Toxicology Letters 258 (2016) 55–70

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

What is the contribution of human FMO3 in the *N*-oxygenation of selected therapeutic drugs and drugs of abuse?

Lea Wagmann[a], Markus R. Meyer[a,b], Hans H. Maurer[a,*]

[a] Department of Experimental and Clinical Toxicology, Saarland University, Homburg, Germany
[b] Department of Clinical Pharmacology and Pharmacoepidemiology, Heidelberg University Hospital, Heidelberg, Germany

HIGHLIGHTS

- Single enzyme incubations best suited for FMO3 substrate identification.
- Integration of FMO3 in the relative activity factor (RAF) approach possible.
- Participation of FMO3 and CYPs in the *N*-oxygenation of therapeutics and DOAs.
- Different contributions of FMO3 to the hepatic clearances of the *N*-oxygenated DOAs.

ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form 12 June 2016
Accepted 13 June 2016
Available online 15 June 2016

Keywords:
FMO3
CYPs
Drugs of abuse
Relative activity factor approach
Hepatic clearance

ABSTRACT

Little is known about the role of flavin-containing monooxygenases (FMOs) in the metabolism of xenobiotics. FMO3 is the isoform in adult human liver with the highest impact on drug metabolism. The aim of the presented study was to elucidate the contribution of human FMO3 to the *N*-oxygenation of selected therapeutic drugs and drugs of abuse (DOAs). Its contribution to the in vivo hepatic net clearance of the *N*-oxygenation products was calculated by application of an extended relative activity factor (RAF) approach to differentiate from contribution of cytochrome P450 (CYP) isoforms. FMO3 and CYP substrates were identified using pooled human liver microsomes after heat inactivation and chemical inhibition, or single enzyme incubations. Kinetic parameters were subsequently determined using recombinant human enzymes and mass spectrometric analysis via authentic reference standards or simple peak areas of the products divided by those of the internal standard. FMO3 was identified as enzyme mainly responsible for the formation of *N*,*N*-diallyltryptamine *N*-oxide and methamphetamine hydroxylamine (>80% contribution for both). A contribution of 50 and 30% was calculated for the formation of *N*,*N*-dimethyltryptamine *N*-oxide and methoxypiperamide *N*-oxide, respectively. However, FMO3 contributed with less than 5% to the formation of 3-bromomethcathinone hydroxylamine, amitriptyline *N*-oxide, and clozapine *N*-oxide. There was no significant difference in the contributions when using calibrations with reference metabolite standards or peak area ratio calculations. The successful application of a modified RAF approach including FMO3 proved the importance of FMO3 in the *N*-oxygenation of DOAs in human metabolism.

1. Introduction

Monooxygenases are responsible for the major part of the human phase I metabolism and convert xenobiotics to more hydrophilic compounds usually leading to inactivation (Cashman, 2005; Zollner et al., 2010). These monooxygenases can be grouped into different enzyme families such as the cytochrome P450 monooxygenases (CYPs) and the flavin-containing monooxygenases (FMOs) (Torres Pazmino et al., 2010). CYPs catalyze oxygenation mainly of carbon but also of nitrogen and sulfur. FMOs usually oxygenate soft nucleophiles, in particular nitrogen, sulfur, phosphorous, and selenium atoms. Both microsomal systems have in common, that they transform a wide range of heteroatom-containing substrates (Cashman, 2005; Cruciani et al., 2014).

* Corresponding author.
E-mail address: hans.maurer@uks.eu (H.H. Maurer).

http://dx.doi.org/10.1016/j.toxlet.2016.06.013
0378-4274/

In comparison to the extensively studied CYPs, FMOs have long been neglected in metabolism investigations. In addition, experimental conditions were usually chosen to optimize CYP activity rather than the activity of other oxidative enzymes leading to underestimation of non-CYP reactions (Strolin et al., 2006). Recently, Fan et al. asked for further investigations that dissect the relative contribution of FMOs compared to CYPs (Fan et al., 2016). Hence, only limited information about interactions of FMO with therapeutic drugs and drugs of abuse (DOAs) in particular is available. Only the oxygenation of amphetamine and methamphetamine by human FMO was described so far, leading to reactive metabolites (Cashman et al., 1999; Szoko et al., 2004). Cashman et al. reported that amphetamine and methamphetamine were *N*-oxygenated by FMO3, the major FMO isoform in the adult human liver. The formed hydroxylamines were also identified as FMO3 substrates leading to *N,N*-dihydroxylation followed by conversion to phenylpropanone oxime and phenylpropanone, respectively. Particularly the first step may pose a significant toxicological threat due to the potential toxic nature of free hydroxylamine (Cashman et al., 1999).

Therefore, the aim of the presented study was to elucidate the contribution of FMO3 to the human, hepatic metabolism of 12 selected, structurally different therapeutic drugs and eight DOAs. Its contribution to the in vivo hepatic net clearance of the *N*-oxygenation products should be calculated by application of an extended relative activity factor (RAF) approach (Crespi and Miller, 1999; Venkatakrishnan et al., 2001) to differentiate from contribution of CYP isoforms. Finally, possible differences in the contributions should be assessed by using calibrations with reference metabolite standards or peak area ratio (PAR) calculations.

2. Materials and methods

2.1. Chemicals and enzymes

N,N-Dimethyltryptamine (DMT) was obtained from THC Pharm (Frankfurt, Germany), methadone from Lipomed (Weil am Rhein, Germany), trimipramine-d_3 and norclozapine-d_8 from Promochem (Wesel, Germany), R,S-methamphetamine, 1-aminobenzotriazole (ABT), isocitrate (IC), isocitrate dehydrogenase (IDH), superoxide dismutase (SOD), potassium dihydrogenphosphate (KH_2PO_4), and dipotassium hydrogenphosphate (K_2HPO_4) from Sigma-Aldrich (Taufkirchen, Germany). All other used drugs (of abuse) were supplied by commercial suppliers (e.g. Fluka, Neu Ulm, Germany; LG Chemicals, Teddington, UK; Lipomed, Weil am Rhein, Germany; Promochem, Wesel, Germany) or by the manufacturers of the marketed drugs. $NADP^+$ was from Biomol (Hamburg, Germany), formic acid (MS grade) from Fluka (Neu-Ulm, Germany), acetonitrile, methanol (both LC–MS grade), and all other chemicals from VWR (Darmstadt, Germany). Methanolic stock solutions (1 mg/mL) of the studied compounds were used. The chemical structures of all tested DOAs are depicted in Fig. 1.

The baculovirus-infected insect cell microsomes (Supersomes) containing human complementary DNA-expressed FMO3 (5 mg protein/mL), CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 (1 nmol/mL), or CYP2E1, CYP3A5 (2 nmol/mL), and pooled human liver microsomes (pHLM, 20 mg microsomal protein/mL, 330 pmol total CYP/mg protein) were obtained from Corning (Amsterdam, The Netherlands). After delivery, the microsomes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. pHLM incubations

Incubations were performed at 37 °C for 30 min with 25 μM of the substrate, regenerating system, and 1 mg protein/mL pHLM according to Michely et al. (2015). Incubations with pHLM were done without pretreatment, after preincubation with the chemical inhibitors ABT (2 mM) for CYPs or methimazole (200 μM) for FMOs, or after preheating. Heat-treated pHLM were prepared by heating at 55 °C for 1 min in absence of $NADP^+$ in accordance to Ring et al. (1999) or at 45 °C for 5 min in absence of $NADP^+$ and cooling on ice for 15 min in accordance to Taniguchi-Takizawa et al. (2015). In addition, blank incubations with buffer instead of enzymes were performed. As described before (Michely et al., 2015), besides enzymes and substrate, the incubation mixtures (final volume 50 μL) contained 90 mM phosphate buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM IC, 1.2 mM $NADP^+$, 0.5 U/mL IDH, and 200 U/mL SOD. Reactions were initiated by addition of the substrate and stopped with 50 μL of ice-cold acetonitrile, containing 5 μM internal standard. Trimipramine-d_3 was used as internal standard for incubations with all analytes, except for amitriptyline, where norclozapine-d_8 was used. The solution was centrifuged for 5 min at 14,000*g*, 70 μL of the supernatant phase was transferred to an autosampler vial and 10 μL injected onto the liquid chromatography (LC)-ion trap (IT)-mass spectrometry (MS) system described below. The amounts of formed metabolites in untreated and

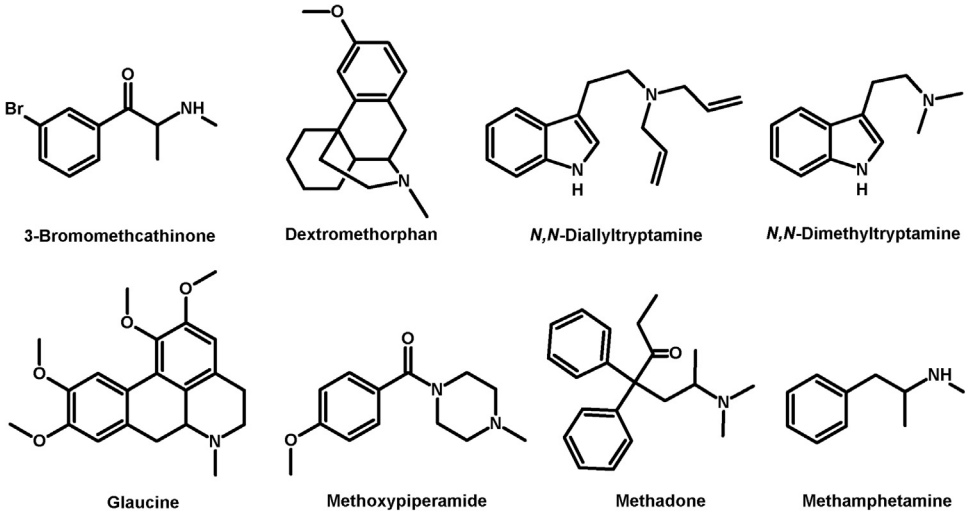

Fig. 1. Chemical structures of tested drugs of abuse in alphabetic order.