity of CYP2D6 is again most sensitive to the effect of CBD [132]. Its IC_{50} values were 2-4 times lower than those of $Δ^9$-THC and CBN. The CYP2D6 inhibition potency of these two is similar. Similarly to the influence on the enzymes of the CYP1A family, CYP3A enzymes are differentially sensitive to the effect of the major phytocannabinoids. CBD inhibited the activity of CYP3A4 and CYP3A5 most potently, while the influence of all three substances on the activity of CYP3A7 was comparable [133]. The inhibition of 3A4 can also be indirectly evidenced by the suppression of cyclosporine A metabolism in both mouse and human liver microsomes preincubated with CBD [134]. The effect of phytocannabinoids on the activity of 17α-hydroxylase (CYP17) was tested in the model of rat testis microsomes. However, CBD was the most potent inhibitor of CYP enzymes in most of the documented experiments, its inhibitory effect on the CYP17 activity was the weakest, and required IC_{50} concentrations over 290 μM [135]. On the other hand, $Δ^9$-THC and CBN inhibited the enzyme's activity with EC_{50} values of 42.8 μM and 32.9 μM, respectively. The inhibition of individual CYPs by phytocannabinoids is in accor-

dance with the older data obtained with less selective CYP substrates [136-138].

The clinical relevance of the presented data is questionable, due to the high concentrations of the tested drugs used in *in vitro* studies and their correspondence to plasma levels reached when phytocannabinoids are used therapeutically or abused. When a marijuana cigarette (15.8 mg $Δ^9$-THC) is smoked, the peak plasma concentrations of $Δ^9$-THC is reported to be only 268 nM [139]. Similarly after CBD and CBN (20 mg) administration by smoking a cigarette, the levels reached 363 nM and 406 nM, respectively [140, 141]. Moreover, the plasma levels of synthetic $Δ^9$-THC dronabinol reached a nanomolar concentration when administered in the recommended therapeutic doses [124]. The review of inhibition constants (Ki) values are presented in Table **5**. It is obvious that clinically relevant inhibition of CYP by phytocannabinoids is likely for enzymes of the CYP1 family with CBN and for the CYP1A1, 2B6, 2C19, and 3A5 enzymes with CBD. The inhibition of CYP enzymes by THC is probably too weak to cause a clinically significant interaction with the co-administered drugs.

3.2. The Influence of Cannabinoids on CYP Metabolic Activity – *In vivo* and Cell Culture Models

The possible discrepancy in the results of direct interaction experiments and the systemic administration of drugs can be demonstrated in the work of Bornheim *et al*. [142]. Different analogues of THC were tested for both direct interaction with naïve mouse liver microsomes, and microsomes sampled 2 hours after the systemic administration of THC analogues to mice. While in the direct interaction part of the study, some of the tested substances inhibited the activities of CYP3A and CYP2C, the same molecules produced no effect after systemic drug administration.

An important factor influencing the result of the study is the duration of drug pre-treatment before the activity is assessed. The results after a single dose of a drug and after the repeated administration can be different. After the repeated administration of a drug, higher values of plasma/tissue concentrations can be reached than with a single dose. Moreover, there is probably interplay between cannabinoids and endocannabinoid CB receptors, which can lead to changes in signal pathways including CYP liver regulation mechanisms. The subsequent change could therefore be time-dependent, such as for instance the induction of the enzyme activity by the mechanism of increased gene transcription, and *de novo* protein synthesis usually takes at least several hours from the drug administration. An example can be found in the study concerning the effect of the synthetic cannabinoid receptor agonist CP 55,940 on CYP activity in rats [143]. The only parameter that changed after a single intraperitoneal dose of the drug (0.4 mg/kg) was an increase in the oxygen consumption by the brain and liver. However after 11 days of treatment with the same dose of the substance the increased brain and hepatic mitochondrial respiration disappeared, and the P-450 reductase, benzo(A)pyrene hydroxylase, and ethoxycoumarin deethylase activities as well as the protein content of the liver microsomes were increased.

The results of the *in vivo* experiment undoubtedly also depend on the experimental model used. When CP 55,940 was administered to mice (intraperitoneally, 0.5 mg/kg/day) for 5 or 24 days, the microsomal protein content was decreased after the latter type of administration [144], in contrast to the previous results with the same substance in rats. Nevertheless, the activity of CYP2E1, measured as p-nitrophenol oxidation, was unaffected. These data correspond with the results of Yang *et al*. from HepG2 cells incubated with a natural CB receptor agonist CBD [145]. Other researchers reported an increase in the expression of CYP2E1 and CYP2C6, together with an increased amount of total CYP hepatic content in mice after a single dose or repeated administration of hashish [146].

Table 5.   Inhibition of CYP metabolic activity *in vitro* [124, 125, 127-129, 131-133, 135].

	1A1	**1A2**	**1B1**	**2A6**	**2B6**	**2C9[1]**	**2C11[2]**	**2C19**	**2D6[1]**	**3A4**	**3A5**	**3A7**	**17[3]**
CBD	0.16[C]	2.69[C]	3.63[C]	55.0[N]	0.69[M]	5.6[C]	19.9 - 21.6[C]	0.793[M]	2.42	1.0[C]	0.195	12.3[C]	124.4[M]
Δ^9-THC	4.78[C]	7.54[C]	2.47[C]	28.9[N]	2.81[M]	1.5[M]	none	1.93[M]	17.1[A]	>50[A]	35.6[A]	30.3[A]	15.9[M]
CBN	0.54[C]	0.08[C]	0.15[C]	39.8[N]	2.55[M]	0.93[C]	none	no data	12.3[A]	>50[A]	>50[A]	23.8[A]	4.5[M]

[A]Half maximal inhibitory concentration (IC$_{50}$) in μM. Other reported values are inhibition constants (Ki) in μM; [M]Mixed type of inhibition; [C]Competitive type of inhibition; [N]Non-competitive type of inhibition; Enzymes are recombinant human proteins, if not indicated different; [1] Human liver microsomes.; [2] Rat liver microsomes; [3] Rat testis microsomes.

The variability in the experimental design thus leads to a high variation in the results obtained. However, if the data from the experiments with the same designs are compared, the variability disappears. The first data regarding the influence of phytocannabinoids on the activity of CYP in the animal models were homologous. An extract from cannabis prolonged the sleeping time of mice treated with the CYP substrate pentobarbitone, documenting the inhibition of its metabolism [147]. Similar results were obtained with CBD [148], the most studied cannabinoid in terms of CYP interactions. Moreover, CBD inhibited the enzyme activities of various more or less selective CYP substrates including p-nitroanisole O-demethylase [137], aniline hydroxylase [137], hexobarbital hydroxylase [149], erythromycin N-demethylase [149], 6β-testosterone hydroxylase [149], and amino-pyrine N-demethylase [150].

The influence of CBD on single CYPs was evaluated in a number of studies. CBD administered to mice at a dose of 120 mg/kg inactivated CYP2C and CYP3A proteins by covalent binding after a single dose [151]. Whereas after repeated administration of the same dose, the expression of the mRNA of these two enzymes increased as well as the protein content, while the activity remained unchanged [152], probably because of the inactivation reported previously. Similarly, in rats CBD decreased the total CYP hepatic content after repeated administration [153]. Further research revealed decreased activities of CYP17 and CYP2C [154]. The influence of CBD on CYP2C activity is probably also sex-dependent [155].

The most recent results from human cell cultures described the induction of CYP1A enzymes by marijuana smoke [156, 157], Δ^9-THC [157-159], and CBD [159], probably mediated by the aryl hydrocarbon receptor (AhR) [157, 159].

One of the great advantages of animal models is the possibility of studying the effects of prenatal exposure to drugs. Maternal exposure to Δ^9-THC, CBD, and CBN increased the levels of hepatic CYP content, whereas postnatal exposure had the opposite effect in male rat offspring [160].

3.3. Drug-Drug CYP-Mediated Interactions of Cannabinoids

The interspecies differences in the CYP system [161] creates a great barrier to the clinical approximation of data obtained from animals. Nevertheless, in most preclinical studies either with Δ^9-THC or CBD, the results correspond to *in vitro* experiments, and generally these cannabinoids are reported to be CYP inhibitors. CBD significantly decreased the metabolism of CYP substrates, e.g. cocaine [162], anandamide [163], cyclosporine A [134], or THC [162, 164, 165]. However, the dose of CBD necessary to evoke the effect was 30 mg/kg in mice, which is higher than any dose of CBD used in clinical practice.

Although there is enough evidence of the influence of cannabinoids on the total hepatic amounts of CYPs and their activities from preclinical studies, the clinical data on the topic are scarce. The risk of interaction is significantly dependent on the dose administered. Rimonabant (40 mg/day for 8 days) did not affect the steady-state concentration of co-administered digoxin, midazolam, warfarin,

nicotine or oral contraceptives [166]. The effect of medicinal cannabis (Bedrocan®), containing 18 % Δ^9-THC and 0.8 % CBD administered for 15 days, on the levels of irinotecan and docetaxel were tested in oncologic patients [167]. Similarly, no significant change in the clearance or exposure to the monitored drugs was observed. Finally, the summary of medicinal product characteristics of the synthetic Δ^9-THC and CBD mixture (Sativex®) declares that no interactions with CYP3 substrates are expected [168]. It has to be stressed, that these results describe the risk of interaction of low doses of CBD. Nadulski *et al.* tested the effect of 5.4 mg of CBD on the pharmacokinetics of 10 mg of Δ^9-THC [169] and concluded that the inhibitory effect of CBD on CYP in this dose is small compared to the variability of CYP activity caused by other factors. This conclusion could be generalized for the clinical use of CBD at doses of up to 5 mg per day. On the other hand, higher doses of CBD in the range 8-25 mg/kg/day were described to markedly inhibit the metabolism of hexobarbital [170] or clobazam [171], both CYP3A4 substrates. The interaction potential of the higher doses of CBD with CYP3A4 substrates is therefore clinically relevant.

4. POSSIBLE INVOLVEMENT OF ENDOCANNABINOID SYSTEM IN THE REGULATION OF CYP EXPRESSION AND CYP METABOLIC ACTIVITY IN THE LIVER

The regulation of CYP metabolic activity is complex in nature, including many endogenous and exogenous factors determining the actual amounts of enzymes and their catalytic activities. Besides the exogenous ones, genetic polymorphisms, and the role of hormones are known to be endogenous factors regulating the expression and activity of CYPs. Recently, the involvement of some neuronal systems was reported [172-174]. The regulatory role of the endocannabinoid system raises the question of its participation in this process, too. Here, we hypothesize that the central and peripheral pathways of the endocannabinoid system and interplay between cannabinoid ligands and various receptors are probably involved in CYP regulation.

4.1. The Role of Central Endocannabinoid System in the CYP Regulation

It is known that genes coding for various CYPs are regulated by endogenous hormones, which are under the control of the central nervous system. It has been also shown that changes in the brain dopaminergic, noradrenergic, and serotonergic systems can affect hepatic CYP expression [175]. The central endocannabinoid system modulates neurotransmission at inhibitory and excitatory synapses, and therefore could be also involved in the regulation of CYP activity. Thus the endocannabinoid system and possible interactions with other neuronal systems, its impact on the hypothalamic-pituitary axis (HPA) and on the levels of circulating hormones are reviewed.

4.1.1. The Brain and Endocannabinoid System

Most central cannabinoid effects are mediated by the CB1 receptors widely expressed throughout the brain, where they are the most abundant in regions controlling a number of key functions [30, 176]. Therefore, CB1 receptors are present at a high density in the basal ganglia, frontal cortex, hippocampus, and cerebellum, and at a

moderate/low density in the nucleus accumbens, hypothalamus, and amygdala [177].

The predominant localization of CB1 receptors at the presynaptic terminals of neurons plays an important regulatory role, because they can influence the release of a number of different neurotransmitters [178, 179]. The postsynaptic localization of CB1 receptors has been also observed, but only rarely [177]. The endocannabinoids are synthesized and released by postsynaptic neurons, and they act as retrograde neuronal messengers at presynaptic CB1 receptors. The activation of CB1 receptors by endocannabinoids suppresses the presynaptic release of γ-aminobutyric acid (GABA), glutamate, acetylcholine, serotonin, and noradrenaline [179].

Another reason for the increased complexity of endocannabinoid signaling is the evidence that CB1 receptors form heteromers with a variety of other GPCRs [180]. CB1 receptors can form functional heterodimers with μ-opioid receptors [181], orexin-1 receptors [182, 183], adenosine A_{2A} receptors [184, 185], serotonergic (mainly 5-HT$_3$) receptors [186, 187], or dopaminergic D$_2$ receptors [188]. Another possible interaction between the dopaminergic and endocannabinoid system is indirectly via the GABAergic [189, 190] and glutamatergic system [191, 192].

Endocannabinoid signaling in the brain may influence liver CYP activity, but the signal has to be somehow mediated from the CNS to the periphery. It is known that such signal transduction could be found in hormones released from HPA and leading to subsequent changes in the hormonal levels released from peripheral organs. Hormones influence not only hepatic glucose or lipid metabolism, but also the expression of genes coding for different CYP liver enzymes [193-196].

Dopaminergic pathways which could possibly contribute to the release of hormones are the mesolimbic and the tuberoinfundibular pathways. It was reported that stimulation of the dopaminergic system increases growth hormone (GH) [197, 198], adrenocorticotropic hormone (ACTH), and corticosterone levels [199, 200]. In contrast, the levels of thyroid-stimulating hormone (TSH) after activation of the dopaminergic system were decreased [198, 201]. The ability of the brain dopaminergic system to affect liver CYP expression by altering the levels of pituitary hormones was first reported in the studies of Wójcikowski *et al.* [174, 202]. Dopaminergic D$_2$ receptors were identified to be involved in the regulation of hormones and liver CYP enzymes in the mesolimbic pathway [174].

Noradrenaline is one of the main neurotransmitters controlling the release of GH [172, 203]. It also controls the production and release of corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) [204]. The release of somatostatin is regulated by noradrenaline, among other neurotransmitters and neuropeptides. It was reported that a damage of the noradrenergic innervation in the arcuate nucleus (ARC) or periventricular hypothalamic nucleus (PVN) proved an opposite effect on the regulation of CYP expression [172]. This can be explained by the fact that destruction of the noradrenergic innervation in the ARC leads to a decrease of the noradrenaline level and also to a decrease of the level of GH. While destruction of the noradrenergic innervation in the PVN causes a decrease of the level of noradrenaline, but the plasma concentration of GH are increased probably due to the decreased secretion of somatostatin. The involvement of the noradrenergic system in CYP regulation was confirmed by Kot *et al.* [205]. Again the hormones were identified to be the mediators of the effect from the brain to the liver. The same author reported the serotonergic system to also take part in CYP neurohumoral regulation [173, 206, 207].

The influence of exogenous cannabinoids on the secretion of pituitary hormones has been known for a long time, but the role of endocannabinoids in the neuroendocrine system is not fully understood yet [208, 209]. Based on available studies, two options for

how endocannabinoids influence HPA are suggested: (i) a direct effect mediated by endocannabinoids receptors and/or (ii) an indirect effect when cannabinoids change the activity of the endocannabinoid system and this modulates the activity of other neuronal systems controlling HPA. CB1 receptors are expressed in various regions of the brain, and were also detected in the hypothalamus and pituitary gland [210, 211]. Many studies describe the influence of cannabinoids or endocannabinoids on the levels of CRH, GH, TSH, prolactin (PRL), and luteinizing hormone (LH), but their findings are often contradictory [208, 212-216]. It seems that the main structure of endocannabinoid influence on neuroendocrine functions is the hypothalamus, where they act as retrograde messengers activating the CB1 receptors. Importantly, it was revealed that endocannabinoids are involved in the rapid negative feedback actions of glucocorticoids (GCs) in parvocellular neurons of the hypothalamic paraventricular nuclei (PVN) containing CRH. GCs, after binding to glucocorticoid (GR) receptors localized in the PVN, activate the postsynaptic GPCRs. This leads to the synthesis and release of endocannabinoids. These endocannabinoids act as retrograde messengers to the CB1 receptors located at presynaptic glutamate terminals and inhibit glutamate release [217]. These findings thus provide a possible mechanism for the rapid feedback inhibition of the hypothalamic pituitary adrenal axis by GCs. Moreover, the CB1 receptors and endocannabinoids are found throughout all of the extrahypothalamic sites that regulate PVN neuronal activation, such as the hippocampus, prefrontal cortex, amygdala, bed nucleus of the stria terminalis, and midbrain monoaminergic nuclei, such as the locus coeruleus and dorsal raphe [218]. These brain regions are the most likely sites of interaction between the endocannabinoid system and other nervous systems [190, 219-221].

Once the CB ligands directly change the activity of the endocannabinoid system or indirectly the activity of other neurotransmitters and the HPA is changed, hormones start the signal transduction towards the liver (Fig. **9**). The regulation of liver CYP enzymes by hormones involves binding the hormone to the nuclear receptor and translocation of the ligand-receptor complex into the cell nucleus. The expression of specific genes including CYP enzymes is activated or inhibited. GH, GCs, and TSH are ligands of nuclear receptors able to change the expression of CYP genes [222-224]. The influence of hormones on the transcription activity of CYP genes is described at a glance in Table **6**.

4.2. The Involvement of Peripheral Cannabinoid Receptors in the CYP Regulation

When administered systemically, cannabinoids are able to target both the regulation centers in the brain and the receptors in peripheral tissues including the liver. Except for the direct interaction with CYPs (see chapter 3.1.) there is a possibility of the influence of cannabinoids on the receptors of target cells. The receptor specificity of cannabinoids is broad due to their high structure variability, therefore there are many receptors which might be activated or inhibited with regard to the properties of the ligand. Here we describe the evidence of interaction between cannabinoids and peripheral receptors involved in the signal pathways of CYP regulation and the role of these receptors in CYP regulation.

The key ligand-activated transcriptional regulators of CYPs are the pregnane X receptor (PXR), constitutive androstane receptor (CAR), retinoid X receptors (RXRs), peroxisome proliferator-activated receptors (PPARs), glucocorticoid receptors (GRs), and aryl hydrocarbon receptor (AhR) [222].

Briefly, PXR plays a key role in the regulation of the CYP2B6, CYP2C, CYP2A6, CYP3A, and CYP4F12 genes [238-245]. Agonists of PXR induce these CYP enzymes. In addition to the induction of CYP enzymes, PXR activation also represses CYP7A1 expression as a protective feedback in response to the accumulation of bile acids in the liver [224, 246].

Table 6.   Hormonal regulation of CYP genetic transcription [175, 193-195, 225-237].

GC	T3	GH	PRL
↑2A6[b]	↓1A1/2[a]	↑1A2 [b]	↓2C11[a]
↑2B6 [b]	↓2A1/2[a]	↑2A1[a]	↓2D1[a]
↑2B8 [b]	↓3A1/2[a]	↑2C7[a]	↓3A4[a]
↑2C8 [b]	↓3A4 [b]	↑2C12[a]	
↑2C9 [b]	↑7A1 [b]	↑2C11[a]	
↑2C19 [b]		↓2C19 [b]	
↑3A4 [b]		↑2D1[a]	
↑3A5 [b]		↓2E1[a]	
		↑3A1[a]	

[a]Data from preclinical experiments on rats. [b]Data obtained from human cell lines.

Fig. (9). Endocannabinoid system and cannabinoids in the regulation of CYP activity.

The activation of CAR is linked to the induction of CYP2B [247]. There is signaling cross-talk between PXR and CAR. These receptors control the expression of human CYP2A6 [238], CYP2B6 [248], CYP2C8/9 [230, 249], CYP2C19 [232], CYP3A4 [250], CYP3A5 [251], and CYP3A7 [252].

AhR is not a true nuclear receptor; it belongs to a family of transcription factors that contain the basic-helix-loop-helix and Per-ARNT-Sim domains. AhR requires the AhR nuclear translocator as its heterodimerization partner to be translocated into the nucleus and turn on the CYP gene transcription. AhR triggers the expression of CYP1A and CYP1B [222, 223].

The PPAR family currently has four members - PPARα, PPARβ, PPARγ, and PPARδ - which differ in their localization [253]. After activation by appropriate ligands, PPARs bind as heterodimers with RXR (PPAR/RXR) to peroxisome proliferator response elements. It has been shown that CYP2B, CYP3A, and

Table 7.   Cannabinoid ligands of nuclear receptors [157, 159, 257, 258].

PPARα	PPARγ	PPARδ	AhR
N-oleoylethanolamine	AEA, 2-AG	N-oleoylethanolamine	Δ^9-THC
palmitoylethanolamide	NADA, Δ9-THC		CBD
virodhamine	ajulemic acid		
noladin ether	CP 55,940, HU-210		
	WIN 55,212-2		

CYP4A are activated by PPAR, and CYP2C11 is suppressed by the PPAR agonist [254, 255].

CYP3A and CYP2B proteins are distinctly regulated by GRs [256]. GRs may induce the expression of a gene that does not contain GRE in its promoter. This is exerted by indirect "trans-regulation". Moreover, GRs contribute to functional cross-talk between the PXR, CAR, AhR, and RXR signaling pathways [222, 226].

RXR is directly or indirectly involved in the regulation of many enzymes and can be considered to be a limiting factor in the overall regulation of hepatic gene expression patterns [253].

Some endocannabinoids, phytocannabinoids, and synthetic cannabinoids are ligands of different PPARs and the AhR. An overview of the selectivity of drugs to individual receptors is given in Table **7**.

5. CONCLUSION

Cannabinoids are a structurally and pharmacodynamically heterogeneous group of drugs with great potential for therapeutic use in the near future. The involvement of CYP in their metabolism is clear and indisputable, whereas the clinical significance of their drug-drug interactions has yet to be evaluated in detail. These interplays may have various mechanisms from the direct interaction of two substrates at the same enzyme, through different types of antagonism with the CYP protein to the activation of various receptors and changes in hormonal levels leading to an alteration in the expression of CYP genes. The latter describes the suggested involvement of the endocannabinoid system in the central regulation of hepatic CYP activity. This hypothesis is based on indirect evidence, and could be proved or refuted by further studies. Moreover, we suggest that changes in liver CYP metabolic activity could be time-dependent. Our idea is based on the signal transduction from the brain to the liver via hormones which are under HPA control, and a negative feedback mechanism plays a significant role here. Therefore, the hormonal changes induced by drug administration can be short-lived as well as the changes in CYP activity. To the best of our knowledge, the factor of time was not studied in any of the previous works focusing on the role of the central nervous system in the regulation of liver CYP activity.

LIST OF ABBREVIATIONS

Δ^9-THC	=	(-)-trans-Δ^9-tetrahydrocannabinol
2-AG	=	2-arachidonoylglycerol
5-HT	=	5-hydroxytryptamine, serotonin
AA	=	Arachidonic acid
ABHD	=	α,β-hydrolase domain
ACEA	=	Arachidonyl-2′-chloroethylamid
ACPA	=	Arachidonyl-cyclopropylamide
ACTH	=	Adrenocorticotropic hormone
AhR	=	Aryl hydrocarbon receptor
ARC	=	Arcuate nucleus
CAR	=	Constitutive androstane receptor
CBC	=	Cannabichromene
CBD	=	Cannabidiol
CBG	=	Cannabigerol
CBN	=	Cannabinol
CYP	=	Cytochrome P450
DA	=	Dopamine
diHETE	=	Dihydroxyeicosatrienoic acids
EET	=	Epoxyeicosatrienoic acids
EET-EA	=	Epoxyeicosatrienoic acid ethanolamide
EET-G	=	Epoxyeicosatrienylglycerol derivatives
FAAH	=	Fatty acid amide hydrolase
GABA	=	γ-aminobutiric acid
GCs	=	Glucocorticoids
GH	=	Growth hormone
GPCRs	=	G-protein coupled receptors
GRs	=	Glucocorticoid receptors
HEET-EA	=	20-hydroxy-epoxyeicosatrienyl ethanolamide
HETE	=	Hydroxyeicosatrienoic acid
HETE-DA	=	Hydroxyeicosatetraenyl dopamine
HPA	=	Hypothalamic-pituitary axis
LH	=	Luteinizing hormone
NA	=	Noradrenaline
NADA	=	N-arachidonoyldopamine
PPARs	=	Peroxisome proliferator-activated receptors
PRL	=	Prolactin
PVN	=	Paraventricular nucleus
RXRs	=	Retinoid X receptors
SC	=	Synthetic cannabinoid
T3	=	Triiodothyronine
T4	=	Thyroxine
TSH	=	Thyroid-stimulating hormone

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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5.2.2 The effect of oleamide on the activity of rat and human P450

As was mentioned above, xenobiotics used or intended to be used as therapeutic agents have to be checked for their interactions with P450. Therefore the interactions of rimonabant, THC, or cannabidiol with P450 are known. Rimonabant and its peripherally acting analogue LH-21 are both *in vitro* inhibitors of CYP3A4 (Alonso et al., 2012; Wustrow et al., 2008). Also other synthetic cannabinoids act as P450 inhibitors in direct interactions studies (Ashino et al., 2014). Similarly, herbal cannabinoids inhibit various P450 enzymes with various potencies *in vitro* (Zendulka et al., 2016). There is limited data on the effect of cannabinoids on P450 *in vivo*, and moreover published studies give divergent results. Both a decrease and increase in the activity/amount of P450 can be found (Bornheim et al., 1993; Sheweita, 2003).

In our study, we described the effect of the endocannabinoid oleamide on selected P450 enzymes in different experimental models using rat and human liver microsomes. We focused on the main P450 enzymes involved in drug metabolism: rat CYP1A2, CYP2A, CYP3A, CYP2B, CYP2C6, CYP2C11, and CYP2D2 and human CYP1A2, CYP2B6, CYP2C9, and CYP3A4.

The oleamide was administered intraperitoneally, dissolved in propylene glycol, to male Wistar albino rats at doses of 0. 1, 1 or 10 mg/kg/day for 7 days. The RLM were prepared by differential ultracentrifugation and the amounts and activities of individual P450 enzymes were assessed. Spectrophotometric methods were used to measure the total protein and total P450 content. Testosterone, dextromethorphan, phenacetin, and diclofenac were used as probe substrates for measuring metabolic activities. Animals administered the highest dose of oleamide exhibited a significantly decreased content of both total protein and P450 in RLM. The metabolic activities of CYP1A, CYP2B, CYP2C11, and CYP2D2 were significantly decreased, and with CYP3A the decrease was not significant, even though the activity dropped to 77 % of the control value. The activities of CYP2A and CYP2C6 were unchanged.

Further tests were performed to determine the underlying mechanism of this effect. Oleamide was tested for a possible direct interaction with RLM isolated from the naïve animals, the levels of hormones involved in the regulation of P450 activity was measured, and the amounts of enzymes were quantified with western blotting. Oleamide was determined to be an inhibitor of rat CYP1A2, CYP2D2, and CYP2C6 *in vitro*. CYP1A2 and CYP2D2 were inhibited weakly, while the effect on CYP2C6 was stronger, and so the Ki was calculated. The values of Ki varied from 28.7 to 71.6 µM with respect to the model of inhibition used. Nevertheless, direct inhibition of the enzyme could not explain the observed effect *in vivo*, as the isolated RLM were thought to be oleamide free and CYP2C6 was not influenced *in vivo*. The levels of hormones (corticosterone, prolactine, and triiodothyronine) were

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The oleamide was administered intraperitoneally, dissolved in propylene glycol, to male Wistar albino rats at doses of 0. 1, 1 or 10 mg/kg/day for 7 days. The RLM were prepared by differential ultracentrifugation and the amounts and activities of individual P450 enzymes were assessed. Spectrophotometric methods were used to measure the total protein and total P450 content. Testosterone, dextromethorphan, phenacetin, and diclofenac were used as probe substrates for measuring metabolic activities. Animals administered the highest dose of oleamide exhibited a significantly decreased content of both total protein and P450 in RLM. The metabolic activities of CYP1A, CYP2B, CYP2C11, and CYP2D2 were significantly decreased, and with CYP3A the decrease was not significant, even though the activity dropped to 77 % of the control value. The activities of CYP2A and CYP2C6 were unchanged.

Further tests were performed to determine the underlying mechanism of this effect. Oleamide was tested for a possible direct interaction with RLM isolated from the naïve animals, the levels of hormones involved in the regulation of P450 activity was measured, and the amounts of enzymes were quantified with western blotting. Oleamide was determined to be an inhibitor of rat CYP1A2, CYP2D2, and CYP2C6 *in vitro*. CYP1A2 and CYP2D2 were inhibited weakly, while the effect on CYP2C6 was stronger, and so the Ki was calculated. The values of Ki varied from 28.7 to 71.6 µM with respect to the model of inhibition used. Nevertheless, direct inhibition of the enzyme could not explain the observed effect *in vivo*, as the isolated RLM were thought to be oleamide free and CYP2C6 was not influenced *in vivo*. The levels of hormones (corticosterone, prolactine, and triiodothyronine) were

measured to check the probable relation between the central effect of oleamide and the liver. Oleamide did not influence the levels of hormones in treated rats, and this possible mechanism can be also excluded. Finally, the amounts of P450 in the isolated microsomes were again decreased in terms of CYP1A2, CYP2B, CYP2C11, and CYP3A1 in animals with the highest dose of oleamide. Together with the fact that the metabolic activity recalculated to the amount of P450 remained unchanged, we concluded that the downregulation of P450 is the mechanism through which oleamide influences P450 enzymes in the rat liver.

Because of known interspecies differences, the effect of oleamide on rat P450 was compared with the effect on human recombinant CYP1A2, CYP2B6, CYP2C9, and CYP3A4 *in vitro*. Additionally, oleamide (1 - 30 µM) was incubated together with primary human hepatocytes and tested for its interaction with human nuclear receptors in HepG2 cells. Oleamide neither changed the activity nor the mRNA levels of the tested P450 in primary human hepatocytes. It also did not influence the metabolic activity of recombinant P450 and neither exhibited agonistic nor antagonistic properties on human CAR, PXR, and AhR nuclear receptors.

The results of the study document that there are differences in the effect of the endocannabinoid oleamide between rat and human P450. The precise mechanism of how oleamide causes the downregulation of rat liver P450 has not been detemined yet. Nevertheless, our study indicates that ECS could be involved in the regulation of P450 activity, as the effect of oleamide is not caused by a simple drug-enzyme interaction.

Dovrtělová G., **Zendulka** O., Nosková K., Juřica J., Peš O., Dušek J., Carazo A., Zapletalová I., Hlaváčová N., Pávek P. Effect of endocannabinoid oleamide on rat and human liver cytochrome P450 enzymes in in vitro and in vivo models. Drug Metabolism and Disposition, 46, 913-923, 2018.

Impact factor: 3.64; Pharmacology & Pharmacy Q1   Citations WoS: 0

Author's contribution: 20%, corresponding author

Effect of Endocannabinoid Oleamide on Rat and Human Liver Cytochrome P450 Enzymes in In Vitro and In Vivo Models

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ABSTRACT

The endocannabinoid system is important for many physiologic and pathologic processes, but its role in the regulation of liver cytochromes P450 (P450s) remains unknown. We studied the influence of the endocannabinoid oleamide on rat and human liver P450s. Oleamide was administered intraperitoneally to rats at doses of 0.1, 1, and 10 mg/kg per day for 7 days. The content and activity of key P450s were evaluated in rat liver microsomes. Moreover, interactions with nuclear receptors regulating P450 genes and serum levels of their ligands (prolactin, corticosterone, and free triiodothyronine) were tested in in vitro P450 inhibition assays. Decreased protein levels and metabolic activities of CYP1A2, CYP2B, and CYP2C11, along with a drop in metabolic activity of CYP2D2, were observed in animals treated with oleamide (10 mg/kg per day). The activities of CYP2C6, CYP2A, and CYP3A and the levels of hormones were not altered. In vitro, oleamide exhibited a weak inhibition of rat CYP1A2, CYP2D2, and CYP2C6. The activities of rat CYP2A, CYP2B, CYP2C11, and CYP3A and human CYP1A2, CYP2B6, CYP2C9, and CYP3A4 were not altered. Oleamide did not interact with human pregnane X, constitutive androstane, or aryl hydrocarbon receptors in reporter gene experiments and did not regulate their target P450 genes in primary human hepatocytes. Our results indicate that oleamide caused the downregulation of some rat liver P450s, and hormones are not mediators of this effect. In vitro oleamide inhibits mainly rat CYP2C6 and is neither an agonist nor antagonist of major human nuclear receptors involved in the regulation of xenobiotic metabolism.

Introduction

The endocannabinoid system (ECS) is an essential regulator of many physiologic processes, such as memory, brain plasticity, thermogenesis, nociception, energy balance, digestion, motility, and fertility (Aizpurua-Olaizola et al., 2017). Therefore, it is not surprising that changes in ECS activity are involved in many pathophysiological processes in the central nervous system, as well as in the peripheral tissues (Pertwee, 2015). The ECS is a signaling system that consists of cannabinoid receptors CB_1 and CB_2; their endogenous ligands (endocannabinoids); and associated proteins for their synthesis, transport, and degradation. Endocannabinoids are metabolized mainly by enzymatic hydrolysis via fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, but some endocannabinoids can also be sensitive to oxidative metabolism by cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes (P450s) (Sugiura et al., 2002; Snider et al., 2010). N-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoyl-glycerol are two of the best studied endocannabinoids (Di Marzo et al., 1998). Apart from these two, oleamide (syn. cis-9,10-octadecenoamide, cis-9-octadecenamide, oleic acid amide) is another molecule of interest because of its role in sleep regulation (Obal and Krueger, 2003). It was first identified in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995). Because of their similar structures, oleamide and anandamide evoke similar effects (Divito and Cascio, 2013) including sleep induction (Herrera-Solis et al., 2010), hypothermia, hypomotility, and analgesia (Smith et al., 1994; Fedorova et al., 2001). Oleamide is an agonist of CB_1 receptors (Leggett et al., 2004). Moreover, it can act as an alternate substrate for FAAH and thus increases the relative half-life and effects of anandamide (Fowler et al., 2001). Synthetically produced oleamide has been used in industry for a long time as a lubricant in the production of plastic materials (Garrido-Lopez et al., 2006) and as a thickener and emulsifier in cosmetics (Flick, 2012).

P450s are the major enzymatic systems of drug metabolism. In particular, the subfamilies CYP3A, CYP2C, CYP2D, CYP2B, and CYP1A are essential for the biotransformation of xenobiotics in humans. It is known that many cannabinoids are not only substrates of P450s, but they can also change the activity of these enzymes. Nowadays, therapeutically used phytocannabinoids

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ANOVA, analysis of variance; CAR, constitutive androstane receptor; CB, cannabinoid; CITCO, (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime); DMSO, dimethylsulfoxide; ECS, endocannabinoid system; ELISA, enzyme-linked immunosorbent assay; FAAH, fatty acid amide hydrolase; HPLC, high-performance liquid chromatography; LBD, ligand binding domain; P450, cytochrome 450; PXR, pregnane X receptor; RLMs, rat liver microsomes.

(Δ^9-tetrahydrocannabinol, cannabidiol) and synthetic cannabinoids (dronabinol and nabilone) are known to modulate the activity of P450s in in vitro and in vivo models (Zendulka et al., 2016). Their main indications in clinical practice include nausea and vomiting associated with cancer chemotherapy, pain, and spasticity (May and Glode, 2016; Keating, 2017). In addition, the ECS is a promising target for new therapeutic strategies for other diseases (e.g., psychiatric disorders, obesity, diabetes, and osteoarthritis (Aizpurua-Olaizola et al., 2017). Thus, its possible role in the regulation of the activity of P450s would be of great interest. Nevertheless, none of the endocannabinoids have been tested for their influence on liver P450s yet.

We focused on oleamide as a candidate endocannabinoid for our study because of its known ability to activate the ECS by various mechanisms (CB_1 receptor and FAAH enzyme). Moreover, the wide use of oleamide in the plastics industry has made it a possible pollutant, and therefore knowledge of its effect on P450s would be beneficial.

The aim of this work was to investigate the influence of the endocannabinoid oleamide on the activities of key rat and human P450s involved in drug metabolism. For this purpose, in vitro P450 interaction assays and an in vivo rat model were used. To obtain additional data about the possible mechanism of oleamide's influence on P450s we also measured the serum concentrations of hormones known to be involved in the regulation of P450 metabolic activity, namely, prolactin, corticosterone, and free triiodothyronine. Finally, the interactions of oleamide with human nuclear receptors that regulate the expression of P450 genes, namely, the pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), and constitutive androstane receptor (CAR), were also tested. The ability to induce their target P450 genes was examined in primary human hepatocytes.

Material and Methods

Chemicals

The following compounds were provided by Sigma-Aldrich (St. Louis, MO): oleamide (CAS no. 301-02-0), prednisone, testosterone, chlorpropamide, phenacetin, diclofenac, 4'-hydroxydiclofenac, acetaminophen, ibuprofen, dextromethorphan, dextrorphan, laudanosine, triethylamine, NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $MgCl_2$, EDTA, KH_2PO_4, Na_2HPO_4, sucrose, NH_4HCO_3, CH_3COOH, NH_3, KCl, bovine serum albumin, dimethylsulfoxide (DMSO), α-naphthoflavone, ketoconazole, fluconazole, ticlopidine, rifampicin, CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime), and 3-methylcholanthrene. Propylene glycol was obtained from Fagron (Olomouc, CZE). The metabolites of testosterone, namely, 2β-, 2α-, 7α-, 6β-, 16α-, and 16β-hydroxytestosterone, were purchased from Steraloids Inc. (Newport, RI). The primary and secondary antibodies, which were used for Western blotting analyses, are described in detail in Table 1. Chemicals and organic solvents for high-performance liquid chromatography (HPLC) analysis were purchased from Lach-ner (Neratovice, CZE) and they were of an HPLC-gradient or LC-mass spectroscopy (MS) grade. Cell culture media, nonessential amino acids, and fetal bovine serum were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA).

Animals

The experiments were carried out on male Wistar albino rats (230 ± 20 g), which were purchased from the Masaryk University breeding facility (Brno, CZE). The rats were housed under standard laboratory conditions (22 ± 2°C room temperature; 55% ± 5% room humidity; 12-hour light/dark cycle). Animals had free access to water and food. All experiments were performed in accordance with Czech act no. 246/1992 and with the approval of both the regional and national Czech Central Commission for Animal Welfare (MSMT-35 473/2012-30).

Experimental Design

After 5 days of acclimatization, the rats were randomly divided into five experimental groups. All animals were treated intraperitoneally for 7 consecutive days. Three groups ($n = 10$/group) were administered oleamide dissolved in propylene glycol at doses of 0.1, 1, and 10 mg/kg per day The doses of oleamide used in our experiment were selected according to preclinical studies in which oleamide evoked various kinds of biologic activities (Fedorova et al., 2001; Huitron-Resendiz et al., 2001). The control group was treated with the appropriate volume of the vehicle (propylene glycol 1 ml/kg, $n = 6$). Because there were no data on the influence of propylene glycol on P450s, a group of animals administered saline (1 ml/kg, $n = 10$) was also used. All animals were sacrificed under ether anesthesia by decapitation 24 hours after the last drug/vehicle administration, and the samples of liver tissue were drawn immediately without any liver perfusion and frozen at $-80°C$.

Preparation of Rat Liver Microsomes and Determination of Total Protein and P450 Content

Rat liver microsomes (RLMs) were isolated from 3 g of liver tissue from each individual animal. Each liver tissue sample was homogenized in 20 mM Tris/KCl buffer (pH 7.4). RLMs were then isolated from the homogenate by differential ultracentrifugation (19,000g, 20 minutes and 2× 105,000g, 60 minutes) in 20 mM Tris/KCl buffer (pH 7.4), including washing with 0.15 M KCl. The final pellet was reconstituted with 0.25 M Tris/sucrose buffer (pH 7.4) to yield 1 ml of RLMs from each gram of isolated tissue and stored at $-80°C$. The total protein content in the microsomal preparations was assessed according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Determination of total P450 content was assessed using a CO difference spectroscopy method according to Omura and Sato (1964). The total protein content and total P450 content are expressed as milligrams of protein per milliliter and nanomoles of P450/ml of RLMs, respectively. RLMs were reconstituted in volume of Tris/sucrose buffer to resemble the original liver tissue content.

TABLE 1

Primary and secondary antibodies used for Western blotting

Antibody	Biologic Source	Species[a]	Catalog No.	Company	Dilution
Anti-CYP1A2	Mouse	m, r, h	sc-53241	Santa Cruz Biotechnology[b]	1:500
Anti-CYP2B1/2B2	Mouse	m, r	sc-73546	Santa Cruz Biotechnology	1:500
Anti-CYP2C11	Goat	r	R-PAP 121	Cypex[c]	1:5000
Anti-CYP3A1	Mouse	m, r, h	sc-53246	Santa Cruz Biotechnology	1:1500
Anti-β-actin	Rabbit	m, r, h, b, p, k	sc-4970	Cell Signaling Technology[d]	1:500
Anti-mouse	Goat		A4416	Sigma-Aldrich[e]	1:5000
Anti-goat	Rabbit		A5420	Sigma-Aldrich	1:5000
Anti-rabbit	Goat		A6667	Sigma-Aldrich	1:5000

[a]m, mouse; r, rat; h, human; b, bovine; p, pig, k, monkey.
[b]Dallas, TX.
[c]Dundee, GBR.
[d]Danvers, MA.
[e]St. Louis, MO.

Determination of P450 Activity in RLM

Assessment of the relative activity of CYP1A2, CYP2B, CYP2C, CYP2D2, and CYP3A enzymes were based on the rate of biotransformation of a specific marker in RLMs with an NADPH-generating system according to the previously described method of Wojcikowski et al. (2008), with a slight modification. The incubation mixture of the final 0.5 ml volume contained a phosphate buffer (50 mM; pH 7.4), EDTA (1.1 mM), NADP (1.2 mM), glucose-6-phosphate (4.4 mM), MgCl$_2$ (3.2 mM), glucose-6-phosphate dehydrogenase (1 U/ml), RLMs (1 mg/ml of total protein content), and a specific marker for determination of the selected P450 enzymes activity. The reaction was stopped after incubation at 37°C in a shaking water bath by the addition of 50 μl of methanol and cooling in ice. The concentrations of markers and their specific metabolites were measured with one of two HPLC systems: a Shimadzu LC-10 (Shimadzu, Tokyo, Japan) with a DAD detector (SPD-M10AVP) and a fluorescence detector (RF 10AXL) or a Dionex UltiMate 3000RS LC (Thermo Fisher Scientific) with a DAD detector. Reaction rates exhibited a linear relationship to the incubation time and protein concentration under the above-mentioned conditions. The relative metabolic activities of all P450s were studied by measuring the rates of P450-specific reactions and were expressed as a molar concentration of the metabolite per minute per milligram of the total protein in RLMs. The specific metabolic activities were also calculated with respect to the total P450 content and are expressed as the molar concentration of the metabolite per minute per nanomolar of total P450 content. The accuracy and precision of the HPLC methods were below 10% of relative standard deviation within the calibration range. The calibration range always included the concentrations of analytes in the measured samples.

Determination of CYP1A2 Activity. The rate of phenacetin *O*-deethylation was used for CYP1A2 activity assessment. The substrate concentration was 300 μM and the incubation period was 20 minutes. After the addition of the internal standard (chlorpropamide) to the incubation mixture, the analytes were extracted from the microsomal suspension with diethyl ether (4 ml, 10 minutes of horizontal vortexing, 1400 rpm), the organic phase was evaporated, and the residue was dissolved in 250 μl of the mobile phase. The following gradient mode of separation was applied: 0:00–5:00 minutes, 90:10 (v/v 0.1% NH$_4$CH$_3$COO/ acetonitrile, pH 4.6); from 5:00 to 6:00 minutes, 35:65; and from 6:00 to the end of the analysis at 8:00 minutes, 90:10. The separation of acetaminophen, phenacetin, and chlorpropamide was achieved using Kinetex, 2.6 μm PFP 100A column (150 × 4.60 mm; Phenomenex, Torrance, CA) thermostatted at 45 ± 0.1°C, at a flow rate of 1.2 ml/min, and UV detection at 245 nm.

Determination of CYP2A, CYP2B, CYP2C11, and CYP3A Activity. Testosterone 7α-, 16β-, 2α-, and 16α-, 2β-, and 6β-hydroxylations were used for the activity assessment of CYP2A, CYP2B, CYP2C11, and CYP3A, respectively. The substrate concentration was 400 μM, prednisone was used as the internal standard, and the incubation time was 15 minutes. The separation conditions for testosterone metabolite determination have been described in detail previously (Dovrtelova et al., 2015).

Determination of CYP2C6 Activity. Diclofenac 4′-hydroxylation was used for measuring CYP2C6 activity, and the substrate concentration was 100 μM. Ibuprofen was used as the internal standard, and the incubation time was 20 minutes. The separation conditions for diclofenac and 4′-hydroxydiclofenac determination have been described in detail previously (Noskova et al., 2016).

Determination of CYP2D2 Activity. Dextromethorphan *O*-demethylation was used for measuring CYP2D2 activity. The substrate concentration was 500 μM, and the incubation time was 20 minutes. After addition of the internal standard (laudanosine) and Na$_2$CO$_3$ (250 μl, 0.5 M) to the incubation mixture, the analytes were extracted from the microsomal suspension with 4 ml of 1-butanol: hexane mixture (1:9). The organic phase was decanted, and 300 μl of 0.01 M HCl was added. After 10 minutes of horizontal shaking at 1400 rpm, the samples were frozen. The organic layer was discarded, and a 5 μl portion of the aqueous phase was injected into the HPLC system. The separation of dextrorphan, dextromethorphan, and laudanosine was achieved in an isocratic separation mode with fluorescence detection according to the method of Zimova et al. (2000).

Western Blotting

Western blot analyses were used to detect the protein expression of CYP2B1/2, CYP3A1, CYP1A2, and CYP2C11 in RLMs. Individual samples were mixed with a loading buffer (0.125 M Tris pH 6.8, 20% glycerol, 10% mercaptoethanol, 4% SDS, 0.004% bromphenol blue), boiled for 5 minutes at 95°C, and separated on a 12% SDS-PAGE gel; 10 μg of RLMs was loaded per lane. After electrophoresis, proteins were electrotransferred (100 V, 75 minutes) onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, DEU), which were blocked with 5% nonfat milk in Tris-buffered saline with 0.08% Tween 20 at room temperature for 1 hour. The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate secondary antibodies for 1 hour at room temperature. The antibodies were dissolved according to Table 1, and the β-actin antibody was used as a loading control for normalization. Where necessary, the membranes were stripped in a stripping buffer (74.3:25:10:0.7 H$_2$O:1 M Tris (pH 7.4):20% SDS:β-mercaptoethanol), washed, and reblotted with another antibody. The intensities of the bands on the membranes were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Watford, GBR) and FUSION SL (Vilber Lourmat, DEU). The Western blots were quantified using the software ImageJ (version 1.48; National Institutes of Health, Bethesda, MD). Statistical analyses were done using at least three repetitions of individual animals of each group.

P450 Inhibition Assay In Vitro

RLMs from drug-naïve control animals, which were pooled from four individuals, were used for a direct inhibition assay in vitro. The incubation mixtures were processed as described in the section *Determination of P450 Activity in R*, except the final volume was 1 ml and the total protein content in RLMs was 0.25 mg/ml. Each sample was prepared in triplicate. After 10 minutes of preincubation with a different concentration of oleamide (see to follow) at 37°C in a shaking water bath, reactions were initiated by the addition of specific markers. The incubation times were 40 minutes (phenacetin and dextromethorphan), 20 minutes (testosterone), and 30 minutes (diclofenac).

First, pilot experiments were performed with single concentrations of the substrates phenacetin (300 μM), testosterone (100 μM), diclofenac (8 μM), or dextromethorphan (28 μM), and three concentrations of oleamide (0.1, 1, and 100 μM). Based on this experiment, we decided to perform only the inhibition assay of CYP2C6 (with diclofenac as a probe substrate). The kinetic parameters of CYP2C6 metabolic activity were determined using Michaelis-Menten kinetics. For construction of the Dixon plot and determination of the inhibition constant (K_i), diclofenac was used at concentrations of 4, 8, and 16 μM, oleamide at 0, 10, 25, 37.5, and 50 μM.

P450 Enzymatic Activity Assays

Human recombinant CYP3A4, CYP2B6, CYP2C9, and CYP1A2 enzymes expressed in a microsomal fraction of P450 cDNA baculovirus-infected insect cells (CYP450-Glo CYP3A4 Assay, CYP450-Glo CYP2B6 Assay, CYP450-Glo CYP2C9 Assay, and CYP450-Glo CYP1A2 Assay; Promega, Hercules, CA) were used to evaluate the interaction of oleamide with these enzymes in vitro according to the manufacturer's protocols. Ketoconazole, ticlopidine, fluconazole, and α-naphthoflavone were used as prototypical inhibitors of CYP3A4, CYP2B6, CYP2C9, and CYP1A2 enzymes.

Nuclear Receptors and Cell Viability Assays

Cell Lines. A HepG2 human Caucasian hepatocyte carcinoma cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, GBR) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 2 mM L-glutamine (Sigma-Aldrich).

Cell Viability Assay. To evaluate the toxicity of oleamide in HepG2 cells, a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol.

Plasmids. The construction of the PXR-responsive p3A4-luc construct containing XREM and basal promoter sequences and pSG5-hourPXR expression construct have been described by Rulcova et al. (2010). The AHR reporter plasmid (pXRE-luc) was described in our previous report (Dvorak et al., 2008). The pRL-TK construct was purchased from Promega. The constructs pCAR-C/VP16 and pCAR-N/GAL4 for the CAR assembly assay have been described in a previous paper of ours (Carazo Fernandez et al., 2015).

Reporter Gene Assays (PXR, AhR). All transient transfection gene reporter assays were performed with LipofectAMINE 3000 transfection
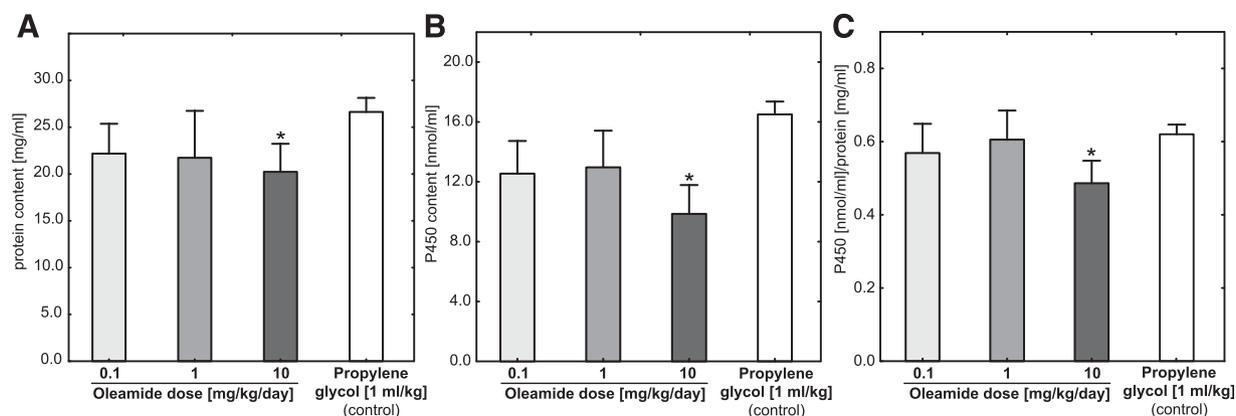
Fig. 1. Changes in total protein content (A), total P450 content (B), and relative content of P450 (C) (nanomoles per milligram of microsomal protein) after systemic administration of oleamide. The total protein content in individual RLMs was assessed according to the method of Lowry et al., and the total P450 content was determined using a CO difference spectroscopy method according to Omura et al. Animals were treated intraperitoneally with oleamide 0.1 ($n = 10$), 1 ($n = 10$), 10 mg/kg per day ($n = 10$), and propylene glycol (control group, $n = 6$). Bars and whiskers express the mean (S.D.). Statistical significance was assessed by Kruskal-Wallis test followed by multiple comparisons of mean ranks (A and C) and one-way ANOVA test followed by Tukey's significant difference test for unequal sample sizes (B). Statistical significance with respect to the control group (propylene glycol) is indicated with *$P \leq 0.05$.

reagent (Invitrogen/Life Technologies, Carlsbad, CA) in HepG2 cells. Briefly, cells were seeded onto 48-well plates and 24 hours later were transfected with a luciferase reporter construct (150 ng/well), PXR expression plasmid (100 ng/well, for PXR experiments), and Renilla reniformis luciferase transfection control plasmid (pRL-TK) (30 ng/well). Then, the cells were stabilized for 24 hours before treatment and further
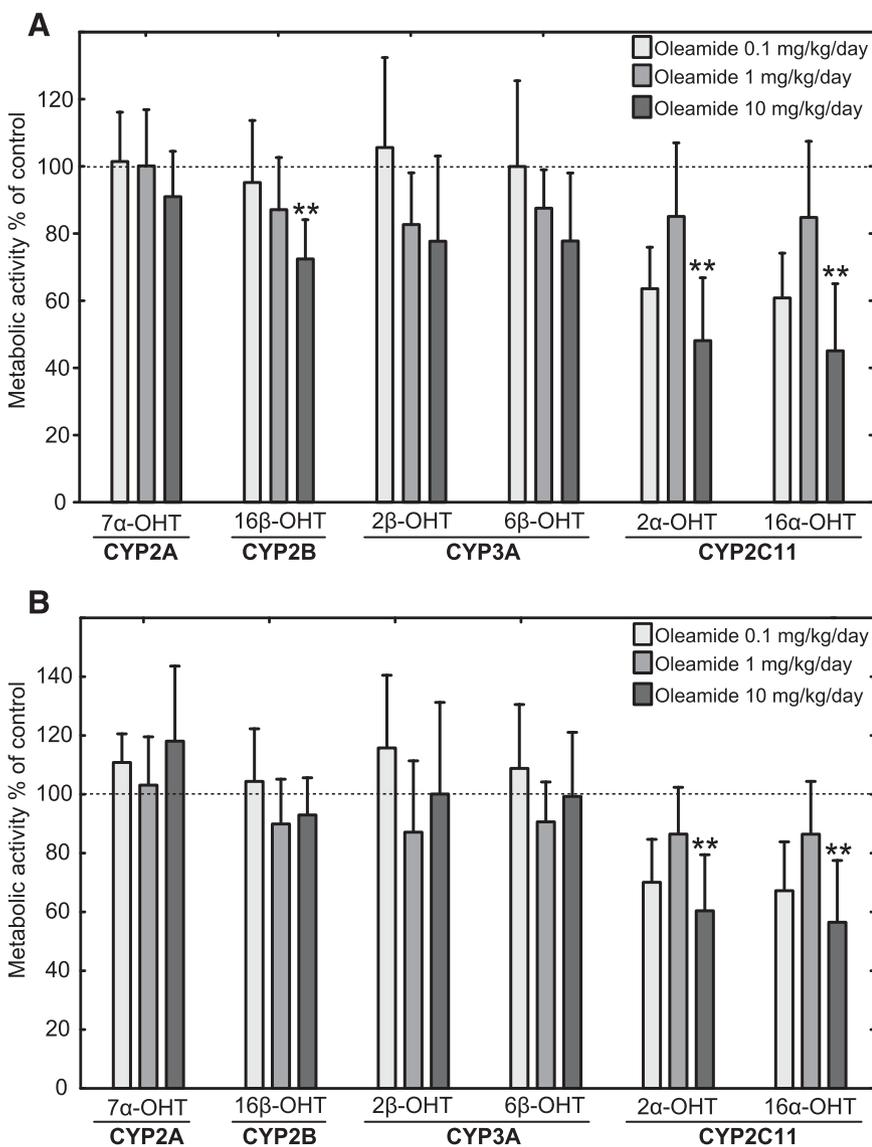
Fig. 2. (A) Relative metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 after systemic administration of oleamide, measured as the rate of testosterone hydroxylation and shown as the percentage of the rate of reaction in the control group. The absolute control values (picomoles per minute per milligram of total protein content) were as follows: 217.4 ± 23.7, 37.5 ± 6.1, 76.2 ± 14.8, 614.8 ± 92.0, 3303.0 ± 779.0, and 2934.5 ± 791.6 (testosterone 7α-, 16β-, 2β-, 6β-, 2α-, and 16α-hydroxylations, respectively). Animals were treated intraperitoneally with oleamide 0.1 ($n = 10$), 1 ($n = 10$), 10 mg/kg per day ($n = 10$), and propylene glycol (control group, $n = 6$). Reactions were performed in the presence of testosterone (400 μM) in RLMs (1 mg/ml of the total protein content) with an NADPH-generating system in a final volume of 1 ml at 37°C for 20 minutes. (B) Specific metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 after systemic administration of oleamide measured as the rate of testosterone hydroxylation per nanomoles of total P450 content per minute and shown as the percentage of the rate of reaction in the control group. The absolute control values (picomoles per minute per nanomoles of total P450 content) were as follows: 350.8 ± 38.8, 60.3 ± 8.2, 122.5 ± 20.2, 989.5 ± 127.0, 5321.8 ± 1188.7, and 4726.4 ± 1209.4 (testosterone 7α-, 16β-, 2β-, 6β-, 2α-, and 16α- hydroxylations, respectively). All values are expressed as bar graphs of the mean (S.D.). Statistical significance was assessed by the Kruskal-Wallis test, followed by multiple comparisons of mean ranks (A) and one-way ANOVA test (B). Statistical significance with respect to the control group (propylene glycol, $n = 6$) is indicated with **$P \leq 0.01$.

maintained in phenol red–free medium (150 μl) containing the evaluated compounds at the indicated concentrations for an additional 24 hours. After treatment, the cells were lysed, and luciferase activity was measured (Dual Luciferase Reporter Assay; Promega). Two modes were used as follows: 1) agonistic mode, in which the cells were treated with 0.1% DMSO or prototype ligand 5 μM or three concentrations of oleamide 1 or 10 or 30 μM; 2) antagonistic mode, in which the cells were treated with 0.2% DMSO or with prototype ligands (5 μM) in combination with oleamide at concentrations of 1 or 10 or 30 μM. Rifampicin (PXR activator) and 3-methylcholanthrene (AhR activator) were used as the prototype ligands. The data are expressed as a fold-change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated control means, which were set to 1. Statistical analyses were done using at least three independent assays ($n = 3$) performed in triplicate.

Assembly Assay (CAR). The CAR ligand binding domain (LBD) assembly assay is based on two hybrid expression constructs encoding the C- (pCAR-C/VP16) and N- (pCAR-N/GAL4) terminal parts of human CAR LBD that are cotransfected together with the pGL5-luc luciferase reporter plasmid (Promega) containing upstream activating sequence binding sites. A ligand promotes the connection of the two parts of the CAR LBD, resulting in luciferase trans-activation. Transient transfection assays were carried out using LipofectAMINE

3000 transfection reagent (Invitrogen/Life Technologies) in HepG2 cells. Cells were seeded into 48-well plates and transfected with a pGL5-luc luciferase reporter construct (80 ng/well), the expression constructs pCAR-C/VP16 and pCAR-N/GAL4 (80 ng/well), and the Renilla reniformis luciferase transfection control plasmid (pRL-TK) (30 ng/well) 24 hours later. Cells were maintained in a phenol red–free medium (150 μl) and treated with the tested compounds. After treatment, the cells were lysed, and luciferase activity was measured with a luciferase detection system (Dual Luciferase Reporter Assay; Promega). Two modes were used as follows: 1) agonistic mode, in which the cells were treated with 0.1% DMSO or CITCO (CAR activator) 5 μM or three concentrations of oleamide, 1 or 10 or 30 μM; and 2) antagonistic mode, in which the cells were treated with 0.2% DMSO or CITCO 5 μM in combination with oleamide at concentrations of 1 or 10 or 30 μM. The data are expressed as the fold-change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated control means, which were set to 1. Statistical analyses were done using at least three independent assays ($n = 3$) performed in triplicate.

Primary Cultures of Human Hepatocytes and Quantitative Reverse Transcription Polymerase Chain Reaction. Human long-term hepatocytes in a monolayer (batch HEP220971; Biopredic International, Saint Grégoire, France) were prepared from the livers of a 46-year-old Caucasian man. The medium was replaced with a serum-free medium the day after delivery of the primary human hepatocytes, and the cultures stabilized for an additional 48 hours before
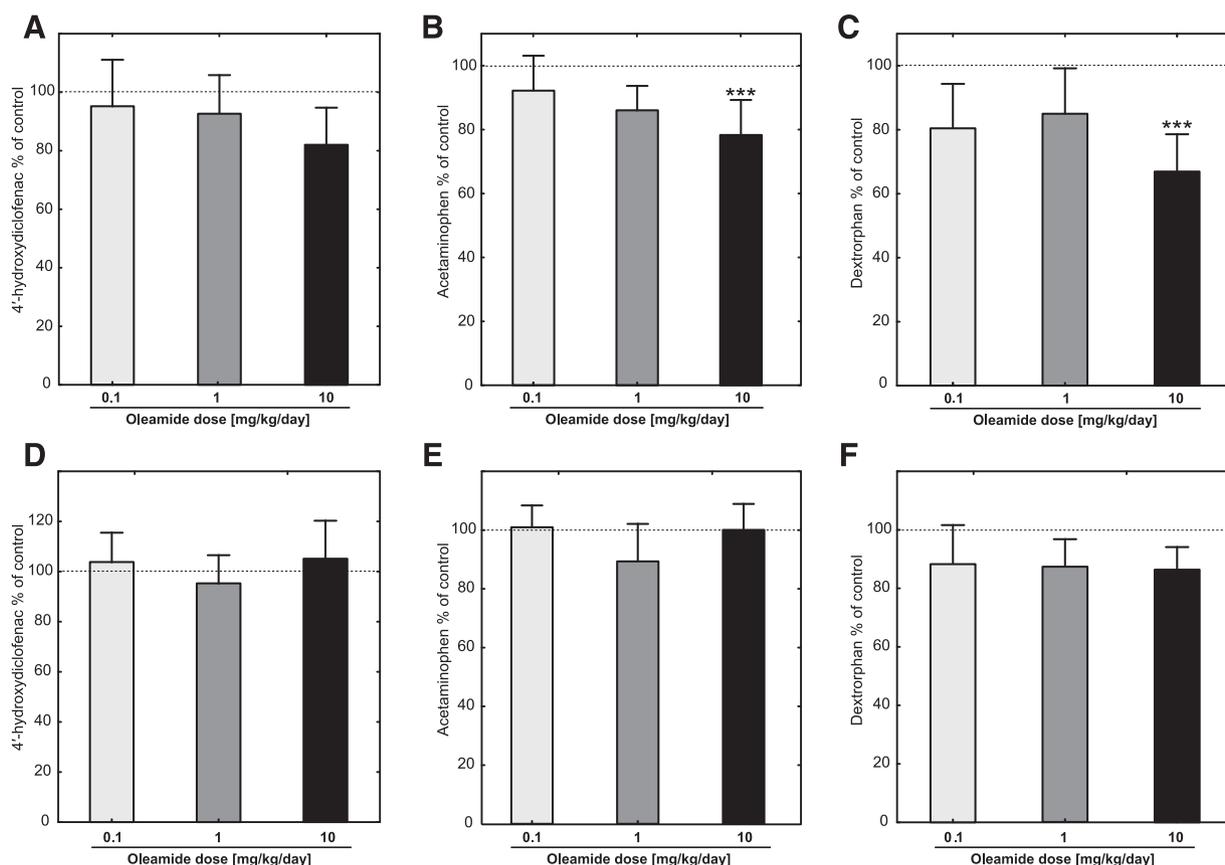
**Fig. 3.** (A) Relative metabolic activity of CYP2C6 after systemic administration of oleamide, measured as rate of diclofenac 4′-hydroxylation and shown as the percentage of the rate of reaction in the control group. The absolute control value was 1194.4 $\pm$ 184.4 pmol/min per milligram of total protein content. (B) Relative metabolic activity of CYP1A2 after systemic administration of oleamide, measured as the rate of phenacetin O-deethylation and shown as the percentage of the rate of reaction in the control group. The absolute control value was 361.3 $\pm$ 21.5 pmol/min per milligram of total protein content. (C) Relative metabolic activity of CYP2D2 after systemic administration of oleamide, measured as rate of dextromethorphan O-demethylation and shown as the percentage of the rate of a reaction in the control group. The absolute control value was 1154.9 $\pm$ 135.0 pmol/min per milligram of total protein content. Animals were treated intraperitoneally with oleamide, 0.1 ($n = 10$), 1 ($n = 10$), 10 mg/kg per day ($n = 10$), and propylene glycol (control group, $n = 6$). Reactions were performed in the presence of diclofenac (100 μM), phenacetine (400 μM), or dextromethorphan (500 μM) in RLMs (1 mg/ml of the total protein content) with the NADPH-generating system in a final volume of 1 ml at 37°C for 15 minutes (phenacetine) or 20 minutes (diclofenac and dextromethorphan). (D–F) Specific metabolic activities of CYP2C6, CYP1A2, and CYP2D6 after systemic administration of oleamide measured as the rate of testosterone hydroxylation per nanomoles of total P450 content per minute and shown as the percentage of the rate of reaction in control group. The absolute control values (picomoles per minute per nanomoles of total P450 content) were as follows: 1926.3 $\pm$ 292.4, 582.48 $\pm$ 23.4, and 1859.5 $\pm$ 169.2 (diclofenac 4″-hydroxylation, phenacetin O-deethylation, and dextromethorphan O-demethylation, respectively). Bars and whiskers express the mean (S.D.). Statistical significance was assessed by one-way ANOVA test, followed by Tukey's significant difference test for unequal sample sizes. Statistical significance with respect to the control group (propylene glycol, $n = 6$), is indicated with ***$P \leq 0.001$.

treatment. The cultures were maintained at 37°C in 5% CO_2 in a humidified incubator and treated with oleamide (10 μM) together with prototype CAR (CITCO, 1 μM), PXR (rifampin, 10 μM), and AHR (3-methylcholantrene, 10 μM) ligands for 48 hours before the total RNA isolation. cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were performed as described previously (Rulcova et al., 2010).

Hormone Assays

Serum concentrations of corticosterone were analyzed by radioimmunoassay after dichloromethane extraction as described previously (Jezova et al., 1994). The intra-assay and interassay coefficient of variation values were 6% and 8%, respectively, and the sensitivity of the assay was 0.5 μg/100 ml of plasma. The concentrations of free triiodothyronine and prolactin in serum were measured using commercially available enzyme-linked immunoassay (ELISA) kits (rat-free triiodothyronine ELISA kit and Prolactin rat ELISA; Cusabio Technology, Houston, TX, respectively). The intra- and inter-assay precision of the kits (coefficient of variation %) were less than 15%. The sensitivity and detection range reported by the manufacturer were 0.125 and 0.125–50 ng/ml for prolactin and 0.38 and 2–32 pmol/liter for triiodothyronine, respectively.

Data Analysis

The results of the determination of P450 activities were statistically evaluated using either 1) parametric one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference test for unequal sample sizes with a normal distribution of data; or 2) nonparametric Kruskal-Wallis test followed by multiple comparisons of mean ranks for all groups with a non-normal distribution of data. The statistical evaluation of the results from Western blot analyses was performed using the Wilcoxon signed-rank test. One-way ANOVA with a Dunnett's post hoc test were used for data from nuclear receptor assays, human P450 inhibition assays, and primary hepatocytes assays. The nonparametric Mann-Whitney U test was used to compare the hormone levels in two sets of samples. All values are expressed as means (S.D.), and they were computed using the software Statistica 13 (TIBCO Software, Palo Alto, CA). Results were considered statistically significant when $P \leq 0.05$. The K_i value was determined using a Dixon plot and calculated with the software SigmaPlot (SPSS, Inc., Chicago, IL). The Michaelis-Menten parameters (K_m and V_{max}) and the kinetic pattern of inhibition were also assessed using SigmaPlot.

Results

Effect of Oleamide on Liver P450 Activity In Vivo

In preliminary experiments, we evaluated how propylene glycol (a vehicle of oleamide) influenced the total microsomal protein, P450 levels, and their metabolic activity. We found no statistical difference comparing these results with experiments with saline-treated animals (data not shown). Hence, propylene glycol-treated animals were further used as controls for oleamide-administered animals.

Total Protein and Total P450 Content. Animals administered the highest dose of oleamide exhibited a significant decrease in the content in both total protein ($P < 0.05$, Kruskal-Wallis test, Fig. 1A) and P450 ($F_{(4,41)} = 4.89$ $P < 0.01$, one-way ANOVA test; Fig. 1B) in RLMs compared with controls. The relative content of P450, representing the ratio of P450 to total protein content (nanomoles per milligram of isolated protein in RLMs) was calculated, and again there was a significant decrease ($F_{(4,41)} = 4.89$, $P < 0.01$, one-way ANOVA test) in the group treated with the highest dose of oleamide compared with the group treated with propylene glycol alone (Fig. 1C).

P450s Metabolic Activity. The incubation of RLMs isolated from oleamide-treated animals with selective markers of P450 metabolic activity resulted in a variety of effects, depending on the P450 isoform. None of the tested oleamide doses influenced the relative or specific metabolic activities of CYP2A and CYP2C6 (Fig. 2, A and B; Fig. 3, A and D respectively) (for definitions of *relative* and *specific* metabolic activity, see section *Determination of P450 Activity in RM*). The rate of testosterone 2β and 6β-hydroxylation decreased to 77% of the control value. Nevertheless, the change in CYP3A relative activity was not statistically significant (Fig. 2A). In contrast, the relative activities of the CYP1A ($F_{(4,41)} = 6,33$ $P < 0.001$, one-way ANOVA test, Fig. 3B), CYP2B, CYP2C11 ($P < 0.01$, Kruskal-Wallis test, Fig. 2A), and CYP2D2 ($F_{(4,40)} = 7,42$ $P < 0.001$, one-way ANOVA test, Fig. 3C) were significantly decreased in animals treated with the highest dose of oleamide. Lower doses of oleamide were incapable of decreasing the relative metabolic activity of the P450s. Specific metabolic activities with respect to the total content of P450 did not exhibit any differences between the oleamide-treated animals and controls in their CYP1A2 (Fig. 3E), CYP2D6 (Fig. 3F), or CYP2B (Fig. 2B). An exception was CYP2C11 (Fig. 2B), where the activities of both the 2α-, and 16α-hydroxylations of testosterone were decreased in the group treated with the highest dose of oleamide (one-way ANOVA test $F_{(4,39)} = 8,22$ $P < 0.001$ and $F_{(4,41)} = 5,31$ $P < 0.001$, respectively).

Content of Distinct P450s. The amount of distinct P450 proteins was determined only for P450s with a significant change in the relative metabolic activity, with the exception of CYP2D2, because the monoclonal antibody was not available. In addition, the protein content was
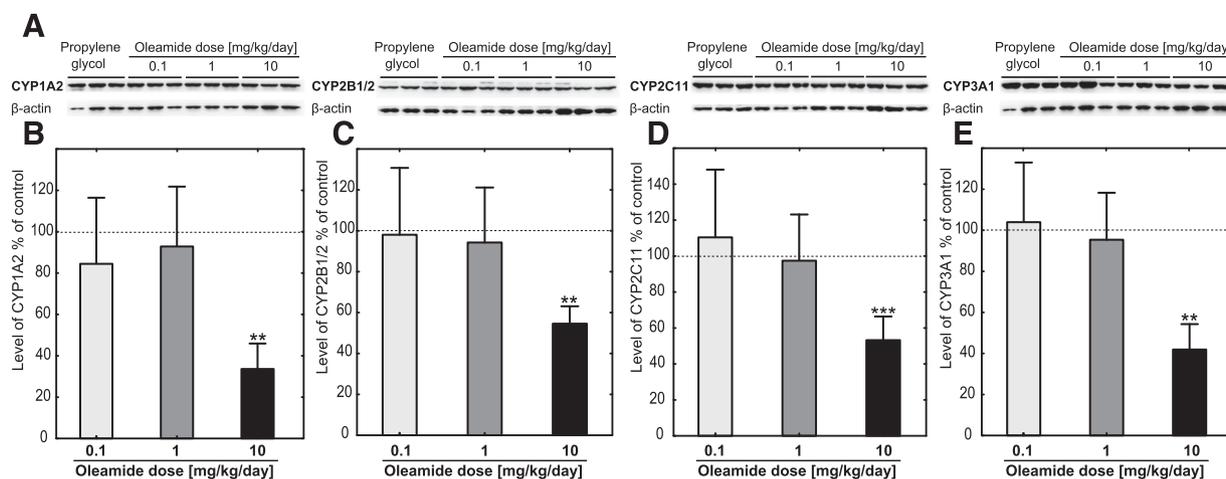
Fig. 4. Western blot analysis showing the expression of CYP1A2, CYP2B, CYP2C11, and CYP3A1 enzymes in RLMs after systemic administration of oleamide. Animals were treated intraperitoneally with oleamide 0.1 ($n = 10$), 1 ($n = 10$), 10 mg/kg per day ($n = 10$), and propylene glycol (control group, $n = 6$). (A) Representative blots of three individual animals of each group show expression of each indicated protein (10 μg in all cases). (B–E) Bar graphs show quantification of blots for CYP1A2, CYP2B, CYP2C11, and CYP3A1 protein, respectively. The bars represent means (S.D.) of individual animals of each group in three repetitions. Blots were quantified relative to the loading control (β-actin). Statistical significance was assessed by Wilcoxon signed-rank test and is shown as **$P \leq 0.01$; ***$P \leq 0.001$ compared with the control.

TABLE 2

P450s interactions with oleamide in vitro (data from the pilot experiment)

The metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 were measured as the rate of testosterone hydroxylation. The absolute control values (picomoles per milligram of protein per minute) were as follows: 1866.7 ± 0.9, 1773.7 ± 0.4, 2080.4 ± 3.5, 2123.9 ± 5.0, 1773.6 ± 0.8, 1825.6 ± 1.3 (testosterone 7α-, 16β-, 2α-, 16α-, 2β-, and 6β- hydroxylation, respectively). The metabolic activity of CYP1A2 was measured as the rate of phenacetin O-deethylation and control value of 53.3 ± 1.2 pmol/mg of protein per minute. The metabolic activity of CYP2C6 was measured as the rate of diclofenac 4′-hydroxylation and the control value 203.8 ± 11.3 pmol/mg of protein per minute. The metabolic activity of CYP2D2 was measured as the rate of dextromethorphan O-demethylation and a control value of 320.2 ± 7.6 pmol/mg of protein per minute. Experiments were performed with single concentrations of substrates phenacetin (300 μM), testosterone (100 μM), diclofenac (8 μM), or dextromethorphan (28 μM) and three concentrations of oleamide in RLMs from naïve animals (0.25 mg/ml of the total protein content) with the NADPH-generating system in a final volume of 1 ml at 37°C. After 10 minutes of preincubation with a different concentration of oleamide, reactions were initiated by the addition of specific markers. The incubation times were 40 (phenacetin and dextromethorphan), 20 (testosterone), and 30 minutes (diclofenac). All reactions were performed in triplicate.

| Oleamide | CYP450 Metabolic Activity (% Activity of Control) | | | | | | | | |
	1A2	2C6	2D2	2A	2B	2C11		3A	
0.1 μM	99.7	110.5	110.0	99.9[a]	100.0[b]	100.1[c]	99.6[d]	100.0[e]	100.1[f]
1 μM	108.6	94.2	96.8	100.1[a]	100.0[b]	100.8[c]	100.6[d]	100.0[e]	100.2[f]
100 μM	774	44.2	70.4	98.4[a]	99.9[b]	100.9[c]	99.4[d]	100.0[e]	100.0[f]

[a]Testosterone 7α-hydroxylation.
[b]16β-hydroxylation.
[c]2α-hydroxylation.
[d]16α-hydroxylation.
[e]2β-hydroxylation.
[f]6β-hydroxylation.

measured for CYP3A, where the decrease in metabolic activity was on the threshold of statistical significance. Blots were quantified, and the decrease in protein levels was present in all evaluated P450s after the 10 mg/kg per day treatment with oleamide (Fig. 4). The most influenced enzyme was CYP1A2, with a decrease in protein content to 33.6% ± 12% of the amount determined in the control group, followed by CYP3A (41.9% ± 12% of the control group). The amount of CYP2B and CYP2C11 decreased similarly to 54.5% ± 8% and 53.2% ± 1.3% of the control group, respectively.

Effect of Oleamide In Vitro

Rat P450 Inhibition Assays In Vitro. The results of the pilot study when oleamide was incubated with RLMs from naïve animals showed that oleamide did not influence the activities of CYP2A, CYP2B, CYP3A, and CYP2C11 (Table 2). The metabolic activities of these enzymes ranged from 97% to 101% of the activity of the control group in all analyzed testosterone metabolites and at all concentrations of oleamide used. Nevertheless, oleamide weakly inhibited phenacetin O-demethylation, indicating a weak inhibition of CYP1A2 activity. The rate of the reaction was suppressed to 77% of the rate of the control at a 100 μM concentration of oleamide in the incubation mixture. With CYP2D2, it was similarly observed that the metabolic activity of the enzyme was suppressed up to 70% of the rate of the control at a 100 μM concentration of oleamide. Such a high concentration of any drug is usually clinically irrelevant. Together with the fact that the inhibition of metabolic activities was very weak, the K_i value was not assessed for CYP1A2 and CYP2D2.

CYP2C6 was the most sensitive P450 to the inhibitory effect of oleamide in the pilot study (Table 2). We determined the kinetic parameters of CYP2C6 metabolic activity by using Michaelis-Menten kinetics (K_m = 7.2 ± 0.94 μM, V_{max} = 1.03 ± 0.04 nmol/min per milligram of protein). The 4′-hydroxylation of diclofenac was inhibited by 56%, with a final concentration of oleamide in the incubation mixture of 100 μM. Thus, further studies to determine the type of inhibition were done. Competitive, noncompetitive, and uncompetitive models of inhibition were evaluated; the respective K_i values were 28.7, 71.6, and 28.9 μM. The Akaike's Information Criterions corrected (AICc) in the individual models were used to evaluate the suitability of each model. The values of AICc were −652.591, −648.078, and −634.012 for the competitive, noncompetitive, and uncompetitive models, respectively.

Therefore, the competitive mode of inhibition seems to be an appropriate model with the best fit (Fig. 5).

Nuclear Receptors and Cell Viability Assays. Oleamide was tested at a range of concentrations from 1 to 30 μM for interaction with PXR, CAR, and AhR in HepG2 cells by gene reporter assays. We found no significant activation by the compound for any of these receptors (Fig. 6). Consistently, we did not observe inhibition of these nuclear receptors in antagonistic experiments with prototype PXR, CAR, and AhR ligands (rifampicin, CITCO, 3-methoxychlorantrene, respectively) (Fig. 6).

In addition, potential cytotoxicity was studied with the CellTiter 96 assay in HepG2 cells. The results clearly showed that oleamide is not toxic at the highest concentration assayed (30 μM) in HepG2 cells (data not shown).

Human P450 Inhibition Assays and Induction Experiments in Primary Human Hepatocytes by Oleamide. In the next set of experiments, we studied whether oleamide could inhibit human
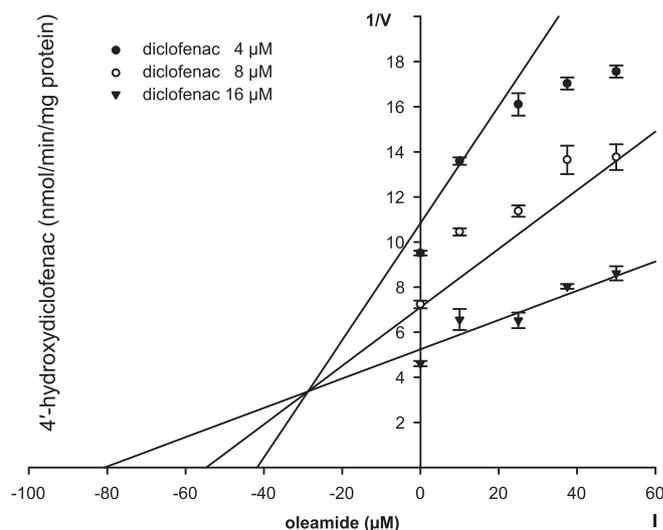
Fig. 5. Dixon plot of the effect of oleamide on 4′-hydroxydiclofenac formation in RLMs from naïve animals in a competitive model of inhibition. Reactions were performed in the presence of diclofenac (4, 8, 16 μM) and various concentrations of oleamide (0, 10, 25, 37.5, 50 μM) in RLMs (0.25 mg/ml of the total protein content) with the NADPH generating system in a final volume of 1 ml at 37°C for 30 minutes after 10 minute preincubation. The reaction was performed in triplicate. V = velocity of the reaction (nanomoles of 4′hydroxydiclofenac per minute per milligram protein) and I = concentration of oleamide (μM).
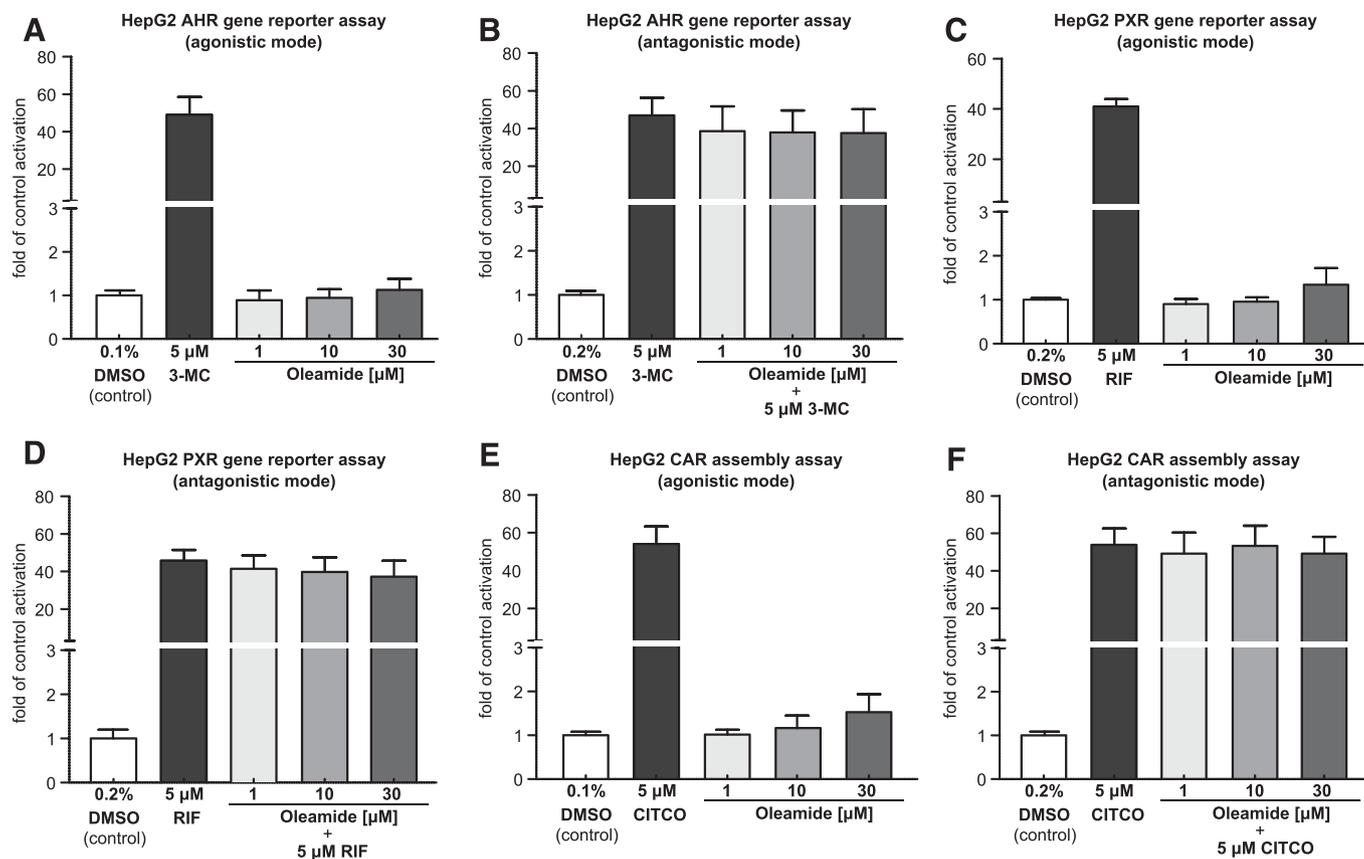
Fig. 6. Interaction of oleamide with PXR, AhR, or CAR in luciferase assays in HepG2 cells in agonistic (A, C, and E) and antagonistic (B, D, and F) modes. In the agonistic mode, cells were treated with 0.1% DMSO (control) or with prototype ligands (5 μM) or three concentrations of oleamide 1, 10, or 30 μM. In the antagonistic mode, cells were treated with prototype ligands (5 μM) in combination with oleamide at concentrations of 1, 10, or 30 μM. Rifampicin (RIF, PXR activator), 3-methylcholanthrene (3-MC, AHR activator), and CITCO (CAR activator) were used as prototype ligands. The data are expressed as the fold-change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO)-treated control means, which were set to 1. Statistical analyses were performed using at least three independent assays ($n = 3$) performed in triplicate, and statistical significance was assessed by one-way ANOVA followed by Dunnett's test.

CYP2B6, CYP2C9, CYP3A4, and CYP1A2 enzymes in vitro and whether it could induce key P450 genes regulated by the xenobiotic nuclear receptors CAR, PXR, and AHR.

We found that oleamide inhibits none of tested human P450s up to a concentration of 30 μM (Fig. 7, A–D). With CYP2B6, we observed an insignificant inhibition of metabolic activity at a 30 μM concentration of oleamide (Fig. 7A.).

We then treated primary human hepatocytes with 10 μM oleamide and CAR (CITCO, 1 μM), PXR (rifampicin, 10 μM), and AhR (3-methylcholantrene, 10 μM) ligands. We observed a significant induction of CYP2B6 mRNA by CITCO, CYP3A4 mRNA by rifampicin, and CYP1A2 mRNA by 3-methylcholantrene after 48 hours of treatment (Fig. 7E). Oleamide slightly upregulated CYP2B6, CYP3A4, and CYP1A2 mRNA (2.0-, 2.5-, and 3.3-fold, respectively), but the effects were not statistically significant.

Serum Hormone Concentrations

We found no statistical differences between control and oleamide-treated animals in serum levels of any of the hormones measured (Fig. 8).

Discussion

In this study, we demonstrate the effect of the endocannabinoid oleamide on rat liver content and activities of key liver P450s, namely, CYP1A2, CYP2A, CYP2B, CYP2C, CYP2D2, and CYP3A. The in vivo effect was assessed after the repeated administration of three

different doses of oleamide to rats (0.1, 1, and 10 mg/kg per day). The doses were selected with respect to available data for oleamide biologic activity, including hypnogenic (10–20 mg/kg, i.p.) (Huitron-Resendiz et al., 2001), analgesic ($ED_{50} = 66$ mg/kg, i.p.) (Fedorova et al., 2001), hypothermic ($ED_{50} = 14$ mg/kg, i.p.; 2.5–20 mg/kg, i.p, respectively) (Fedorova et al., 2001; Huitron-Resendiz et al., 2001), anxiolytic (5 mg/kg, i.p.) (Fedorova et al., 2001), and antidepressant-like (5 mg/kg, i.p.) (Ge et al., 2015) effects in animal experiments. Our results showed that oleamide at a dose of 10 mg/kg per day decreased the total protein content, as well as the total P450 protein levels in liver (Fig. 1); however, the metabolic activities of the individual tested P450s varied (Figs. 2 and 3). The overall decrease in the relative metabolic activity could be interpreted in two ways. It could be caused by an inhibition of P450 enzymes in terms of a decreased ability to catalyze the conversion of substrate to the appropriate metabolite with the preserved amounts of enzyme. The other possibility is the downregulation of P450s, meaning decreased amounts of distinct P450 proteins with the preserved metabolic activity. In our study, the relative metabolic activities of P450s were assessed after the standardization per total protein content, but not per individual amount of P450s. Decreased levels of distinct P450s (Fig. 4) corresponded to their decreased relative metabolic activities (Figs. 2 and 3). When relative metabolic activities were recalculated to specific metabolic activities with respect to the total P450 content in the incubation mixtures, there were no significant changes in the rate of marker reactions between the tested groups, except for the decreased activity of CYP2C11 with the highest dose of oleamide (Fig. 2). The reduction in CYP2C11 activity was
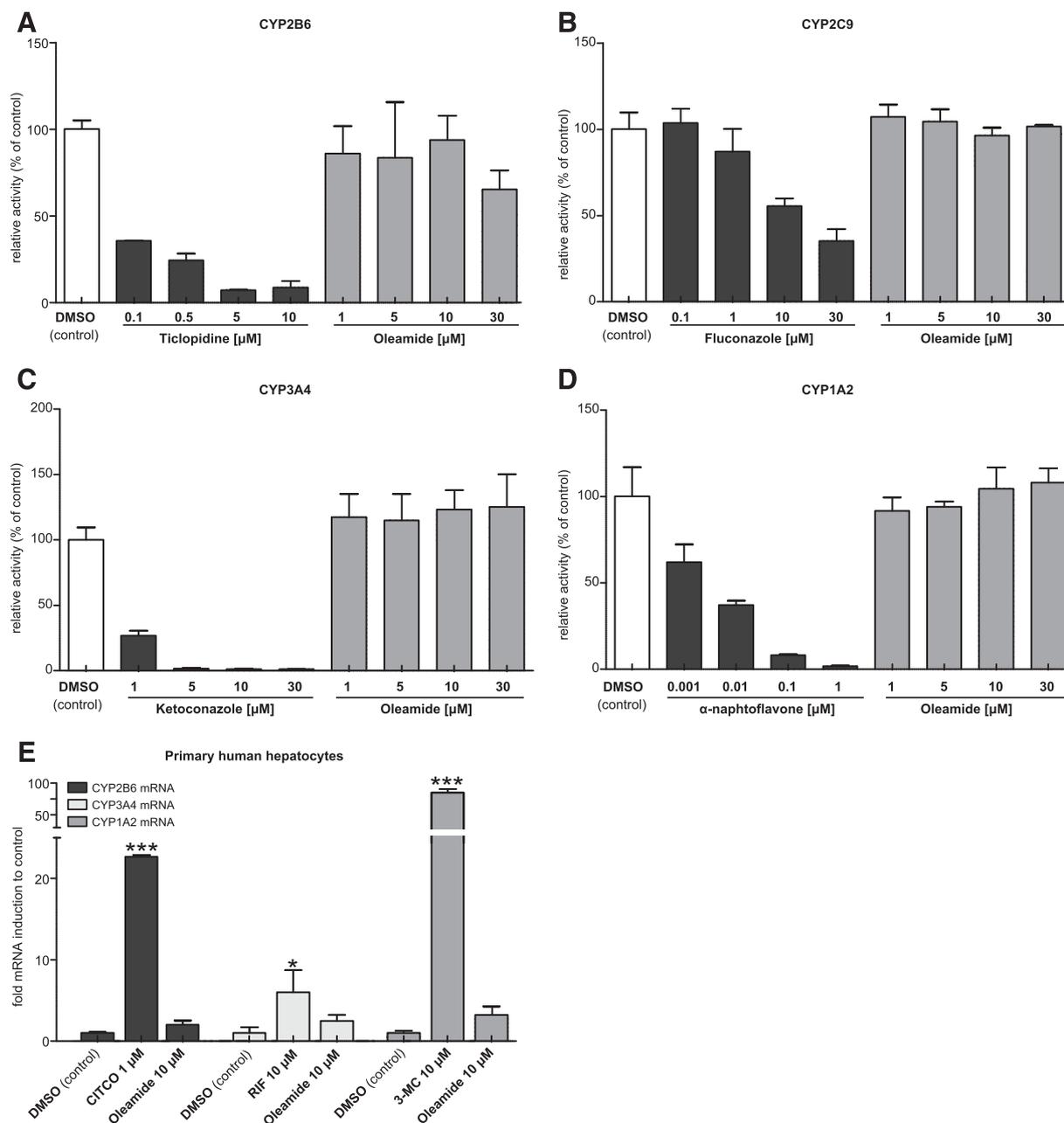
Fig. 7. Oleamide does not affect CYP2B6, CYP2C9, CYP3A4, and CYP1A2 enzymatic activities in vitro and mRNA expression in primary human hepatocytes. It does not inhibit metabolic activities of human CYP2B6 (A), CYP2C9 (B), CYP3A4 (C), and CYP1A2 (D), enzymes expressed in the microsomal fraction of P450 cDNA baculovirus-infected cells. Assays were performed in three independent experiments ($n = 3$) measured in triplicate. (E) qRT-PCR experiment in primary human hepatocytes treated with oleamide (10 μM), CITCO, RIF, or 3-methylcholantrene (3-MC) for 48 hours before total RNA isolation and qRT-PCR analysis were done. Data are presented as the means (S.D.) of triplicates from a representative batch of primary human hepatocytes (human long-term hepatocytes in monolayer, batch HEP220971, Biopredic) and are expressed as the fold mRNA upregulation relative to the vehicle-treated controls (set to 100%). Statistical significance was assessed by one-way ANOVA, *$P \leq 0.05$; ***$P \leq 0.001$.

greater than could be explained by a simple decrease in total P450 content, but the exact mechanism of this influence is still under investigation. Based on the experimental data, we concluded that the metabolic activity of most enzymes in the in vivo experiment had not been affected. The decreased relative activities measured in our experiment were caused by P450 downregulation rather than by inhibition of enzymes, which is in line with the results of Western blot testing.

The effects of other endocannabinoids on the activity of P450s are unknown, and the influence of exogenous cannabinoid receptor ligands is diverse. In detail, the decrease in the microsomal protein content in experimental animals was reported after administration of the CB_1 ligand (CP 55,940) to mice (Fontanellas et al., 2005) and rats (List et al., 1977; Dalterio et al., 1986; Costa et al., 1996). Cannabidiol also decreased the microsomal protein levels in human HepG2 cells (Yang et al., 2014) and inhibited metabolism of the selected P450 substrates in mice (Watanabe et al., 1981; Bornheim and Correia, 1989). On the other hand, studies suggesting that cannabinoids (CP55,940, hashish, and cannabidiol) would increase the content and/or activity of liver P450s in rodents have been published (Dalterio et al., 1986; Costa et al., 1996; Sheweita, 2003). The fact that these substances are usually of a different chemical structure with different biologic activity limits generalization of the results obtained using different cannabinoids as a general "class
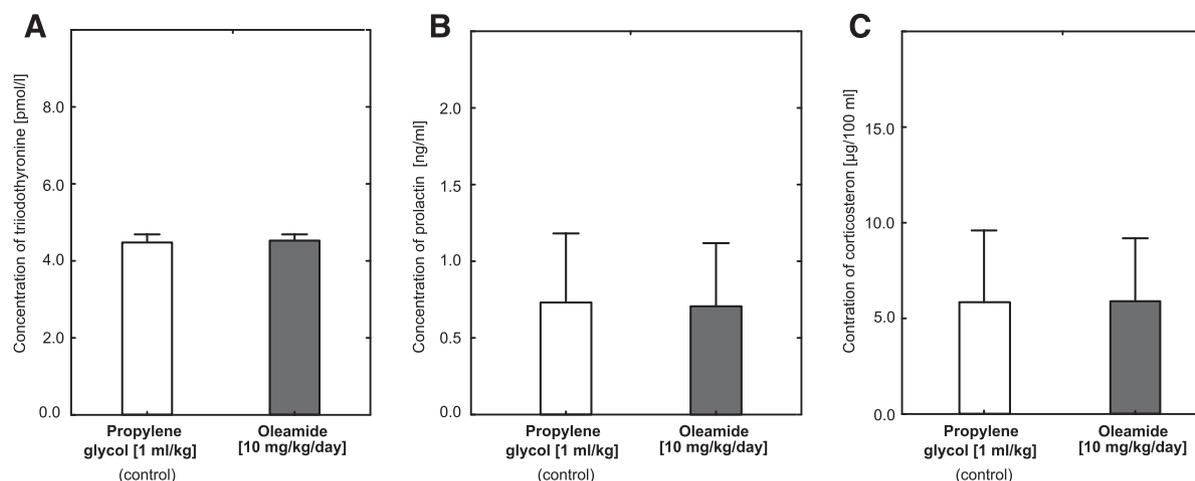
Fig. 8. Effect of systemic administration of oleamide on hormone concentrations in rat blood serum. Animals were treated intraperitoneally with oleamide 10 mg/kg per day ($n = 10$) and propylene glycol (control group, $n = 6$). Bar graphs show concentrations of free triiodothyronine (A), prolactin (B), and corticosterone (C). The bars represent means (S.D.) of individual animals of each group in three repetitions. Statistical significance was assessed by Mann-Whitney U test.

effect" of cannabinoids. Moreover, the ECS could have reacted differently to the same drug at different time points during drug administration, as mechanisms of desensitization, or receptor down-regulation or upregulation might have been involved (Daigle et al., 2008; Burston et al., 2010).

The ECS, as a retrograde modulator, is associated with different neuronal systems within the brain, and therefore it can change the activities of several neurotransmitters in various brain regions. It has been documented that changes in the activity of these specific neuronal circuits could influence the activity of liver P450s. Hormones were identified as a probable link between the brain and liver (Wojcikowski et al., 2008; Wojcikowski and Daniel, 2011; Bromek et al., 2013; Rysz et al., 2016). Although exogenous cannabinoids have long been known to be modulators of pituitary hormones (Pagotto et al., 2006), the influence of endocannabinoids on the neuroendocrine system has not been fully understood. Endocannabinoids modulate the levels of growth hormone (Pagotto et al., 2006), thyrotropin-releasing hormone (Deli et al., 2009), corticotrophin-releasing hormone (Steiner and Wotjak, 2008; Hill and Tasker, 2012), prolactin, and luteinizing hormone (de Miguel et al., 1998), probably via the hypothalamus, where the ECS acts as a retrograde messenger system. Hormones then bind to nuclear receptors in hepatocytes and regulate P450 gene expression. Therefore, the ECS is probably involved in the regulation of the metabolic activity of liver P450s as well. This hypothesis was described in detail elsewhere (Zendulka et al., 2016). In our study, oleamide did not influence the levels of prolactin, corticosterone, or free triiodothyronine (Fig. 8). Therefore, we expect these hormones not to be included in the mechanism of liver P450 activity being influenced by oleamide.

The interactions of oleamide with the key human nuclear receptors involved in regulation of major P450s were further tested to obtain additional data on the possible influence of this endocannabinoid in humans. The results showed that oleamide did not interact with human AhR, PXR, or CAR nuclear receptors either in an agonistic or antagonistic mode (Fig. 6). Consistently, oleamide did not induce CYP2B6, CYP2C9, or CYP3A4 mRNA expression in a representative primary human hepatocyte culture (Fig. 7E).

The direct inactivation of rat P450s by oleamide in RLMs was tested in our in vitro model. Weak inhibition of CYP1A2, CYP2D2, and, more noticeably, CYP2C6 are described in Table 2. Similarly, the direct inhibition of P450s by phytocannabinoids, such as cannabidiol, cannabinol, and Δ^9-THC, were reported (Zendulka et al., 2016). All

three substances inhibited the metabolic activity of P450s with various potencies. Differences between the results obtained from two models used in our study can be explained by the divergent modes of how the metabolic activity of P450s can be influenced. In an in vitro experiment, the drug could have bound either to the active or to the allosteric site of P450s and decreased the metabolic activity by direct enzyme in-activation. Livers isolated from animals treated with oleamide were drawn 24 hours after the last dose of drug was administered and microsomes were washed out several times with buffers during the process of isolation. Therefore, the microsomes were thought to be "drug free." This was proved by there being no change in the metabolic activity of CYP2C6 detected in the in vivo model compared with the results of the in vitro model, where CYP2C6 was inhibited, and conversely by a decreased metabolic activity of CYP2C11 assessed in vivo, with no influence in vitro. Furthermore, the direct interaction with enzymes could not explain the decreased P450 protein levels, which were detected for CYP1A2, CYP2B, CYP2C11, and CYP3A after repeated systemic administration of oleamide (Figs. 2–4). These findings further support our conclusion that the influence of oleamide on rat P450s was mediated by the mechanism of downregulation. Besides the direct interaction of oleamide with RLMs, we tested its interaction with human recombinant P450s (CYP2B6, CYP2C9, CYP3A4, and CYP1A2), finding insignificant effects (Fig. 7, A–D).

In conclusion, we hypothesize that the repeated intraperitoneal administration of oleamide (10 mg/kg per day) caused the down-regulation of CYP1A2, CYP2B, CYP2C11, and CYP2D2 in rats. The effect is unlikely to be caused by the direct inhibition of P450 enzymes or by oleamide influencing the hormonal levels. The mechanism of this effect has not yet been determined. Although oleamide inhibited rat CYP1A2, CYP2D2, and CYP2C6 enzymes, it did not interact with the tested recombinant human cytochromes in vitro. We observed no effect on human AhR, PXR, and CAR nuclear receptors in the regulation of main human P450s in the primary human hepatocytes. To the best of our knowledge, we are the first to report the effects of the endocannabinoid CB$_1$ agonist and the FAAH inhibitor oleamide on rat and human P450s. Our results suggest the possible involvement of the endocannabinoid system in the regulation of rat liver cytochrome activity. Still, oleamide affected neither the activity of human recombinant P450s nor the expression of mRNA of P450s in human primary hepatocytes. The effects observed in rat P450 enzymes are likely to be species-specific, and the clinical impact needs to be elucidated in future studies.

Acknowledgments

Authorship Contributions

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6.   P450 phenotyping in clinical practice

It is hard to predict the pharmacokinetic variability of drug metabolism due to numerous influences that could participate in this phenomenon. On the one hand, the majority of drugs are so safe that even huge interindividual differences in plasma levels after the same dose of the drug administered to different patients lead to the expected therapeutic effect. On the other hand, if we were able to predict the plasma levels of the drug prior to the drug's administration, it could prevent patients from experiencing adverse effects or signs of toxicity in drugs with a narrow therapeutic range. One possible way to at least partially predict the metabolic pharmacokinetic variability is P450 genotyping. Some P450 are genetically polymorphic. A polymorphism is an occurrence of various alleles at one locus with at least two of them occurring with a greater than 1% frequency in the population (de Leon et al., 2009). The population can be classified into up to four groups with respect to the number of alleles coding the functional enzyme. Poor metabolizers have both alleles null, intermediate metabolizers have partially defective alleles or a combination of null and a partially defective allele, extensive metabolizers have normal alleles, and ultrarapid metabolizers have duplicated normal alleles (Zanger and Schwab, 2013). The higher the number of normal alleles, the faster the speed of drug elimination, or for pro-drugs their bioactivation. A drug could be ineffective in ultrarapid metabolizers due to rapid elimination while the same dose can cause toxicity in poor metabolizers. The variability in the P450 activity between phenotypes can be as high as 30- to 40-fold for CYP3A, 100-fold for CYP2D6, 50- to 60-fold for CYP2B6, and 40- to 50-fold for 2C9 (Tracy et al., 2016). Not all polymorphic P450 enzymes exhibit all four of these types of phenotypes. CYP2C19 genes code enzymes of extensive and poor metabolizer phenotypes (Chaudhry et al., 2008), while all four classes are described for CYP2D6 (Kiss et al., 2018). There are simple commercial assays for P450 genotyping using polymerase chain reaction methods. CYP3A5, CYP2C19, CYP2C9, and CYP2D6 can be genotyped. The genotype describes just one of the factors that determine the P450 phenotype. In some P450 enzymes, the role of genotype on the final P450 phenotype is significant while in others it could be much weaker. P450 enzymes with a known strong influence of gene polymorphism on the phenotype are CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C19, and CYP2D6 (Zanger and Schwab, 2013). The reliability of genotype-based predictions of phenotype therefore varies. For CYP2D6, it is described to be 67% (Kiss et al., 2018). Nevertheless, the frequency of the genotype also depends on the population studied, as there are different frequencies of P450 alleles across various ethnic groups (McGraw and Waller, 2012).

The best option for predicting metabolic variability is therefore phenotyping. The principle of the assay is simple. A known probe substrate of P450 is administered to a patient and biological fluids

(blood, urine, saliva) are sampled at specific time intervals. The amounts of P450-specific metabolites are measured and the metabolic ratio (MR), the ratio of the concentration of metabolite to the concentration of probe substrate, is calculated and evaluated. For some probe substrates there are alternative evaluations to MR, but the administration of the probe substrates remains the same. The speed of metabolism can be evaluated with a breath test using radiolabelled probes (Furuta et al., 2009) or by measurement of the drug's biological activity (pupilometry after tramadol use as a probe of CYP2D2) (Matouskova et al., 2011).

Table 2. Overview of P450 selective probe substrates. Adapted with the permission of the author (Dovrtělová, 2018).

Marker activity/reaction	Human CYPs	CYP subfamily	Rat CYPs	Marker activity/reaction
caffeine/N-demethylation phenacetine/O-deethylation theophylline/N-demethylation,8-hydroxylation 7-ethoxyresorufin/O-deethylation	CYP1A2	**CYP1A**	CYP1A2	7-methoxyresorufin/ O-dealkylation phenacetin/O-deethylation
coumarin/7-hydroxylation cotinine/3'-hydroxylation	CYP2A6	**CYP2A**	CYP2A1	testosterone/7α-hydroxylation
			CYP2A2	testosterone/15α-hydroxylation
bupropion/4-hydroxylation efavirenz/8-hydroxylation S-mephenytoin/N-demethylation	CYP2B6	**CYP2B**	CYP2B1	pentoxyresorufin/O-deethylation testosterone/16β-hydroxylation
diclofenac/4'-hydroxylation tolbutamide/methylhydroxylation S-warfarin/7-hydroxylation	CYP2C9	**CYP2C**	CYP2C11	testosterone/2α-hydroxylation testosterone/16α-hydroxylation
S-mephenytoin/4´hydroxylation omeprazole/5-hydroxylation	CYP2C19		CYP2C6	diclofenac/4'-hydroxylation S-warfarin/7-hydroxylation
codeine/O-demethylation debrisoquine/4-hydroxylation dextromethorphan/O-demethylation metoprolol/α-hydroxylation sparteine/dehydrogenation tramadol/O-demethylation	CYP2D6	**CYP2D**	CYP2D1	bufuralol/1´-hydroxylation
			CYP2D2	dextromethorphan/O-demethylation debrisoquine/4-hydroxylation propranolol/7-hydroxylation bufuralol/1´-hydroxylation
alfentanil/demethylation midazolam/1-hydroxylation dextromethorphan/N-demethylation erythromycin/N-demethylation testosterone/6β-hydroxylation cortisol/6β-hydroxylation	CYP3A4	**CYP3A**	CYP3A1	testosterone/6-hydroxylation testosterone/2-hydroxylation
testosterone/6β-hydroxylation midazolam/1'-/4-hydroxylation alprazolam/4-/1-hydroxylation	CYP3A5		CYP3A2	midazolam/4-hydroxylation
			CYP3A1	triazolam/1'-hydroxylation

Different probe substrates are used in preclinical and clinical tests. In both, the basic requirements for the probes are: i) to be metabolized via a distinct P450 for the specific metabolite; ii) the metabolism should be the main process of probe elimination; iii) the probe and its metabolite should be easily measured in the biological samples. The safety of the probe substrates is the limiting requirement for clinical and *in vivo* experimental use. An overview of common P450 probes for rat and human P450 can be found in Table 2. There are single-probe assays as well as cocktail approaches. The great advantage of cocktail phenotyping is the possibility of assessing multiple P450 enzymes in one test and thus eliminate the influence of inter- and intra-individual variability in testing. On the other hand decreased sensitivity, risk of adverse effects, toxicity, or drug-drug interactions are the most common disadvantages (Zhou et al., 2004a).

6.1 Phenotyping of CYP1A2 with caffeine as a probe substrate

The activity and expression of CYP1A2 in human liver exhibits an approximately 40-fold inter-individual variability (Ghodke-Puranik and Lamba, 2017). Even though the number of drug substrates of CYP1A2 is not as high as with CYP3A4/5 or CYP2D6, it still contributes in the metabolism of approximately 9% of drugs biotransformed via the P450 system (Zanger and Schwab, 2013). Besides clozapine, tacrine, or theophylline, it is involved in the metabolism of endogenous substrates (melatonin, estradiol) and activates toxic compounds and procarcinogens (aflatoxin B1, aromatic hydrocarbons) (Koonrungsesomboon et al., 2017).

The methylxanthine derivate caffeine, a phosphodiesterase inhibitor and antagonist of the adenosine receptor, is a well-established probe substrate for P450 CYP1A2 phenotyping *in vivo*. It undergoes extensive metabolism by CYP1A2 in the liver. The urinary MR of caffeine and its several metabolites can be used for the assessment of CYP1A2 metabolic activity (Turesky et al., 2015).

In the article "Determination of caffeine and its metabolites in saliva and urine as a measure of CYP1A2 metabolic activity", we present a fast, sensitive, and reproducible method for CYP1A2 phenotyping. Briefly, the caffeine was administered at a dose of 3 mg/kg orally in a gelatin capsule. The urine was collected for the following 6 hours and aliquots were drawn 3 and 6 hours after administration of the probe. Saliva was sampled similarly 3 and 6 hours after caffeine administration. The best recovery and sensitivity for sample extraction was reached with a 8.5 : 1.5 (v/v) mixture of chloroform and isopropylacohol. An HPLC method was developed for the analysis of extracted samples. The analytes were separated in a reversed-phase analytical column. Isocratic elution was used with a mobile phase consisting of 15 mM sodium acetate and methanol 85 : 15 (v/v) and a flow rate of 0.8 mL/min. Caffeine and its metabolites were detected with a diode array detector at various wavelengths characteristic for individual analytes. A good correlation between urine and saliva

paraxantine/caffeine MR for both the 3 and 6 hour sampling points was found. There are many advantages to our method over other published methods. Firstly, it is a method which can be performed with a conventional HPLC system with diode array detector. It provides the possibility of selecting urine or saliva for the assessment of CYP1A2 activity. Finally, the method can be used for determination of the activity of other enzymes involved in the metabolism of caffeine, including xanthinoxidase and N-acetyl transferase.

Turjap M., **Zendulka** O., Glatz Z., Brejcha S, Madr A., Juřica J. Determination of caffeine and its metabolites in saliva and urine as a measure of CYP1A2 metabolic activity. Current Pharmaceutical analysis, 12 (4), 325-332, 2016.

Impact factor: 0.75; Pharmacology & Pharmacy Q4   Citations WoS: 2

Author's contribution: 10%

Determination of Caffeine and its Metabolites in Saliva and Urine as a Measure of CYP1A2 Metabolic Activity

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Abstract: ***Objective***: The aim of this work was to develop a new, simple, rapid, sensitive and reproducible RP-HPLC method for the determination of caffeine, 1,7 dimethylxanthine, 1,7-dimethyluric acid, 5-acetylamino-6-formylamino-3-methyluracil, 5-acetylamino-6-amino-3-methyluracil, 1-methylxanthine, and 1-methyluric acid in human urine and saliva as a method of determining the metabolic activity of the human enzyme CYP1A2.

Methods: A Luna C18(2) (150 × 4.6 mm i.d.) analytical column was used for the separation. The mobile phase consisted of sodium acetate trihydrate (pH 5.0) and methanol 85:15 (v/v). The flow rate was maintained at 0.8 mL/min. The absorbance of the eluent was monitored at 263 nm (5-acetylamino-6-amino-3-methyluracil), 285 nm (5-acetylamino-6-formylamino-3-methyluracil, 1,7-dimethyluric acid, 1-methyluric acid), 272 nm (caffeine, 1,7 dimethylxanthine) and 268 nm (1-methylxanthine). Acetaminophen as an internal standard was used to ensure the precision and accuracy of this method, and it was monitored at 245 nm.

Results: All compounds, including the internal standard, were eluted within 18 min. Analytes were extracted by liquid-liquid extraction. Limits of quantitation varied from 7.2 to 74.2 µg/L for individual analytes in saliva and from 8.4 to 82.4 µg/L in urine.

Conslusion: This method may become a useful alternative to urine caffeine metabolic ratio measurement with respect to CYP1A2 metabolic activity assessment in clinical practice.

Keywords: Caffeine, CYP1A2, saliva, urine, phenotyping, metabolic ratio.

1. INTRODUCTION

Caffeine, 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione, is a methylxanthine derivative, a phosphodiesterase inhibitor, adenosine receptor antagonist, used as a psychostimulant and analgesic adjuvant. Caffeine is extensively metabolized *via* cytochrome P450 enzymes, *N*-acetyltransferase and xanthinoxidase (Fig. **1**) [1]. CYP1A2 plays a major role in caffeine biotransformations, especially in *N3* - demethylation, and thus it is used as a measure of CYP1A2 metabolic activity. The theoretical assumption that genotype is only one of many factors influencing the endophenotype of CYP enzymes was confirmed at least for CYP1A2 [2]. For instance, the allele CYP1A2*1F predicts a higher susceptibility to enzyme induction than the *wt* allele, and thus the impact of CYP1A2 gene mutations also depends on environmental factors [3]. CYP1A2 metabolic activity seems to be a better

predictor of treatment outcomes than the CYP1A2 genotype [4] and therefore CYP1A2 phenotype determination is superior to genotype determination in terms of clinical validity and utility. Several substrates have been used as "specific markers" of CYP1A2 metabolic activity including phenacetin [5], nonetheless caffeine is the most widely used, despite other enzymes also being involved in its biotransformation (xanthinoxidase, *N*-acetyltransferase and to a lesser extent also CYP2E1, CYP2B6, CYP3A4, CYP3A5) [6, 7].

The molar concentration ratio of marker to metabolite(s) in urine, plasma or saliva was used in phenotyping studies. Several ratios of molar concentrations of caffeine's metabolites are used to estimate the actual metabolic activity of CYP1A2 in humans as well as in rats [6, 8, 9]. The following metabolic ratios which are believed to reflect the metabolic activity of CYP1A2 have been reported [10-12] with "caffeine metabolic ratio" [CMR: (AFMU + 1X + 1U)/17U] as probably the best proved measure of CYP1A2 metabolic activity [8]. Due to the low concentrations of most caffeine metabolites in saliva, except for 1, 7X, a simple 1,7 dimethylxanthine (paraxanthine) to caffeine molar concentration

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Fig. (1). Biotransformation pathways of caffeine.

ratio assessed in saliva or serum/plasma is also frequently used [9]. Moreover, a good correlation has been reported between a 6-hour salivary molar ratio of 1,7X to 1,3,7X and immunoreactive CYP1A2 liver content, and an excellent correlation between a 4-hour salivary ratio of 1,7X to 1,3,7X and apparent caffeine clearance [13, 14].

Many methods for determining caffeine in the pharmaceutical industry, beverages, food supplements, coffee beans and even coffee flowers have been reported [10, 11, 15]. Several methods for determining caffeine metabolites have been reported to date. Briefly, Holland *et al.* assessed paraxanthine and caffeine in serum samples after simple sample preparation using an RP analytical column and UV detection, a single run was approx. 10 min and LOQ was 50 µg/L [16]. Krul and Hageman reported a HPLC - UV method for the determination of caffeine and its five metabolites in urine, but the analysis length was 30 min, which is unacceptable for the batch analysis of large sets of samples [17]. Similarly, Bendriss and colleagues published an HPLC-UV method for the determination of caffeine and fourteen of its metabolites, with limits of detection of 2 ng/mL. However, the method utilized gradient elution (both in the flow and composition of the mobile phase) and a single run was 60 min, not counting column equilibration between runs [10]. Atia *et al.* published a HPLC-UV method for the determination of caffeine and 8 of its metabolites in urine with LOQs in the range 14 to 41 ng/mL, analysis length of 15 minutes, however only spiked blank urine samples were analyzed [18]. Zydron *et al.* published a method for the determination of caffeine and 9 of its metabolites in urine. A single run was about 30 minutes, LOD were in the range 50-60 ng/mL and the authors used a gradient elution [19]. The literature is scarce in terms of methods for the determination of caffeine and metabolites in saliva. The method of Perera *et al.* enables only 1,3,7X and 1,7X determination in saliva and plasma, a single run is 18 minutes and the limit of detection in saliva is 15 µg/L for both 1,7X and 1,3,7X [20].

Due to the instability of AFMU in biological samples at neutral or alkaline conditions, when AFMU is non-enzymatically transformed to AAMU, some authors prefer the determination of both (AAMU+AFMU) [12, 21], other authors quantitatively transformed AFMU to AAMU. CMR an then be calculated with use of molar concentrations as follows: (AAMU + AFMU + 1X + 1U)/1,7U [12].

HPLC-MS methods were also used in CYP1A2 metabolic activity determination [12, 22, 23] as well as capillary electrophoretic methods [24]. In general, LC-MS methods give lower LOQ and have the advantage of a shorter analytical run.

However, none of the above methods enable the determination of caffeine and its major metabolites in saliva and urine samples with an acceptable length of a single analysis run, without gradient elution, which requires the equilibration of the column.

Here we present a simple, sensitive, reproducible and also universal HPLC method for the determination of caffeine and its metabolites in saliva and urine suitable for the determination of CYP1A2 metabolic activity in humans.

2. MATERIALS AND METHODS

2.1. Chemicals

Caffeine, (1,3,7X), paraxanthine (1,7X), 1-methylxanthine (1X), 1,7-dimethyluric acid (1,7U), 1-methyluric acid (1U), N-(4-hydroxyphenyl)acetamide (syn. acetaminophen, paracetamol, PAR), acetonitrile, methanol, sodium acetate trihydrate and hydrochloric acid were purchased from Sigma-Aldrich (Prague, Czech Republic). 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 5-acetylamino-6-amino-3-methyluracil (AAMU) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Chlorophorm and isopropylalcohol

were purchased from Chromservis (Prague, Czech Republic).

All reagents used were of analytical grade, except for methanol and acetonitrile, which were of HPLC grade. All water solutions were prepared with Ultrapure water (Premier MFG Systems, Phoenix, Arizona, USA) filtered through a 0.45 μm nylon membrane filter.

2.2. Standard Stock Solutions, Calibration Standards and Quality Controls

The standards 1,3,7X, 1,7X, 1X, AAMU and PAR (IS) were dissolved in methanol, AFMU was dissolved in metha-nol: 0.1 M acetic acid (50:50 *v/v*), and 1,7U and 1U were dissolved in a methanol: 0.1 M NaOH solution (50:50 *v/v*) to prepare stock solutions. The stock solutions were stored at −18 °C. Working standard solutions of AAMU; AFMU; 1U; 1X; 1,7 U; 1,7X and 1,3,7X (80.0, 60.0, 51.7, 48.9, 56.7, 70.0, 63.33 and 86.7 mg/L respectively) were made by dilut-ing the stock solutions with water-methanol (1:1, *v/v*). The linearity of the detector response was evaluated with sets of standard solutions in the mobile phase to obtain the final concentrations specified in Table **1**. The calibration stan-dards were prepared by spiking fresh blank urine/saliva sam-ples with the working standard solutions.

A working internal standard solution was prepared at a final concentration of 630 mg/L. Quality control samples were prepared by spiking drug-free saliva and urine with standard stock solutions at three different concentrations of each analyte for the method validation.

2.3. Equipment

The HPLC system was a Shimadzu LC-10 series set (Shimadzu, Kyoto, Japan), i.e. LC-10ADvp gradient pump with DGU-14A degasser, SIL10ADvp autosampler, CTO10ACvp column oven, SPD-M10Avp DAD detector and SCL10Avp system controller. Data from the detector were collected and analyzed using LabSolution 1.03 SP3 software (Shimadzu, Kyoto, Japan). A MultiReax vortex shaker (Heidolph, Schwabach, Germany) and Universal 32R centrifuge (Hettich, Tuttlingen, Germany) were used for sample preparation.

3. EXPERIMENTAL

3.1. Sample Preparation

Blank human saliva and urine samples from 6 healthy volunteers were drawn after 72 h of a caffeine-free diet. These blank samples were spiked and used for calibration, precision and accuracy determination. Aliquots (500 μL) of human saliva or urine samples (3 and 6 h postdose) or spiked blank human urine or saliva were transferred into screw-capped glass tubes. 25 μL of the IS solution (630 mg/L) and 100 μL of 0.1M HCl were added. Then, 5 mL of chloro-phorm:isopropanol (85:15 v/v) was added and samples were vortexed for 10 min at 2,000 rpm and centrifuged for 5 min at 3,000 g at 4°C. After that, a 4.5 mL of organic layer was transferred into a glass conical tube and organic solvent was evaporated under a gentle stream of nitrogen at 30 °C. The residues after evaporation were dissolved in 250 μL of mo-bile phase, were transferred into vials for automated HPLC analysis, and 50 μL was injected into the system. Due to the previously reported instability and non-enzymatic conversion of AFMU towards AAMU [25], which was also observed by ourselves and confirmed by UPLC-ESI(+)-MS/MS, AAMU was assessed along with AFMU.

3.2. Chromatographic Conditions

HPLC separations were carried out in a Luna C18(2) re-versed phase analytical column (150 × 4.6 mm i.d.) protected with a SecurityGuard (8.0 × 3.0 mm i.d.), both packed with 5 μm particles (Phenomenex, Torrance, CA, USA). The mo-bile phase consisted of 15 mM sodium acetate (pH 5.0; ad-justed with 10% CH_3COOH) and methanol 85:15 (v/v) and was filtered through a 0.45 μm filter (Supelco, Bellefonte, PA, USA) before use. The flow rate was maintained at 0.8 mL/min. Absorbance of the eluent was monitored with use of diode array detector at absorption maximum of each ana-lyte in the mobile phase: 285 nm (AFMU; 1,7U; 1U), 272 nm (1,3,7X and 1,7X) and 268 nm (1X). The bandwidth of all wavelengths was 4 nm. All compounds, including the internal standard, were eluted within 18 min.

3.3. Method Validation

The method was validated for its selectivity, accuracy, precision, recovery, linearity and stability according to the

Table 1.   **Parameters of linearity of calibration curve.**

Analyte	Slope	Intercept	Determination Coefficient	Calibration interval [mg/l]
AFMU	216.98	102.5	0.999	0.119 – 40.0
AAMU	82.15	-3.57	0.991	0.089 - 30.0
1U	119.43	-33.04	0.999	0.077 - 51.7
1X	145.66	-27.30	0.999	0.072 - 48.9
1,7U	108.02	-25.61	0.999	0.0839 - 56.7
PAR (I.S.)	152.86	-28.14	0.999	0.104 - 35.0
1,7X	69.47	-14.51	0.999	0.094 – 31.7
1,3,7X	84.85	-23.95	0.999	0.128 – 43.4

principles of the relevant Food and Drug Administration Guidance [26].

3.4. Human Saliva and Urine Samples

Sixteen volunteers after at least 24 hours of abstinence from caffeine received orally a capsule with caffeine at a dose of 3 mg/kg at 07:00. Urine was collected in a glass bottle until 13:00. Aliquots (3x 500 µL) were drawn at 10:00 and 13:00. Saliva samples were taken at 10:00 and 13:00 into Salivette plastic tubes (Sarstedt AG & Co., Nümbrecht, Germany) and centrifuged at 2000 g for 10 minutes. Samples were frozen at -40 °C until analysis (max. 4 weeks).

4. RESULTS AND DISCUSSION

4.1. Extraction

The extraction procedure was optimized for extraction recovery and sensitivity. We tested diethylether (100 %), diethylether + isopropylalcohol 8.5 : 1.5, hexane + butanol 9 : 1 and chloroform + isopropylalcohol 8.5 : 1.5 (v/v). The best results were obtained using the latter mixture of solvents. Along with the extraction procedure, protein precipitation using 100 µL of acetonitrile + vortexing + centrifugation was also tested as a sample pre-treatment, but was evaluated as insufficient due to observed interferences. The extraction recoveries for all analytes were measured at three levels of concentration in spiked saliva and urine (Table **2**). Three

replicates of these samples were extracted and analyzed according to the method described above. Standard samples were prepared along with spiked saliva and urine samples by diluting standard stock solutions with the mobile phase. Recovery was calculated using the following equation: Recovery = (peak area after extraction / peak area of standard sample of the equal nominal concentration) x 100 (%). Despite the simple extraction, the extraction recoveries of all analytes except for AFMU, which is extremely unstable in solution, were within the acceptable range of 75.2 - 93.3 % when extracted from urine. Extraction recovery from saliva was in the range 75.8 - 95.3 %.

Compared to other methods for determining caffeine and metabolites in biological samples and utilizing liquid-liquid extraction [10], the extraction recoveries of the analytes were comparable, with the exception of AFMU. Nonetheless, if we assume that there is an equimolar conversion of AFMU to AAMU, and the metabolic activity of CYP1A2 is expressed by means of (17X+17U)/137X, (AAMU + AFMU + 1X + 1U)/17U or simply 1,7X/1,3,7X, this instability and thus analytical imprecision is of negligible clinical importance. Moreover, the differences in the recoveries of the analytes had negligible impact on the accuracy of the measured concentration due to the preparation of calibration samples (standards in blank urine/saliva) concurrent extraction with the experimental samples and use of an internal standard.

Table 2. Intraday and interday precision and accuracy data for caffeine and its metabolites in spiked urine, extraction recovery of liquid-liquid extraction of analytes from spiked samples.

Analyte	Nominal concentration (mg/L)	Intraday Accuracy (%)	Intraday RSD (%)	Interday Accuracy (%)	Interday RSD (%)	Saliva Extraction recovery (%)	Urine Extraction recovery (%)
AAMU	10.66	117.80	1.73	114.44	1.94	89.6	87.5
	1.78	118.20	3.64	123.80	1.59	88.7	86.7
	0.59	119.53	5.94	109.55	8.77	95.3	93.3
AFMU	8.00	90.30	0.49	92.02	8.66	58.3	54.3
	1.33	102.89	1.53	92.28	6.80	55.8	52.2
	0.44	99.76	8.23	93.76	5.88	58.3	56.9
1U	6.88	100.30	1.03	96.66	2.15	78.5	77.8
	1.15	108.63	7.31	101.24	1.82	79.2	75.2
	0.38	109.11	7.62	104.79	5.96	80.1	78.4
1X	6.52	103.80	0.17	100.37	1.74	88.2	87.5
	1.09	116.67	2.06	106.12	4.26	84.5	82.3
	0.36	122.81	7.92	109.04	3.30	83.6	80.6
1,7U	7.56	100.50	0.25	97.65	2.13	80.7	81.0
	1.26	103.77	2.00	101.67	1.08	82.4	84.2
	0.42	115.20	7.90	107.92	6.07	83.4	86.6
1,7X	9.32	101.60	0.27	98.10	1.83	87.0	84.8
	1.55	100.65	0.37	101.85	1.07	85.9	82.3
	0.52	106.74	7.31	105.92	7.36	82.9	78.9
1,3,7X	8.44	101.60	0.28	98.62	1.73	86.5	78.7
	1.41	102.62	1.44	101.29	1.03	91.6	82.0
	0.47	106.48	7.76	105.43	7.59	87.0	83.8

4.2. Optimization of the Separation

The HPLC method was optimized to obtain sufficient resolution with an acceptable analysis length. We tested several RP columns (Luna C18 150x4.6 mm, 5 μm; Kinetex C18, 150x4.6 mm 2.6 μm; 5 μm, Kinetex HILIC, 150x4.6 mm 2.6 μm and Gemini C18, 150 x 4.6 mm; 5 μm; Phenomenex, Torrance, CA, USA) and mobile phases (different proportions of methanol or acetonitrile + 0.1% acetic acid, methanol or acetonitrile + 10-15-25 mM acetate buffer pH 4.6 - 4.8 - 5.0 - 5.2 and acetonitrile + KH_2PO_4 buffer pH 4.0 - 4.5 - 4.8) with the proportion of organic phase ranging from 10 to 30 % and mobile phase flow from 0.4 to 1.8 mL/min. The best results in terms of resolution and single run length were obtained with the conditions described in section 3.2.

Despite its simple analytical instrumentation and conditions, the method enables good resolution with acceptable retention times: AAMU 2.3 min, AFMU 2.7 min; 1U 3.5 min; 1X 4.9 min; PAR 6.3 min; 1,7U 6.9 min, 1,7 X 8.9 min; 1,3,7X 17.9 min. Fig. (**2a**) shows a chromatogram of blank urine spiked with internal standard, Fig. (**2b**) shows a chromatogram of blank saliva spiked with internal standard. Typical chromatograms of human urine and saliva samples are shown in Fig. (**3a** and **3b**), respectively. In all of the chromatograms presented, the samples were extracted using the same liquid-liquid extraction procedure as described above in section 3.1.

Current clinical pharmacology still needs simple analytical tools for phenotyping major biotransforming enzymes, such as CYP1A2. CYP phenotyping remains relevant in some clinically difficult situations, where simple therapeutic drug monitoring (TDM) cannot provide a satisfactory explanation of non-response or drug toxicity in drugs being
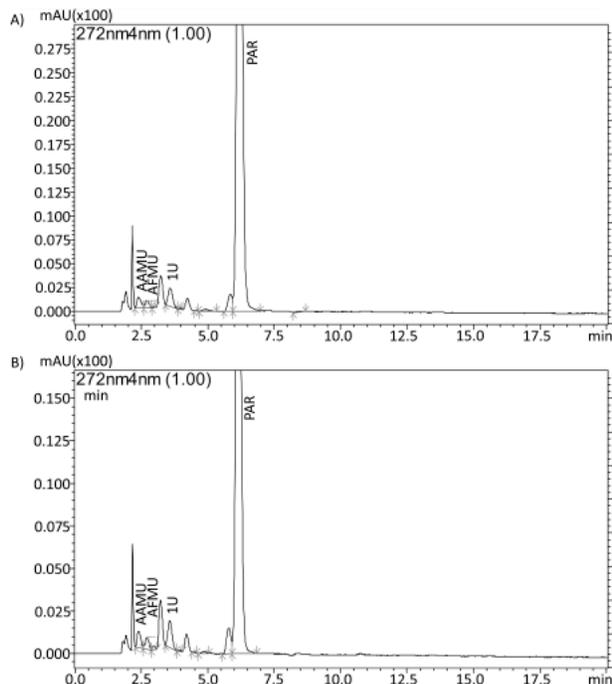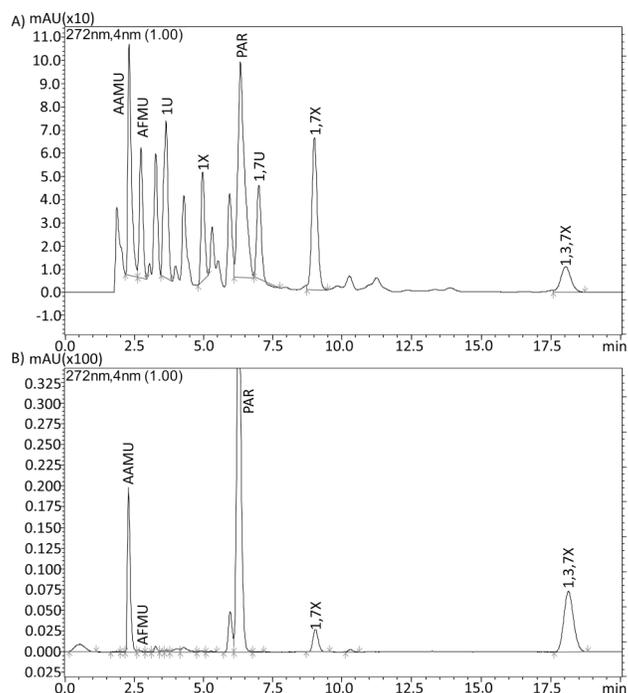

Fig. (3). Typical chromatograms of **a**) human urine and **b**) saliva samples drawn 6 h after caffeine administration.

metabolized by polymorphic enzyme, or the suspected induction/inhibition of CYP enzymatic activity which occurs with co-medication [27]. Analytical methods for clinical practice need to be precise, repeatable, robust, easy to use and affordable. The separation of analytes in our method is good - resolution was at least 1.75 for AAMU and AFMU, in other analytes it is much greater. The analysis length is less than 18 minutes, which is indisputably worse than LC-MS methods, but in light of the number of analytes measured (7+ IS) and compared to other LC-UV methods, is acceptable and allows for routine use in a clinical setting. Moreover, method does not require expensive instrumentation, such as MS detector.

4.3. Linearity

The linearity of the detector response for the solutions of standards in the mobile phase was evaluated using a linear regression model; regression lines were constructed by plotting peak area to concentration in three replicates at six levels of concentration. The parameters of the linear regression equations of detector response [mAU.s] versus concentration [mg/L] are presented in Table **1**.

4.4. Precision and Accuracy

To determine the intra- and inter-day precision and accuracy of the assay, replicate sets (n = 6 for intraday and n = 6 consecutive days for interday, respectively) of three concentrations of each analyte in blank urine were analyzed. Precision was calculated as intra- and inter-day RSD values, and accuracy was expressed as (measured concentration x 100) / nominal concentration [%]. The intraday variations for the measured analytes were < 8.2 %. The interday variations were < 8.8 %, respectively (Table **2**).

Fig. (2). a) chromatogram of blank urine spiked with internal standard b) chromatogram of blank saliva spiked with internal standard.

4.5. Limit of Detection

To determine the limit of detection and limit of quantitation (LOD, S/N > 3:1, LOQ , S/N > 10:1), spiked blank urine and saliva samples were analyzed at the lowest concentration level used for linearity evaluation. Chromatograms from the assay of blank urine and saliva samples were used to establish the "background noise" in the assay. The LODs in saliva were in the range 2.16 (1X) - 22.25 µg/L (AFMU), the LOQs in saliva were in the range 7.2 (1X) - 74.17 µg/L (AFMU). The LODs in urine were in the range 2.52 (1X) - 24.72 µg/L (AFMU) and the LOQs in urine were in the range 8.40 - 82.41 µg/L. The LOD and LOQ are mentioned in the supplementary material (Table S1). These limits of detection and quantitation proved to be sufficient for common CYP1A2 metabolic activity determination, when caffeine is administered at a dose of 1-4 mg/kg and concentrations of caffeine and metabolites observed by ourselves (at the dose of 3 mg/kg) in urine were in units of mg/L. In saliva, concentrations of 1,3,7X and 1,7X are still 3 to 20 times higher than the LOQ, but some metabolites of caffeine in some of the saliva samples (3 or 6 hours after caffeine administration), namely 1X, 1U, 1,7U are close to or below the LOQ. Nevertheless, ratios other than 1,7X to 1,3,7X are not routinely evaluated in saliva samples. The limits of detection of this method are, in general, comparable with the previously published HPLC-UV methods [10, 28], but inferior to LC-MS methods [8, 12, 24] or HPLC with electrochemical detection [29].

4.6. Stability of Sample Extracts

The stability of analytes was evaluated as the percentage of peak area of distinct analyte in the extract of blank saliva samples spiked with standards (at medium concentration level mentioned in Table **1**, which ranged from 1.09 to 1. 93 mg/L) after 7 days in the dark at 4°C. The stability in the mobile phase was poor for AFMU and AAMU, where a 28% increase in the peak area of AAMU was observed, and a 22% decrease in the peak area of AFMU compared with the peak areas of freshly prepared extract (considered as 100 %). In all the other analytes, the stability was acceptable, with the lowest peak area in 1U (96 % of original) and the highest peak area in 1,7X (109 % of original) after 7 days of storage. The data are available in supplementary material (Fig. **S2**).

The instability of AFMU does not allow for sample storage, and thus immediate analysis is necessary once extraction is performed. Similar findings on AFMU instability were also reported by others [12, 25].

4.7. Selectivity

The selectivity (non-interference with endogenous compounds) was tested with pooled blank samples spiked with standards at the concentration near to the LOQ (10 µg/L of each analyte). Moreover, the non- interference with some of frequently used medication (acetylsalicylic acid, diazepam, diclofenac, fluoxetine, ketoconazole, prednisone, alprazolam, phenacetine, chlorpropamide) was proved in the same manner with use of standards of above mentioned drugs.

4.8. Data Obtained from Experimental Samples

This method was used to determine the molar metabolic ratios of caffeine to its metabolites in human saliva and human urine samples obtained from 0-3 h and 0-6 h (urine) or the single point sample collections at 3 h and 6 h (saliva) after a single dose of caffeine (3 mg/kg). Molar metabolic ratios were calculated (1,7X/1,3,7X and AAMU+AFMU+1U+1X/1,7U) in 16 subjects. Descriptive statistical methods were performed for the analysis of metabolic ratios in urine and saliva. Using a linear regression model, a good correlation was found between the 0-3 h urinary and 3 h salivary molar 1,7X/1,3,7X ratio, with the regression equation: y = 0.0744 + 0.093x; r = 0.8553; p = 0.00005 where y = salivary 1,7X/1,3,7X and x = urinary 1,7X/1,3,7X ratio. Similarly, we observed a correlation between the 0-6 h urinary and 6 h salivary molar 1,7X/1,3,7X ratio, with the regression equation: y = 0.1187 + 0.1325x; r = 0.7279; p = 0.0014.

Based on the papers of Perera, Faber and Fuhr [14, 20, 30, 31], the salivary ratio of 1,7X to 1,3,7X has proved its applicability in CYP1A2 metabolic activity assessment. We have confirmed a good correlation between the salivary and urinary 1,7X to 1,3,7X metabolic ratio reported by others [8, 13, 14, 31-33] which could allow for CYP1A2 phenotype determination with the use of saliva samples in clinical settings. Moreover, according to Bendriss *et al.* [10], this method could be also used for determining the metabolic activity of N-acetyltransferase 2 (NAT2), CYP2A6 and xanthine oxidase, since the ratio of 1U/1X+1U is considered a measure of xanthine oxidase metabolic activity, AFMU/(AFMU+1X+1U) as a measure of NAT2 metabolic activity and 17U/(AFMU+1X+1U+1,7X+1,7U) as a measure of CYP2A6 metabolic activity. Since Perera *et al.* reported that a 24h methylxanthine-free diet has a negligible impact on apparent caffeine to paraxanthine clearance and paraxanthine clearance, when compared to no methylxanthine-free diet [34], CYP1A2 phenotyping methods which utilize caffeine are clinically applicable, even if the subjects fail to abstain from methylxanthines.

An advantage of this method is that the analysis can be carried out in a standard analytical laboratory and no special equipment is needed. Our method is simple, cost-effective, has a proven sufficient sensitivity comparable with other LC-UV methods, and enables the assessment of various caffeine metabolic ratios in urine, and without any modification also the salivary caffeine to paraxanthine ratio. The method was fully validated in terms of linearity, precision, accuracy, extraction recovery and the stability of analytes. It may be easily used on any HPLC-UV instrumentation by clinicians or laboratory technicians. The method was successfully applied in the analysis of real samples and therefore has been shown to be clinically useful.

CONCLUSION

Our method has several advantages over published methods. First, the method enables the determination of CYP1A2 metabolic activity using either urine or saliva samples. Nonetheless, the method is universal and can be also utilized for the assessment of CMR, xanthinoxidase xanthinoxidase or NAT2 metabolic activity in urine samples. Second, de-

termination may be performed by laboratory technicians, with a conventional HPLC system with a DAD detector with sufficient sensitivity for clinical usage.

LIST OF ABBREVIATIONS

CYP = cytochrome P450 (e.g. CYP1A2 = 1A2 enzyme of cytochrome P450 family)

NAT2 = N- acetyltransferase 2

PAR = paracetamol, acetaminophen, N-(4-hydroxyphenyl)acetamide

1,3,7X = caffeine, 1,3,7-trimethylpurine-2,6-dione

1,7X = paraxanthine, 1,7-dimethyl-3-hydropurine-2,6-dione

1X = 1-methylxanthine, 1-methyl-3,7-dihydropurine-2,6-dione

1,7U = 1,7-dimethyluric acid, 1,7-dimethyl-3,9-dihydropurine-2,6,8-trione

1U = 1-methyluric acid, 1-methyl-7,9-dihydro-3H-purine-2,6,8-trione

AFMU = 5-acetylamino-6-formylamino-3-methyluracil

AAMU = 5-acetylamino-6-amino-3-methyluracil

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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6.2   Phenotyping of CYP2D6 with dextromethorphan as a probe substrate

Despite CYP2D6 representing only approximately 4% of the total P450 content in the liver, it is involved in the metabolism of 15 - 25% of all drugs used in clinical practice (Zanger and Schwab, 2013). However CYP2D6 is non-inducible, there is an up to 100-fold difference in inter-individual activity (Ning et al., 2018). Opioids, antidepressants, antidysrhythmics, or β blockers are examples of substrates of CYP2D6. Both ultrarapid and poor metabolizers can be at risk of adverse effects of CYP2D6 drug substrates due to the increased activation of opioids (codeine, tramadol) or slow elimination of antidepressants (venlafaxine, fluoxetine) (Haufroid and Hantson, 2015). Both CYP2D6 genotyping and phenotyping is used for the optimization of dosaging.

Dextromethorphan O-demethylation is a well established and safe probe substrate for both *in vitro* and *in vivo* CYP2D6 phenotyping. The urine MR of dextromethorphan to dextrorphan is widely used for evaluating the CYP2D6 phenotype, while plasma or salivary MR are used rarely. The correlation between serum and urine MR was studied in our trial in 30 healthy volunteers. Dextromethorphan was administered orally (30 mg) in a syrup, and urine was collected for the following eight hours. Blood was sampled three hours after the administration of the probe substrate. The samples were enzymatically hydrolysed with *β*-glucuronidase, extracted by two-step liquid-liquid extraction and dextromethorphan and its metabolites dextrorphan, methoxymorphinan, and hydroxymorphinan were analysed by HPLC. A tight non-linear correlation between serum and urine MR was described. The cut-off values for distinguishing between the phenotypes (poor, intermediate, and extensive metabolizers) were calculated using receiver operating characteristic analysis, in the second part of the study. Levels of dextromethorphan and dextrorphan were analysed in serum samples of 30 healthy volunteers and 51 psychiatric drug-naïve patients. The cut-off values for the group of poor metabolizers to extensive or combination of extensive and intermediate metabolizers were calculated to be in the range between 0,215-0,742, with 100% sensitivity and specificity. The distinguishing cut-off value for the groups of extensive metabolizers and of intermediate + poor metabolizers, as well as for the cut-off between extensive and intermediate metabolizers, was 0,02081. Nevertheless, the sensitivity and specificity of these values decreased to 70.03% +70.37% and 61.9% + 70.83%, respectively.

Juřica J., Barteček R., Žourková A., Pindurová E., Šulcová A., Kašpárek T., **Zendulka** O. Serum dextromethorphan/dextrorphan metabolic ratio for CYP2D6 phenotyping in clinical practice. Journal of Clinical Pharmacy and Therapeutics, 37, 486-490, 2012.

Author's contribution: 10%, corresponding author

Pharmacogenetics

Serum dextromethorphan/dextrorphan metabolic ratio for CYP2D6 phenotyping in clinical practice

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SUMMARY

What is known and Objective: **Accurate prediction of actual CYP2D6 metabolic activity may prevent some adverse drug reactions and improve therapeutic response in patients receiving CYP2D6 substrates. Dextromethorphan-to-dextrorphan metabolic ratio ($MR_{DEM/DOR}$) is well established as a marker of CYP2D6 metabolizer status. The relationship between urine and plasma or serum $MR_{DEM/DOR}$ is not well established nor is there evidence of antimode for separation of intermediate and especially poor metabolizers (PM) from extensive metabolizers (EM). This study addressed whether CYP2D6 phenotyping using molar metabolic ratio of dextromethorphan to dextrorphan ($MR_{DEM/DOR}$) in serum is usable and reliable in clinical practice as urinary $MR_{DEM/DOR}$.**
Methods: **We measured $MR_{DEM/DOR}$ in serum and CYP2D6 genotype in 51 drug-naive patients and 30 volunteers. Receiver-operator characteristic (ROC) analysis was used for the evaluation of optimum cut-off value for discriminating between extensive, intermediate and PM. In addition, we studied the correlation of serum $MR_{DEM/DOR}$ with urine $MR_{DEM/DOR}$ in the 30 healthy volunteers.**
Results and Discussion: **A trimodal distribution of log $MR_{DEM/DOR}$ in serum was observed, with substantial overlap between extensive and intermediate metabolizer groups. We obtained an acceptable cut-off serum $MR_{DEM/DOR}$ value to discriminate between PM and either extensive or extensive + intermediate metabolizers. Using serum $MR_{DEM/DOR}$, it seems to be unreliable to discriminate EM from intermediate metabolizers (IM). A strong correlation between serum $MR_{DEM/DOR}$ and urine $MR_{DEM/DOR}$ was found.**
What is new and Conclusion: **Serum $MR_{DEM/DOR}$ (3 h) correlated with $MR_{DEM/DOR}$ in urine (0–8 h). Serum $MR_{DEM/DOR}$ discriminated between extensive and PM and between extensive + intermediate and PM. Our CYP2D6 phenotyping using serum dextromethorphan/dextrorphan molar ratio appears reliable but requires independent validation.**

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WHAT IS KNOWN AND OBJECTIVE

CYP2D6 plays a pivotal role in the metabolism of many drugs. It is a highly polymorphic enzyme, and to date, more than 80 allelic variants alleles have been identified.[1] The variant alleles may encode proteins with normal (e.g. *CYP2D6*2*), decreased (e.g. *CYP2D6*10*) or no (e.g. *CYP2D6*4*) enzyme activity. As a consequence, there is a wide range of CYP2D6 metabolic activity shows wide inter-subject variability. The CYP2D6 metabolic phenotype can be classified into four groups – poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizers (UM).[2] Thus, the same dose of a drug extensively metabolized by CYP2D6 can result in a different clinical response in patients depending on the P450 phenotype.[2] Moreover, CYP2D6–drug interactions may also have clinical consequences. For example, after administration of paroxetine, a CYP2D6 inhibitor, to patients on tamoxifen, a decrease in plasma levels of the active metabolite of tamoxifen was detected.[3] This change may have serious consequences in breast cancer treatment with tamoxifen. CYP2D6 ultrarapid metabolizer phenotype may cause failure of pharmacotherapy because of the very low and thus ineffective drug plasma levels.[4] In antipsychotic treatment, an association has been observed between extrapyramidal adverse effects and the CYP2D6 genotype.[5] Therefore, for drugs with narrow therapeutic indices, it seems to be useful to assess CYP2D6 metabolic activity prior to or during therapy with CYP2D6 substrates and to adjust the individual dosage according to the patient's phenotype. Such personalized pharmacotherapy may prevent adverse effects and improve response. Specific substrates (markers) that are metabolized selectively by CYP2D6 are often used for the assessment of metabolic activity. The concentration of specific substrate and its metabolite in body fluids (a ratio of molar concentrations; metabolic ratio) serves as a measure of the individual CYP activity. Histograms of log-transformed metabolic ratios may show cut-off values of MR which distinguish EM from PM, UM or IM. Suitable substrates for CYP2D6 include debrisoquine, bufuralol, tramadol, dextromethorphan, metoprolol and sparteine.[2,6] Debrisoquine and sparteine are not currently available, and except for dextromethorphan, the other substrates are seldom administered. Dextromethorphan remains the most widely used probe for CYP2D6 metabolic activity

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assessment in vivo.[6] The ratio of molar concentrations of dextromethorphan to dextrorphan ($MR_{DEM/DOR}$) serves as a measure of metabolic activity and phenotype of CYP2D6. A cut-off value for 8-h urinary $MR_{DEM/DOR}$ of 0·3 is widely used to differentiate between extensive and poor metabolizers.[7–9] As 8-h sampling of urine is demanding, alternative procedures have been developed. Saliva or plasma samples can also be used for the determination of $MR_{DEM/DOR}$.[7,10] A non-invasive CYP2D6 phenotype test was developed by Hu et al.[10] using single or multiple doses of controlled-release dextromethorphan tablets. Although these authors have demonstrated that there is a good correlation between single-dose and multiple-dose dextromethorphan as well as between $MR_{DEM/DOR}$ assessed in various kinds of samples, they did not investigate the cut-off value for discriminating EM from PM.[10] Indeed, only two studies have investigated this cut-off value; Shiran et al.[11] suggested a value of 0·1 for differentiating between EM and PM using 3 h post-dose $MR_{DEM/DOR}$, whereas Kohler et al.[12] suggested a value of 0·126 using 1 h post-dose $MR_{DEM/DOR}$. However, the relationship between urine and plasma or serum $MR_{DEM/DOR}$ is not well established, and there is as yet no reliable evidence of antimode for identification of IM and especially PM, from EM patients.

Our aim was to assess the correlation between $MR_{DEM/DOR}$ in serum (3 h) and $MR_{DEM/DOR}$ in urine (0–8 h) and to identify the serum $MR_{DEM/DOR}$ cut-off value for distinguishing between EM, IM and PM.

METHODS

Fifty-one drug-naive patients and 30 healthy volunteers were studied. Participation was voluntary. The study was performed in accordance with the Helsinki declaration, revision 2008, and approved by the ethics committee of the Faculty Hospital Brno. All subjects had given their written informed consent prior to the start of any activities connected with the study.

Inclusion criteria for patients were as follows: treatment at the Department of Psychiatry of Masaryk University, Brno; male and female patients aged 18–70 years; diagnosis of schizophrenia/depression/anxiety disorder according to ICD X; no medication known to affect cytochrome P450 enzyme activity; and no previous pharmacological treatment of schizophrenia/depression/anxiety disorder. Exclusion criteria were the following: patients treated with substrates or inhibitors of CYP2D6; serious somatic condition; patients with risk of suicidal behav-

iour; and pregnant and breastfeeding women. Inclusion criteria for healthy volunteers were men and women aged 18–70 years of age. Exclusion criteria were treatment with substrates or inhibitors of CYP2D6; serious somatic condition; and pregnant and breastfeeding women. All the subjects were Caucasians.

Molecular genetic analysis: Peripheral blood samples were taken from patients into tubes with EDTA. DNA was isolated from lymphocytes using standard procedure of phenol chloroform precipitation or by means of an automatic isolator (Roche, Indianapolis, IN, USA). As a main method, direct automatic sequencing of all the nine exons of the CYP2D6 gene using seven specific pairs of primers was performed using the Primer3plus software (Free Software Foundation, Inc., Boston, MA, USA). Because of the localization of two pseudogenes in close proximity to the CYP2D gene, the product generated by Long Range PCR (Analytik Jena/bio solution, Jena, Germany) was used as a template for sequencing and genotyping.[13] The DNA sequences were compared with the standard sequences, and the resultant allele combinations were evaluated.

Real-time PCR and specific FRET probes were used (TibMol-Biol, Berlin, Germany) as a complementary method for the detection of the most frequent null alleles *3,*4,*6,*7,*8 in Caucasian population.[14] Subsequently, real-time PCR method using FRET probes was replaced by high-resolution melting analysis (Roche). This approach helps us to assess the PM faster than standard sequencing and cheaper than FRET probes. By using high-resolution melting analysis, we could distinguish standard subjects (wt/wt) from variant homozygotes and heterozygotes. Pairs of primers (designed in our laboratory, sequences and lengths of primers are given in Table 1) and an intercalation dye that binds on dsDNA ensured production of very short sequences (max. 148 kb).

Large gene deletions and duplications were detected using agarose electrophoresis or UPL probes (Roche).[15] Subjects carrying a combination of null/null alleles were classified as genetically PM. Subjects with null/full function alleles, null/reduced function alleles or reduced function/reduced function alleles were classified as IM, and subjects carrying either full function/full function alleles or full function/reduced function alleles were classified as EM.

Phenotype analysis: Dextromethorphan hydrobromide (Robitussin syrup; Wyeth, Wien, Austria) in syrup available as OTC drug was administered to subjects orally at 07:00 h at the dose of 30 mg after overnight fasting. Blood samples (10 mL) were

Table 1. Primer sequences for CYP2D6 established alleles (Primer 3 plus free software used as source of all sequences)

Allele	Main nucleotide change	Sequence of primers	Product length
ALLELE *4	1846G>A	F: ACCCCTTACCCGCATCTC R: TTGCTCACGGCTTTGTCC	74 bp
ALLELE *9	2613-2615delAGA	F: GACCTGACTGAGGCCTTCCT R: CTCAACCCACCACCCTTG	80 bp
ALLELE *41	2988G>A	F: GAGCCCATCTGGGAAACAGT R: GGTGTCCCAGCAAAGTTCAT	77 bp
ALLELE *3	2549deA	F: TTCCAAAAGGCTTTCCTGAC R: AAGGCCTCAGTCAGGTCTCG	98 bp
ALLELE *10	100C>T	F: TAGTGGCCATCTTCCTGCTC R: ACCTGGTCGAAGCAGTATGG	148 bp
ALLELE *7	2935A>C	F: GCCTCCTGCTCATGATCCTA R: GTTTCCCAGATGGGCTCAC	55 bp
ALLELE *6 + *8	1758G>T + 1707delT	F: CAAGAAGTCGCTGGAGCAG R: TCCCAGTTCCCGCTTTGT	110 bp

drawn 3 h after ingestion of dextromethorphan (in both patients and volunteers) into BD Vacutainer SST tubes for serum separation. Urine was collected for 8 h after dextromethorphan administration into glass screw-capped tubes. Urine was collected only for volunteers and not for patients because of the unreliability of collection (particularly in schizophrenic patients). Blood samples were centrifuged 10 min at 1000 g at 4 °C. Serum aliquots were transferred into a screw-capped polypropylene tube and were frozen at −75 °C until HPLC analysis. Collected urine samples for each subject were pooled, the volume was measured, and the aliquots were frozen at −75 °C until analysis.

The analysis was carried out according the method of Zimova et al.[16] Prior to the analysis, the samples were enzymatically hydrolysed with β-glucuronidase and extracted with a mixture of n-hexane/1-butylalcohol (9 : 1) and re-extracted into 0·1 M HCl (all chemicals were from SigmaAldrich, Darmstadt, Germany). Aliquots were injected in a Shimadzu LC-10 series HPLC system (Shimadzu, Kyoto, Japan). A Tessek Separon SGX phenyl (150 × 3 mm, 5 μm) reversed-phase analytical column protected with guard column Tessek SGX phenyl (4 × 40 mm; 5 μm) (both Tessek, Prague, Czech Republic) was used for the separation. Dextromethorphan, its metabolites dextrorphan, methoxymorphinan, hydroxymorphinan and internal standard laudanosine were detected using fluorescence detector RF-10Axl (Shimadzu), the excitation wavelength was set at λ_{ex} = 280 nm, and the emission wavelength was set at λ_{em} = 320 nm. Data from the detector were collected and analysed using LabSolution 1.03 SP3 software (Shimadzu). Limits of detection (signal-to-noise ratio = 3) for dextromethorphan and dextrorphan were 186 and 204 ng/L, respectively. Coefficients of variation for intra- and interday precision of assessment were below 10%. Molar concentrations of dextromethorphan and dextrorphan in serum and urine samples were measured and $MR_{DEM/DOR}$ calculated.

Receiver-operator characteristic (ROC) analysis was undertaken to find the $MR_{DEM/DOR}$ cut-off values for separating EM/IM/PM. Cut-off values were assessed on the basis of the highest AUC of the ROC curve.

RESULTS AND DISCUSSION

Histograms of log-transformed serum and urine $MR_{DEM/DOR}$ values are shown in Figs 1 and 2. As can be seen, a bimodal distribution of urine $MR_{DEM/DOR}$ was observed in volunteers, and a trimodal distribution of serum $MR_{DEM/DOR}$ was observed in drug-naive patients with substantial overlap between the EM and IM groups. Despite this, the $MR_{DEM/DOR}$ values for the two groups were different (t test, P = 0·017).

Genotype was analysed in 51 patients and 30 volunteers. Analysis of genotype in 51 patients proved that six subjects were classified and grouped as PM with genotype of *4*4 or *4*5(3-h serum $MR_{DEM/DOR}$ 0·74–4·98), 21 subjects were classified as IM because of their genotype *1*4, *1*3, *4*41, *4*2, *4*10, *10*41, *41*41 (3-h serum $MR_{DEM/DOR}$ 0·0033–0·2133), and the remainder of the subjects were shown to be EM with genotypes *1*1, *1*2, *1*41, *1*9, *2*9, *2*2, *2*41 (3-h serum $MR_{DEM/DOR}$ 0·0032–0·1062).

Only two PM with genotypes *4*4 and *3*4 and with respective 8-h urine $MR_{DEM/DOR}$ of 1·58 and 1·59 were observed. Interestingly, the highest 8-h urine $MR_{DEM/DOR}$ of 30·1 (serum $MR_{DEM/DOR}$ 3·4) was observed in a subject classified as IM based on his *1*4 genotype. Nine subjects were classified as IM
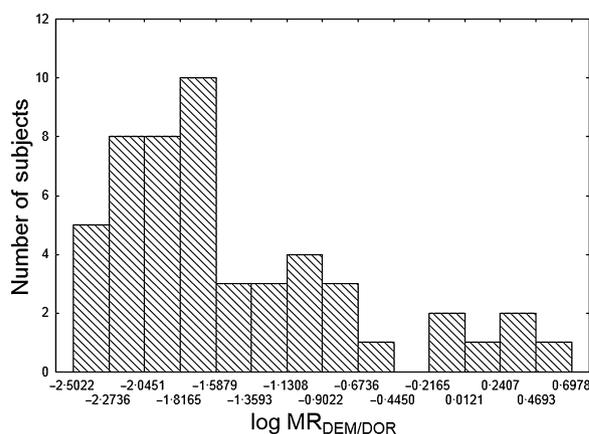

**Fig. 1.** Histogram of log-transformed serum $MR_{DEM/DOR}$ of 51 drug-naive patients.
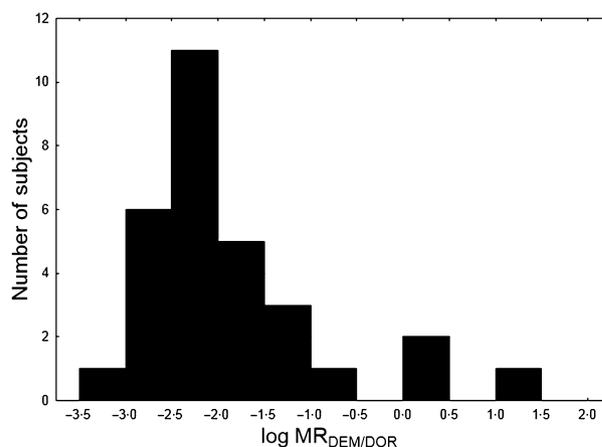
**Fig. 2.** Histogram of log-transformed urine $MR_{DEM/DOR}$ of 30 volunteers.

with genotypes *1*4 or *1*3 (8-h urine $MR_{DEM/DOR}$ 0·00465–30·1), and the other subjects as EM, carrying genotypes *1*1, *1*2, *1*41 or *1*9 (8-h urine $MR_{DEM/DOR}$ 0·00094–0·0819).

Cut-off values were assigned based on the CYP2D6 genotype and serum $MR_{DEM/DOR}$ of 51 drug-naive patients (Fig. 3). From the ROC analysis, the cut-off value for discriminating IM + PM from EM was 0·02081 with the specificity of 70·03% and the sensitivity of 70·37%. The cut-off value for discriminating EM from IM only was also 0·0208 but with only 61·9% sensitivity and 70·83% specificity.

The cut-off value for discriminating IM from PM was between serum $MR_{DEM/DOR}$ 0·215 and 0·742 (100% sensitivity, 100% specificity, AUC of 1·0 within the range). Similarly, the cut-off value for discriminating PM from IM + EM was in the range 0·21–0·742 (100% sensitivity, 100% specificity, AUC of 1·0). There was good correlation of log $MR_{DEM/DOR}$ in serum with that of urine based on 30 volunteers (r^2 = 0·87).
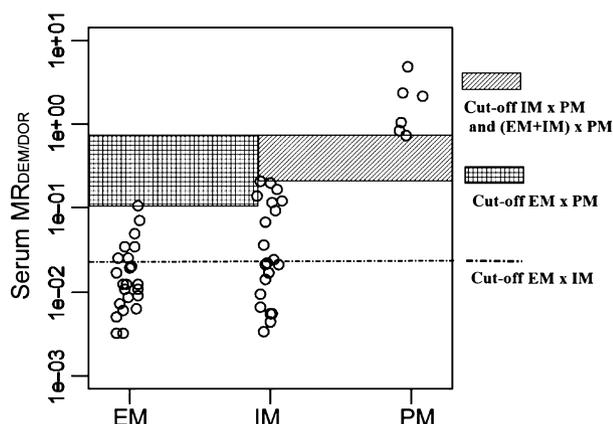
Fig. 3. Grouping of patients according to their genotype and serum $MR_{DEM/DOR}$ in cut-off value assessment.

DISCUSSION

The CYP2D6 genotype does not always predict the metabolic phenotype reliably.[11,17,18] Thus, metabolic activity assessment may be preferable for predicting therapeutic response. The CYP2D6 metabolic phenotype is mostly assessed using urinary $MR_{DEM/DOR}$ (0–8 h post-dose), because it does not require invasive procedure. Collection of urine for 8 h is inconvenient and often not reliable especially in psychiatric patients. Besides urinary samples, serum and salivary samples have also been used for $MR_{DEM/DOR}$ determination.[10,19,20] For salivary samples, greater doses of dextromethorphan are needed,[19] and there is no consensus on the cut-off for discriminating EM from PM.

Moreover, the usefulness of salivary CYP2D6 testing with dextromethorphan seems to be inferior to serum-/urine-based testing.[10,20]

Bimodal or trimodal distribution of the log-transformed urinary $MR_{DEM/DOR}$ is normally observed with dextromethorphan, debrisoquine or sparteine CYP2D6 phenotyping.[2,9,16,21,22] In our study, the distribution of both serum and urine log-transformed $MR_{DEM/DOR}$ was similar. The distribution of serum log $MR_{DEM/DOR}$ demonstrates[11,12] that it can be used for phenotype assessment. A good correlation exists between serum $MR_{DEM/DOR}$ and urine $MR_{DEM/DOR}$. It is well known that the metabolic phenotype of P450 enzymes may be responsible for the high variance seen in plasma concentrations of drug substrates and consequently in their clinical response too.[23] In our opinion, at least some patients may benefit from CYP2D6 phenotyping prior to pharmacological treatment.

WHAT IS NEW AND CONCLUSION

Our data suggest that serum $MR_{DEM/DOR}$ can be used to discriminate PM from either extensive or extensive + intermediate metabolizers, but not EM from IM. However, our results need independent validation.

ACKNOWLEDGEMENT

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7   Conclusions

The system of P450 enzymes plays a crucial role in drug safety and efficacy. Research on this topic is ongoing, and this habilitation thesis adds one of the pieces to the final puzzle of P450. We have reported an interaction between memantine and fluoxetine at the level of CYP2D2 in rats. The activity of this enzyme was inhibited, and there was a difference in the effect between the *in vitro* and *in vivo* model. Similarly, crocin and quercetin decreased the metabolic activity of P450, whereas an increased activity of P450 enzymes was documented after the administration of safranal. The effect of *trans*-resveratrol on the activity of P450 seems to be enzyme- and sex-dependant. Most of our experiments are carried out on animal models and enzymes, therefore their clinical relevance should be carefully interpreted with respect to the known interspecies differences between rat and human P450.

An important part of understanding the P450 system is its endogenous regulation. The bile acids are an excellent example of how the P450 system is involved in the biosynthesis of endogenous metabolites on one hand and how these products can influence P450 activity on the other. The endocannabinoid system seems to be also involved in the endogenous regulation of P450 activity.

The clinical outcomes of the research included in these theses are represented by two methods for P450 phenotyping. Phenotype assessment should be the most relevant method for optimizing drug dosaging, assuming P450 to be an essential enzyme for the pharmacological effect of administered drugs.

The more knowledge about the function, regulation, and interactions of P450 we gain, the safer and more effective pharmacotherapy with its substrates can be, especially with the drugs with narrow therapeutic ranges.

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