1521-009X/44/8/1270–1276$25.00
Drug Metabolism and Disposition
http://dx.doi.org/10.1124/dmd.115.069104
Drug Metab Dispos 44:1270–1276, August 2016

Special Section on Emerging Novel Enzyme Pathways in Drug Metabolism

Microbial Flavoprotein Monooxygenases as Mimics of Mammalian Flavin-Containing Monooxygenases for the Enantioselective Preparation of Drug Metabolites[S]

Turan Gul, Marzena Krzek, Hjalmar P. Permentier, Marco W. Fraaije, and Rainer Bischoff

Analytical Biochemistry, Groningen Research Institute of Pharmacy (T.G., H.P.P., R.B.), Molecular Enzymology Group, Groningen Biomolecular Sciences and Biotechnology Institute (M.K., M.W.F.), and Interfaculty Mass Spectrometry Center (H.P.P.), University of Groningen, Groningen, the Netherlands

Received December 21, 2015; accepted March 15, 2016

ABSTRACT

Mammalian flavin-containing monooxygenases, which are difficult to obtain and study, play a major role in detoxifying various xenobiotics. To provide alternative biocatalytic tools to generate flavin-containing monooxygenases (FMO)–derived drug metabolites, a collection of microbial flavoprotein monooxygenases, sequence-related to human FMOs, was tested for their ability to oxidize a set of xenobiotic compounds. For all tested xenobiotics [nicotine, lidocaine, 3-(methylthio)aniline, albendazole, and fenbendazole], one or more monooxygenases were identified capable of converting the target compound. Chiral liquid chromatography with tandem mass spectrometry analyses of the conversions of 3-(methylthio) aniline, albendazole, and fenbendazole revealed that the respective sulfoxides are formed in good to excellent enantiomeric excess (*e.e.*) by several of the tested monooxygenases. Intriguingly, depending on the chosen microbial monooxygenase, either the (*R*)- or (*S*)-sulfoxide was formed. For example, when using a monooxygenase from *Rhodococcus jostii* the (*S*)-sulfoxide of albendazole (ricobendazole) was obtained with a 95% *e.e.* whereas a fungal monooxygenase yielded the respective (*R*)-sulfoxide in 57% *e.e.* For nicotine and lidocaine, monooxygenases could be identified that convert the amines into their respective *N*-oxides. This study shows that recombinantly expressed microbial monooxygenases represent a valuable toolbox of mammalian FMO mimics that can be exploited for the production of FMO-associated xenobiotic metabolites.

Introduction

Metabolism of xenobiotics in humans and other mammals often starts with oxidation of the target molecule. Most of the phase I metabolism reactions are catalyzed by cytochrome P450 monooxygenases (P450) (Cashman, 2005). However, in addition to P450s, recent studies have shown that the so-called flavin-containing monooxygenases (FMOs) play a crucial role in the biotransformation of a large variety of xenobiotics, including pharmaceuticals and natural products.

Mammals typically employ several FMO isoforms. The human proteome contains five isoforms, FMO1–FMO5, all of which have their typical tissue-dependent expression patterns and roles in metabolism (Cashman and Zhang, 2006). FMOs have been shown to be involved in the oxygenation of heteroatom-containing compounds, such as amines and sulfides (Cashman, 2004; Krueger and Williams, 2005; Cashman and Zhang, 2006). Different from P450s, which contain a heme cofactor, FMOs use a flavin cofactor for oxidations which also translates into a different oxidative mechanism. Furthermore, to discriminate between metabolism by human FMOs or P450s, often differences in stability and specific inhibitors can be used (Taniguchi-Takizawa et al., 2015). FMO enzymes require nicotinamide adenine dinucleotide phosphate (NADPH) for reducing the flavin adenine dinucleotide (FAD) flavin cofactor. The reduced flavin subsequently reacts with molecular oxygen, resulting in the formation of a reactive 4a-hydroperoxyflavin. This reactive flavin intermediate is able to perform a variety of oxygenation reactions, for example, sulfoxidations and *N*-hydroxylations (see Ziegler 1990, 1993; Malito et al., 2004; Cashman, 2005; Krueger and Williams, 2005; Cashman and Zhang, 2006; van Berkel et al., 2006 for mechanistic details).

It has been established that human FMOs are essential in oxidizing a variety of xenobiotics, but biochemical and metabolic studies on these

This work was supported by the Dutch Technology Foundation (STW) [Grant 11957], and the Dutch Foundation for Scientific Research (NWO, ECHO) [Grant 711.012.006].

T.G. and M.K. contributed equally to this work.

dx.doi.org/10.1124/dmd.115.069104.

[S]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: BVMO, Baeyer-Villiger monooxygenase; $BVMO_{Mt1}$, Baeyer-Villiger monooxygenase from *Myceliophthora thermophila*; $BVMO_{Rj}$, Baeyer-Villiger monooxygenase from *Rhodoccocus jostii*; CD, circular dichroism; $CHMO_{Ac}$, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*; *e.e.*, enantiomeric excess; FAD, flavin adenine dinucleotide; FMO, flavin-containing monooxygenase; HPLC, high-pressure liquid chromatography; LC, liquid chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; OD, optical density; P450, cytochrome P450; PAMO, phenylacetone monooxygenase; $PAMO_{M446G}$, phenylacetone monooxygenase Met446Gly mutant; SPE, solid phase extraction; SRM, selected reaction monitoring.

enzymes are hampered by their poor availability. Human FMOs and their mammalian orthologs are typically membrane associated and often thermolabile, which appear to be the major reasons for their problematic isolation from tissue (Cashman et al., 1995; Wu et al., 2004) and inefficient recombinant production. Although human FMOs can be studied using microsomal preparations and some human FMOs were expressed as functional enzymes in heterologous hosts (Motika et al., 2009; Balke et al., 2012; Geier et al., 2015; Shimizu et al., 2015), these enzyme preparations involve costly and cumbersome isolation procedures, and often suffer from low activity and stability (Cashman et al., 1992; Cashman and Zhang, 2006).

Sequence comparison studies have revealed that FMOs are part of a large family of monooxygenases, the so-called class B flavoprotein monooxygenases (Reddy et al., 2010). Intriguingly, many bacteria and fungi contain sequence-related class B flavin-containing monooxygenases (Mascotti et al., 2015), which are typically involved in catalyzing Baeyer-Villiger oxidations to form a subfamily of Baeyer-Villiger monooxygenases (BVMOs). Biocatalytic studies on these microbial monooxygenases confirmed that they employ the same catalytic mechanism as FMOs (Torres Pazmiño et al., 2008); interestingly, they also can catalyze oxygenations of heteroatom-containing compounds. In contrast to human FMOs, many microbial BVMOs are soluble enzymes and can be easily produced in recombinant form (de Gonzalo et al., 2010).

Inspired by the observation that microbial BVMOs are sequence-related to human FMOs and exhibit similar activities, we set out to explore their use as mammalian FMO mimics. By testing a panel of xenobiotic compounds, including drug molecules, with a collection of microbial BVMOs, we discovered that these biocatalysts may serve as tools to prepare metabolites. With the proper choice of monooxygenase, all the tested xenobiotics (nicotine, lidocaine, 3-(methylthio)aniline, albendazole, and fenbendazole, see Fig. 1) could be converted. Chiral liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis showed that sulfides were converted to the corresponding sulfoxides with excellent and complementary enantioselectivities. Our study reveals that recombinant microbial BVMOs, which are relatively easy to produce and robust as biocatalysts, represent attractive alternatives to mammalian FMOs for the preparation of FMO-related metabolites.

Materials and Methods

Materials. We purchased 3-(methylthio)aniline, albendazole, ricobendazole (racemic albendazole sulfoxide), fenbendazole, lidocaine, nicotine, 1,4-dioxane, and Tris from Sigma-Aldrich (Zwijndrecht, the Netherlands). Acetaminophen was purchased from Fluka (Buchs, Switzerland), ultra-pure high-pressure liquid chromatography (HPLC)–grade acetonitrile and HPLC grade methanol were purchased from Biosolve (Valkenswaard, the Netherlands). We purchased catalase from Fluka, and phosphite dehydrogenase was prepared using an established protocol (Dudek et al., 2011). Ultrapure water was obtained from a Milli-Q Advantage A10 Water Purification system (Millipore Corp., Billerica, MA). Oasis HLB 30-mg solid phase extraction (SPE) cartridges were purchased from Waters (Manchester, United Kingdom).

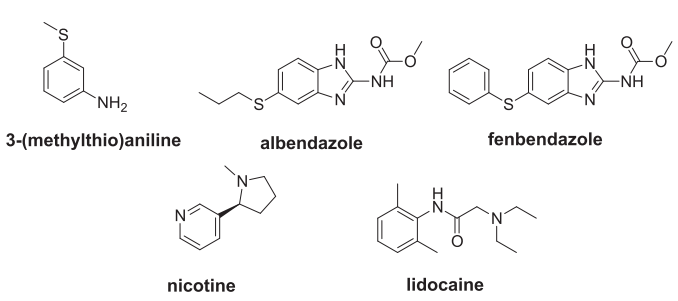

Fig. 1. Substrates used in microbial monooxygenase-catalyzed conversions.

Recombinant Expression of BVMOs and Preparation of Cell Extracts. The enzymes were overexpressed in *Escherichia coli* using previously established conditions and protocols. Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* ($CHMO_{Ac}$), the phenylacetone monooxygenase Met446Gly mutant ($PAMO_{M446G}$), and $BVMO_{Rj24}$ were expressed using the pCRE2 expression vector (Torres Pazmiño et al., 2009), yielding the enzyme fused to His-tagged phosphite dehydrogenase, which facilitates cofactor regeneration. His-tagged PAMO and Strep-tagged FMO_{RjE} were expressed as described previously elsewhere (Fraaije et al., 2005). For expressing $BVMO_{Mt1}$, a pET_SUMO vector was used. Precultures were grown overnight at 37°C with shaking (180 rpm) in lysogeny broth medium containing ampicillin (50 μg/ml). The exception was $BVMO_{Mt1}$ for which cells were grown in the presence of kanamycin (100 μg/ml).

Flasks containing 200 ml of terrific broth medium with the respective antibiotic were inoculated 1:100 (v/v) using the preculture and grown for another 4 hours at 30°C. After that, each flask was supplemented with inducer: 1.0 mM isopropyl β-D-thiogalactopyranoside for $BVMO_{Mt1}$, and 0.02% arabinose for PAMO, $PAMO_{M446G}$, and $CHMO_{Ac}$, and 0.002% arabinose for the remaining enzymes. After 48 hours of growth at 24°C with shaking (130 rpm), the cells were harvested by centrifugation at 4°C, 17,000*g* and resuspended in 50 mM Tris buffer, pH 8.0.

The cells were diluted to an optical density of 600 nm (OD_{600}) of 212 for terrific broth samples and an OD_{600} of 98 for lysogeny broth samples. Subsequently, the cells were disrupted by sonication for 90 seconds using 2-second sonication pulses and 2-second breaks, while on ice.

The prepared cell extracts were supplemented with glycerol (15%), aliquoted (100 μl in Eppendorf tubes), frozen in liquid nitrogen, and stored at −80°C. As the negative control, *E. coli* cells were grown without expression plasmid and used for the preparation of cell extract as described earlier. Overexpression of the enzymes was confirmed with SDS-PAGE by analyzing OD-normalized samples from bacterial cultures.

Monooxygenase-Catalyzed Conversions. For conversions, cell extracts (100 μl) were supplemented with 1.0 mM substrates [except for 3-(methylthio) aniline, for which 3.0 mM was used] using 1,4-dioxane as a cosolvent [1% v/v for all substrates except for 3-(methylthio)aniline (0.6% v/v)], 100 μM NADPH, 20 mM phosphite, 5.0 μM phosphite dehydrogenase, 20 mU catalase, and 50 mM Tris-HCl (pH 8.5) in a total volume of 300 μl. To increase the solubility of albendazole and fenbendazole, 9.6 mM β-cyclodextrine was added. Negative control experiments were performed by incubating substrates with cell extracts that did not contain any expressed monooxygenase. All the conversions were performed in duplicate.

After 135 minutes of incubation at room temperature, a 100-μl sample was taken, and the proteins were precipitated by adding 300 μl of acetonitrile containing 0.2% formic acid. Samples were vortexed for 30 seconds and centrifuged at 13,000 rpm for 6.5 minutes. After centrifugation, 200 μl of the supernatants was evaporated to dryness under nitrogen before SPE.

The SPE was performed on Oasis HLB 30-mg cartridges that were wetted with acetonitrile and equilibrated with H_2O/acetonitrile (95:5). Dried samples were dissolved in 200 μl of water and loaded onto the cartridge. Water (3 × 250 μl) was used to wash the cartridges, and the final elution was performed with acetonitrile (4 × 250 μl). For the LC-MS/MS analysis, samples were diluted 10 times in water containing 10 μM acetaminophen, as an internal standard for LC-MS/MS signal normalization.

Chiral LC-MS/MS in the Selected Reaction Monitoring Mode. LC-MS/MS analyses in the selected reaction monitoring (SRM) mode were performed on an HPLC system with an Accela Autosampler and a Surveyor Pump coupled to a TSQ Quantum AM triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with an electrospray ionization interface in the positive mode (Supplemental Table 1 and Supplemental Table 2). We separated 3-(methylthio)aniline, albendazole, fenbendazole, and their chiral sulfoxide products with an amylose tris(3-chlorophenylcarbamate)-based chiral column (Chiralpak ID, 5 μm particle size, 2.1 × 150 mm; Chiral Technologies Europe, Illkirch, France) at a flow rate of either 100 or 200 μl/min.

The liquid chromatography (LC) separation of lidocaine and its products was performed with a C_{18} reversed-phase column (GraceSmart RP 18, 5 μm particle size, 2.1 × 150 mm; Grace Davison, Lokeren, Belgium) at a flow rate of 250 μl/min. The LC separation of nicotine and its products was performed with a hydrophilic interaction chromatography column (Xbridge amide, 3.5 μm particle size, 2.1 × 150 mm; Waters, Milford, MA) at a flow a rate of 250 μl/min.

The following set of solvents was used for the separations: solvent A (H_2O with 0.1% formic acid), solvent B (acetonitrile with 0.1% formic acid), solvent C (H_2O with 20 mM ammonium bicarbonate [pH 9.0, adjusted with NH_3]), solvent D (acetonitrile), and solvent E (H_2O with 10 mM ammonium formate, pH 5.5). Separation of the two 3-(methylthio)aniline sulfoxide enantiomers was performed isocratically at 100 μl/min (20 minutes) with 90% solvent C/10% solvent D. The albendazole sulfoxide enantiomers were separated isocratically with 50% solvent C/50% solvent D at 200 μl/min (20 minutes), and fenbendazole sulfoxides were separated with 40% solvent C/60% solvent D at 200 μl/min (15 minutes), using the Chiralpak column.

The LC-MS/MS analysis of lidocaine and its *N*-oxide was performed by reversed-phase LC applying a linear gradient starting from 5% to 95% solvent B in solvent A over 11 minutes, which was held for 1 minute. Solvent B was decreased rapidly to 5% in 20 seconds, and the column was re-equilibrated at 5% solvent B for 4 minutes. The LC-MS/MS analysis of nicotine and its *N*-oxide was performed by hydrophilic interaction chromatography applying a linear gradient starting from 10% to 90% solvent E in solvent D (acetonitrile) over 8 minutes, which was held for 2 minutes. Solvent E was decreased rapidly to 10% in 20 seconds, and finally the column was re-equilibrated at 10% solvent E for 3 minutes. Acetaminophen was used as internal standard to normalize the peak areas across the LC-MS/MS runs.

Circular Dichroism. To assign the absolute configuration of the products, we analyzed the samples by circular dichroism (CD) spectroscopy. Samples were purified with SPE, dried by evaporation of acetonitrile, and dissolved in methanol to a nominal concentration of 0.75 mM. For CD analysis, the samples were further diluted 4 times in methanol. CD spectra were recorded on a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using a 1-mm quartz cell cuvette and scanning from 200 to 350 nm at 25°C; methanol was used as a blank.

Results

Conversion of the Sulfide 3-(Methylthio)Aniline. We selected seven microbial flavoprotein monooxygenases originating from three different microorganisms. Besides the three well-studied BVMOs, we included in the study PAMO from *Thermobifida fusca* (Fraaije et al., 2005), a Met446Gly PAMO mutant ($PAMO_{M446G}$) (de Gonzalo et al., 2012), a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* ($CHMO_{Ac}$) (Torres Pazmiño et al., 2009), two recently discovered BVMOs from *Rhodoccocus jostii* ($BVMO_{Rj4}$ and $BVMO_{Rj24}$) (Riebel et al., 2013), and a BVMO from the fungus *Myceliophthora thermophila* ($BVMO_{Mt1}$). Furthermore, we also included a representative of a newly discovered distinct subfamily of microbial monooxygenases, the so-called type II FMOs, which share characteristics of both BVMOs and FMOs: FMO_{RjE} from *R. jostii* (Riebel et al., 2013).

All the studied monooxygenases belong to the class B flavoprotein monooxygenases, so they are distantly related to mammalian FMOs, as evidenced by significant sequence identities (20%–40%) and highly conserved sequence motifs (Krueger and Williams, 2005). Also the obtained crystal structures of a bacterial FMO and several BVMOs have confirmed that class B flavoprotein monooxygenases share structural and mechanistic features (Malito et al., 2004). $CHMO_{Ac}$ and PAMO display complementary and broad substrate acceptance profiles (Pazmiño and Fraaije, 2008). The other monooxygenases have been hardly explored for their substrate scope. Therefore, we anticipated that by studying such a large panel of different microbial monooxygenases, several targeted compounds could be converted by one or more monooxygenases.

All monooxygenases were produced in *E. coli* as the expression host. SDS-PAGE gel analysis confirmed high and quantitatively comparable overexpression in soluble form of all investigated enzymes. In all cell extracts, the expressed monooxygenase was the most prominent protein band on the SDS PAGE gel. Because *E. coli* does not contain any endogenous enzymes with similar activity, BVMO- or FMO-type

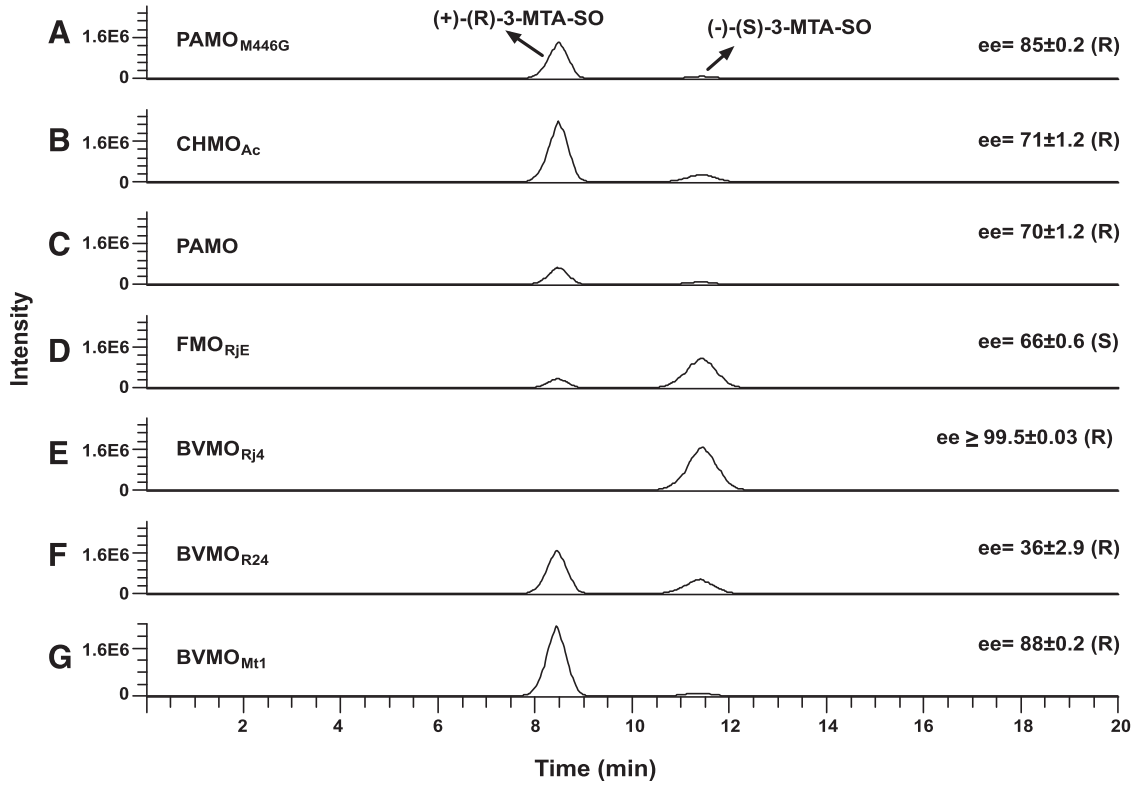

Fig. 2. Chiral LC-MS/MS in the SRM mode of 3-(methylthio)aniline sulfoxide (3-MTA-SO) (SRM transition: 156/93). We incubated 3-(methylthio)aniline for 135 minutes in the presence of the following microbial monooxygenases: (A) $PAMO_{M446G}$, (B) $CHMO_{Ac}$, (C) PAMO, (D) FMO_{RjE}, (E) $BVMO_{Rj4}$, (F) $BVMO_{Rj24}$, and (G) $BVMO_{Mt1}$. Based on CD analysis and data reported in the literature, the first eluting enantiomer at 8.4 minutes was assigned to (*R*)-3-methylthioaniline sulfoxide and the second eluting enantiomer at 11.5 minutes to (*S*)-3-methylthioaniline sulfoxide (Slack et al., 2012). The enantiomeric excess is given as *e.e.*

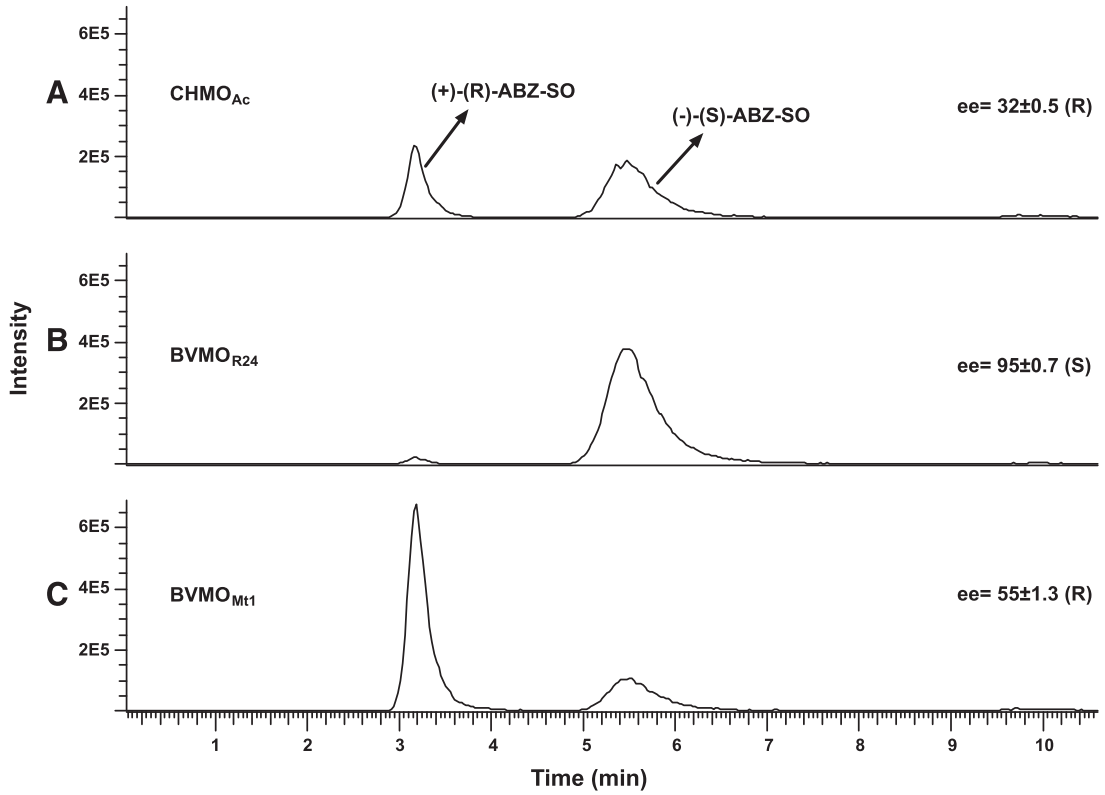

Fig. 3. Chiral LC-MS/MS in the SRM mode of albendazole sulfoxide (ABZ-SO) (SRM transition: 282/240). Albendazole was incubated for 135 minutes in the presence of the following monooxygenases: (A) CHMO$_{Ac}$, (B) BVMO$_{Rj24}$, and (C) BVMO$_{Mt1}$. The first eluting enantiomer at 3.1 minutes was assigned to (*R*)-albendazole sulfoxide and the second eluting enantiomer at 5.5 minutes to (*S*)-albendazole sulfoxide based on literature data (Materazzo et al., 2014).

monooxygenases, no enzyme purification step was required, and the cell extracts were used for performing the conversions. As a first test substrate, 3-(methylthio)aniline was used.

PAMO and PAMO mutants have been shown to be able to efficiently convert aromatic sulfides (de Gonzalo et al., 2005). Human FMOs are also known for their ability to perform sulfoxidations of aromatic sulfides or thioureas (Cashman and Zhang, 2006; Motika et al., 2007). Depending on the type of sulfide and FMO isoform, various enantioselectivities by mammalian FMOs have been described (Moroni et al., 1995; Virkel et al., 2004; Hai et al., 2009). Conversion of this relatively simple aromatic thioether was probed with all seven of the studied monooxygenases.

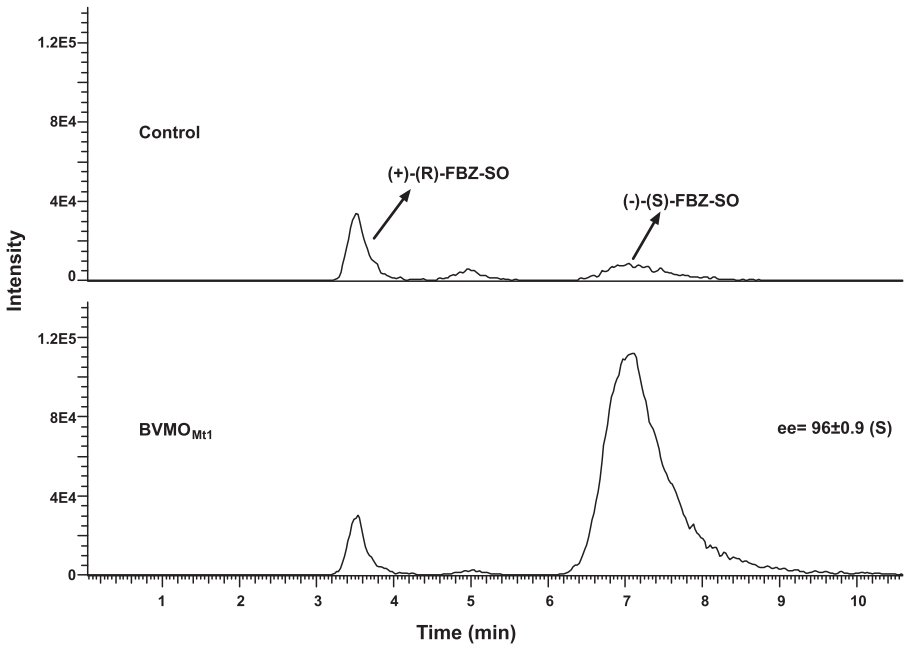

Fig. 4. Chiral LC-MS/MS in the SRM mode of fenbendazole sulfoxide (FBZ-SO) (SRM transition: 316/284). Fenbendazole was incubated for 135 minutes in the presence of the studied monooxygenases, of which only BVMO$_{Mt1}$ showed activity. The first eluting enantiomer at 3.5 minutes was assigned to (*R*)-fenbendazole sulfoxide and the second eluting enantiomer at 7.1 minutes to (*S*)-fenbendazole sulfoxide based on literature data (Materazzo et al., 2014).

For the conversions, 3.0 mM 3-(methylthio)aniline was incubated with cell extracts supplemented with phosphite, NADPH, and phosphite dehydrogenase to regenerate the reduced coenzyme. After 135 minutes of incubation, product analysis was performed by chiral LC-MS/MS in the SRM mode. The absolute configuration of the observed sulfoxides was determined by CD spectroscopy of the isolated enantiomers.

Control reactions also resulted in the formation of low amounts of sulfoxides due to spontaneous reaction with molecular oxygen. These reactions are not enantioselective, and the observed amounts in the enzymatic conversions were corrected for the background oxidation level.

All tested monooxygenases produced significant amounts of sulfoxides but with markedly different enantioselectivity (Fig. 2). CD analysis of the 3-(methylthio)aniline sulfoxides formed in the $BVMO_{Rj4}$ and $BVMO_{Mt1}$ samples gave $[a]_D^{25}$ values of +20.8 and −16.0, respectively (Supplemental Fig. 1). Based on comparison with the literature data for (*R*)-3-(methylthio)aniline, we assigned the first eluting enantiomer (at 8.4 minutes) to (+)-(*R*)-3-(methylthio)aniline sulfoxide and the second eluting enantiomer (at 11.2 minutes) to (−)-(*S*)-3-(methylthio)aniline sulfoxide (Folli et al., 1973; Slack et al., 2012).

Most monooxygenases have a preference for forming the (+)-(*R*)-3-(methylthio)aniline sulfoxide (enantiomeric excess [*e.e.*] values 66%–88%). However, $BVMO_{Rj4}$ produced the (−)-(*S*)-3-(methylthio) aniline sulfoxide with an *e.e.* of >99.5%, showing that this set of monooxygenases allows the synthesis of both sulfoxide enantiomers in very good to excellent enantiomeric excess. Only $BVMO_{Rj4}$ and $BVMO_{Mt1}$ produced an additional sulfone product at less than 2% of the amount of the sulfoxide. The standard addition method was used to quantify the conversion of 3-(methylthio)aniline after 135 minutes of incubation. The conversion reached 72% for $BVMO_{Rj24}$ and 97% for $BVMO_{Mt1}$ (Supplemental Figs. 2 and 4). This indicates that with the current approach 4–5 mg of enantiopure sulfoxide metabolite is produced in 1 hour using a cell extract from a 1-liter culture.

Conversion of the Thioether Drugs, Albendazole, and Fenbendazole. Two drugs that are commonly used to treat worm infestations in mammals, albendazole and fenbendazole, are known to be converted in an enantioselective manner into their sulfoxides by mammalian FMOs (Moroni et al., 1995). Testing the panel of microbial monooxygenases revealed that three monooxygenases ($CHMO_{Ac}$, $BVMO_{Rj24}$, and $BVMO_{Mt1}$) converted albendazole, yielding sulfoxides in significant enantiomeric excess (Fig. 3). Only $BVMO_{Mt1}$ was able to catalyze the sulfoxidation of fenbendazole.

Determination of the $[a]_D^{25}$ values by CD analysis could not be performed because the yields of the albendazole and fenbendazole sulfoxides were too low. However, the sulfoxide enantiomers of albendazole and fenbendazole have been characterized using the same chiral column and a similar solvent system by Materazzo et al. (2014), allowing us to assign the first eluting enantiomers to (*R*)-albendazole sulfoxide (3.1 minutes) and (*R*)-fenbendazole sulfoxide (3.5 minutes), respectively, and the second eluting enantiomers to (*S*)-albendazole sulfoxide (5.5 minutes) and (*S*)-fenbendazole sulfoxide (7.1 minutes), respectively.

Chiral LC-MS/MS in the SRM mode showed that $CHMO_{Ac}$ and $BVMO_{Mt1}$ formed the same product as mammalian FMOs, (*R*)-albendazole sulfoxide (32% and 55% *e.e.*, respectively), whereas $BVMO_{Rj24}$ enzyme produced (*S*)-albendazole sulfoxide with 95% *e.e.* The yields of albendazole sulfoxide were determined with standard addition and reached 55% for $BVMO_{Rj24}$ and 25% for $BVMO_{Mt1}$ (Supplemental Figs. 5 and 7). This corresponds to 1–2 mg/h and per liter of bacterial culture. Less than 1% of the sulfone product was formed by $BVMO_{Rj24}$, $BVMO_{Mt1}$ and $CHMO_{Ac}$ upon conversion. No other side products were detected. Chiral LC-MS/MS in the SRM mode showed that conversion of fenbendazole by $BVMO_{Mt1}$ yields (*S*)-fenbendazole sulfoxide in 96% *e.e.* (Fig. 4).

Conversion of the Amines, Lidocaine, and Nicotine. Lidocaine and nicotine contain a regular and a cyclic tertiary amine group, respectively. Lidocaine is a widely used local anesthetic, and nicotine is a plant alkaloid that acts as a stimulant. Both drugs are known to be oxidized by mammalian FMOs into their corresponding *N*-oxides. LC-MS/MS analysis showed that significant conversion of lidocaine into the *N*-oxide is performed by $BVMO_{Rj24}$, while nicotine is converted by $CHMO_{Ac}$ (Fig. 5). With the applied conditions, the degree of conversion for both substrates was rather low, below 10%.

For lidocaine, no other products (specifically the P450-catalyzed *N*-dealkylation or aromatic hydroxylation products) were observed in significant amounts. The *N*-oxide of lidocaine can be distinguished from other monooxygenation products by its specific SRM transition (*m/z* 251/130). Additionally, its retention time was confirmed using a chemically oxidized lidocaine *N*-oxide standard.

LC-MS/MS analysis of the nicotine conversion samples showed two *N*-oxidation products, which could be assigned on the basis of their fragmentation patterns; the SRM transition of *m/z* 179/117 is unique for nicotine-1′-*N*-oxide (oxidation of nitrogen on the pyrrolidine ring) and the *m/z* 179/148 transition is unique for nicotine-1-*N*-oxide (oxidation of nitrogen on the pyridine ring) (Smyth et al., 2007; Piller et al., 2014).

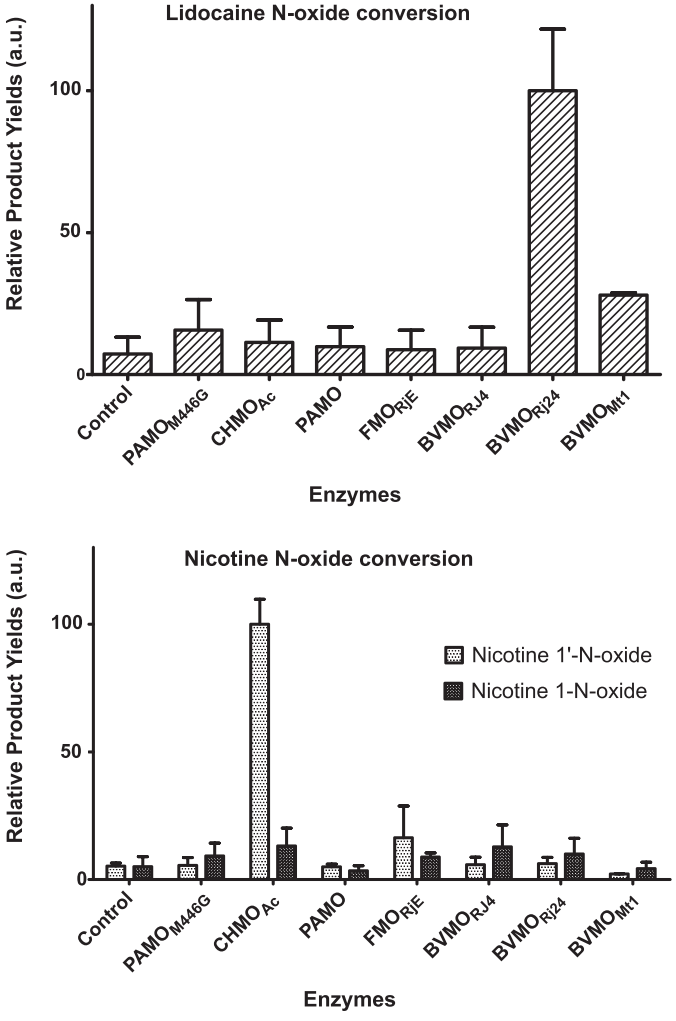

Fig. 5. Relative yields of the *N*-oxide products of lidocaine (top) and nicotine (bottom) in the presence of different bacterial monooxygenases as determined by LC-MS/MS in the SRM mode. Experiments were performed in duplicate.

The nicotine-1′-*N*-oxide product was the major *N*-oxide formed by $CHMO_{Ac}$. The low amount of nicotine-1-*N*-oxide was similar to the amount formed in the control reaction.

Discussion

Human FMOs and other mammalian FMOs play a crucial role in degrading a wide range of xenobiotics, including many drugs. Although they are known for their chemo- and enantioselective oxidations, mammalian FMOs are notoriously difficult to obtain or to use as isolated biocatalysts. To provide an alternative for the biocatalytic production of FMO-derived metabolites, we explored the use of the microbial class B flavoprotein monooxygenases, which are all sequence-related to FMOs.

Besides sequence similarities, members of the class B flavoprotein monooxygenases all share a similar structural fold. They are composed of FAD-binding domain with a tightly bound FAD as a prosthetic group and a NADPH-binding domain which binds NADPH as coenzyme during catalysis (Krueger and Williams, 2005). In addition, kinetic and mechanistic studies on FMOs and BVMOs have revealed that these flavoprotein monooxygenases also share a common catalytic mechanism. This is also reflected in the type of oxygenations reactions that are catalyzed by members of both monooxygenase groups: they overlap and include *N*-oxygenations, sulfoxidations, and Baeyer-Villiger oxidations (Fiorentini et al., 2016).

The catalytic cycle starts with binding of the reduced coenzyme NADPH, which results in reduction of the flavin cofactor. Through a subsequent fast reaction with molecular oxygen, the peroxyflavin intermediate is formed, which is the key to catalyzing substrate oxygenation (Beaty and Ballou, 1981; Torres Pazmiño et al., 2008). The reactive peroxyflavin is stabilized through interactions with active site residues and awaits entry of a suitable substrate in the active pocket. The accessibility, character, and size of the active site pocket determine the substrate specificity and the enantio- and regioselectivity of each monooxygenase.

As a consequence and different from many other flavoprotein monooxygenases and P450 monooxygenases, formation of the reactive oxygenating enzyme intermediate is not dependent on binding of a substrate. Many class B monooxygenases, including human FMOs, have been shown to display a relaxed substrate acceptance profile. This triggered our study to explore the catalytic potential of microbial flavoprotein monooxygenases, which are sequence related to mammalian FMOs, for the conversion of FMO substrates.

One of the advantages of using such enzymes for in vitro conversion of FMO-targeted xenobiotics is the ease of production of the microbial enzymes at high levels and in soluble form in *E. coli*. Upon growth of the recombinant bacteria, the cell extracts could be immediately used for conversion of the targeted xenobiotics. Another advantage of this approach is the fact that in the last decade a large number of recombinant microbial flavoprotein monooxygenases have become available. For example, we have generated an in-house library of >30 different microbial flavoprotein monooxygenases (Fraaije et al., 2005; Riebel et al., 2013). For our study we decided to explore a set of seven monooxygenases that are known to display dissimilar substrate acceptance and oxygenation selectivity profiles.

Five different xenobiotics [3-(methylthio)aniline, albendazole, fenbendazole, lidocaine, and nicotine] were chosen to examine enantio-, region-, and chemoselective oxygenation by using a panel of seven different recombinant microbial flavoprotein monooxygenases. Chiral LC-MS/MS in the SRM mode was instrumental in establishing activity and selectivity of each enzyme toward each test compound. The enzymes that were found to be able to convert albendazole and fenbendazole formed the corresponding sulfoxides with very good enantiomeric excess. Both enantiomers of albendazole sulfoxide were produced in enantiomeric excess [$CHMO_{Ac}$ and $BVMO_{Mt1}$ for the (*R*)-sulfoxide and $BVMO_{Rj24}$ for the (*S*)-sulfoxide].

Others have shown that mammalian FMOs have a preference for forming (*R*)-albendazole sulfoxide from albendazole (Moroni et al., 1995; Virkel et al., 2004). For fenbendazole, only one active enzyme ($BVMO_{Mt1}$) was identified that preferentially formed the (*S*)-sulfoxide. Fenbendazole has been shown to be converted into the (*R*)-sulfoxide by mammalian FMOs with significant enantiomeric excess (Virkel et al., 2004). The observation that only one out of seven enzymes was active on fenbendazole may reflect the fact that fenbendazole differs from albendazole in having a phenyl moiety replacing a propyl moiety which makes it more bulky, sterically hindering oxidation of the thioether.

It is worth mentioning that, except for the formation of low amounts of sulfones, no other oxidation products were formed upon conversion of the tested thioethers, which demonstrates that the monooxygenases are chemo-, regio-, and enantioselective. Monooxygenases that form the *N*-oxides of nicotine ($CHMO_{Ac}$) and lidocaine ($BVMO_{Rj24}$) were also identified. In the literature it was reported that human FMO3 is responsible for selective formation of nicotine-1′-*N*-oxide (Cashman et al., 1992), which was also the major *N*-oxide formed by $CHMO_{Ac}$. This shows that the microbial monooxygenases can also be used for chemo- and regioselective *N*-oxidations of xenobiotics.

Our study illustrates that sequence-related microbial monooxygenases can be used for the production of FMO-related metabolites. As for mammalian FMOs (Cashman et al., 1992; Wu et al., 2004), each tested oxidation was very specific, and no side products were formed in considerable amounts. This makes them interesting biocatalysts for the production of pharmaceutically relevant drug metabolites.

Acknowledgments

The authors thank Assunta Green (Chiral Technologies Europe) for screening chiral columns and Professor Wesley Browne and Yigit Altay (University of Groningen, Stratingh Institute for Chemistry) for CD analysis.

Authorship Contributions

Participated in research design: Gul, Krzek, Permentier, Fraaije, Bischoff.

Conducted experiments: Gul, Krzek.

Performed data analysis: Gul, Krzek, Permentier.

Wrote or contributed to the writing of the manuscript: Gul, Krzek, Permentier, Fraaije, Bischoff.

References

Balke K, Kadow M, Mallin H, Sass S, and Bornscheuer UT (2012) Discovery, application and protein engineering of Baeyer-Villiger monooxygenases for organic synthesis. *Org Biomol Chem* **10**:6249–6265.

Beaty NB and Ballou DP (1981) The reductive half-reaction of liver microsomal FAD-containing monooxygenase. *J Biol Chem* **256**:4611–4618.

Cashman JR (2005) Some distinctions between flavin-containing and cytochrome P450 monooxygenases. *Biochem Biophys Res Commun* **338**:599–604.

Cashman JR (2004) The implications of polymorphisms in mammalian flavin-containing monooxygenases in drug discovery and development. *Drug Discov Today* **9**:574–581.

Cashman JR, Park SB, Berkman CE, and Cashman LE (1995) Role of hepatic flavin-containing monooxygenase 3 in drug and chemical metabolism in adult humans. *Chem Biol Interact* **96**: 33–46.

Cashman JR, Park SB, Yang ZC, Wrighton SA, Jacob P, 3rd, and Benowitz NL (1992) Metabolism of nicotine by human liver microsomes: stereoselective formation of trans-nicotine N′-oxide. *Chem Res Toxicol* **5**:639–646.

Cashman JR and Zhang J (2006) Human flavin-containing monooxygenases. *Annu Rev Pharmacol Toxicol* **46**:65–100.

de Gonzalo G, Mihovilovic MD, and Fraaije MW (2010) Recent developments in the application of Baeyer-Villiger monooxygenases as biocatalysts. *ChemBioChem* **11**:2208–2231.

de Gonzalo G, Pazmiño DET, Ottolina G, Fraaije MW, and Carrea G (2005) Oxidations catalyzed by phenylacetone monooxygenase from *Thermobifida fusca*. *Tetrahedron Asymmetry* **16**: 3077–3083.

de Gonzalo G, Rodríguez C, Rioz-Martínez A, and Gotor V (2012) Improvement of the biocatalytic properties of one phenylacetone monooxygenase mutant in hydrophilic organic solvents. *Enzyme Microb Technol* **50**:43–49.

Dudek HM, de Gonzalo G, Pazmiño DET, Stepniak P, Wyrwicz LS, Rychlewski L, and Fraaije MW (2011) Mapping the substrate binding site of phenylacetone monooxygenase from *Thermobifida fusca* by mutational analysis. *Appl Environ Microbiol* **77**:5730–5738.

Fiorentini F, Geier M, Binda C, Winkler M, Faber K, Hall M, and Mattevi A (2016) Biocatalytic characterization of human FMO5: unearthing Baeyer-Villiger reactions in humans. *ACS Chem Biol* DOI: 10.1021/acschembio.5b01016 [published ahead of print].

Folli U, Iarossi D, and Taddei F (1973) Intramolecular hydrogen bonds in aromatic sulphoxides: 1H nuclear magnetic resonance and acidity constant measurements. *J Chem Soc, Perkin Trans 2* 6:848–853 DOI: 10.1039/P29730000848.

Fraaije MW, Wu J, Heuts DPHM, van Hellemond EW, Spelberg JHL, and Janssen DB (2005) Discovery of a thermostable Baeyer-Villiger monooxygenase by genome mining. *Appl Microbiol Biotechnol* **66**:393–400.

Geier M, Bachler T, Hanlon SP, Eggimann FK, Kittelmann M, Weber H, Lütz S, Wirz B, and Winkler M (2015) Human FMO2-based microbial whole-cell catalysts for drug metabolite synthesis. *Microb Cell Fact* **14**:82.

Hai X, Adams E, Hoogmartens J, and Van Schepdael A (2009) Enantioselective in-line and off-line CE methods for the kinetic study on cimetidine and its chiral metabolites with reference to flavin-containing monooxygenase genetic isoforms. *Electrophoresis* **30**:1248–1257.

Krueger SK and Williams DE (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther* **106**:357–387.

Malito E, Alfieri A, Fraaije MW, and Mattevi A (2004) Crystal structure of a Baeyer-Villiger monooxygenase. *Proc Natl Acad Sci USA* **101**:13157–13162.

Mascotti ML, Lapadula WJ, and Juri Ayub M (2015) The origin and evolution of Baeyer-Villiger monooxygenases (BVMOs): an ancestral family of flavin monooxygenases. *PLoS One* **10**: e0132689.

Materazzo S, Carradori S, Ferretti R, Gallinella B, Secci D, and Cirilli R (2014) Effect of the water content on the retention and enantioselectivity of albendazole and fenbendazole sulfoxides using amylose-based chiral stationary phases in organic-aqueous conditions. *J Chromatogr A* **1327**: 73–79.

Moroni P, Buronfosse T, Longin-Sauvageon C, Delatour P, and Benoit E (1995) Chiral sulfoxidation of albendazole by the flavin adenine dinucleotide-containing and cytochrome P450-dependent monooxygenases from rat liver microsomes. *Drug Metab Dispos* **23**:160–165.

Motika MS, Zhang J, and Cashman JR (2007) Flavin-containing monooxygenase 3 and human disease. *Expert Opin Drug Metab Toxicol* **3**:831–845.

Motika MS, Zhang J, Zheng X, Riedler K, and Cashman JR (2009) Novel variants of the human flavin-containing monooxygenase 3 (FMO3) gene associated with trimethylaminuria. *Mol Genet Metab* **97**:128–135.

Pazmiño DET and Fraaije MW (2008) Discovery, redesign and applications of Baeyer-Villiger monooxygenases, in *Future directions in biocatalysis* (Mastuda T, ed) pp 107–128, Elsevier, Amsterdam.

Piller M, Gilch G, Scherer G, and Scherer M (2014) Simple, fast and sensitive LC-MS/MS analysis for the simultaneous quantification of nicotine and 10 of its major metabolites. *J Chromatogr B Analyt Technol Biomed Life Sci* **951–952**:7–15.

Reddy RR, Ralph EC, Motika MS, Zhang J, and Cashman JR (2010) Characterization of human flavin-containing monooxygenase (FMO) 3 and FMO5 expressed as maltose-binding protein fusions. *Drug Metab Dispos* **38**:2239–2245.

Riebel A, de Gonzalo G, and Fraaije MW (2013) Expanding the biocatalytic toolbox of flavoprotein monooxygenases from *Rhodococcus jostii* RHA1. *J Mol Catal, B Enzym* **88**:20–25 Elsevier B.V.

Shimizu M, Origuchi Y, Ikuma M, Mitsuhashi N, and Yamazaki H (2015) Analysis of six novel flavin-containing monooxygenase 3 (FMO3) gene variants found in a Japanese population suffering from trimethylaminuria. *Mol Genet Metab Rep* **5**:89–93.

Slack RD, Mott BT, Woodard LE, Tripathi A, Sullivan D, Nenortas E, Girdwood SCT, Shapiro TA, and Posner GH (2012) Malaria-infected mice are completely cured by one 6 mg/kg oral dose of a new monomeric trioxane sulfide combined with mefloquine. *J Med Chem* **55**:291–296.

Smyth TJ, Ramachandran VN, McGuigan A, Hopps J, and Smyth WF (2007) Characterisation of nicotine and related compounds using electrospray ionisation with ion trap mass spectrometry and with quadrupole time-of-flight mass spectrometry and their detection by liquid chromatography/electrospray ionisation mass spectrometry. *Rapid Commun Mass Spectrom* **21**: 557–566.

Taniguchi-Takizawa T, Shimizu M, Kume T, and Yamazaki H (2015) Benzydamine *N*-oxygenation as an index for flavin-containing monooxygenase activity and benzydamine *N*-demethylation by cytochrome P450 enzymes in liver microsomes from rats, dogs, monkeys, and humans. *Drug Metab Pharmacokinet* **30**:64–69.

Torres Pazmiño DE, Baas BJ, Janssen DB, and Fraaije MW (2008) Kinetic mechanism of phenylacetone monooxygenase from *Thermobifida fusca*. *Biochemistry* **47**:4082–4093.

Torres Pazmiño DE, Riebel A, de Lange J, Rudroff F, Mihovilovic MD, and Fraaije MW (2009) Efficient biooxidations catalyzed by a new generation of self-sufficient Baeyer-Villiger monooxygenases. *ChemBioChem* **10**:2595–2598.

van Berkel WJH, Kamerbeek NM, and Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J Biotechnol* **124**:670–689.

Virkel G, Lifschitz A, Sallovitz J, Pis A, and Lanusse C (2004) Comparative hepatic and extrahepatic enantioselective sulfoxidation of albendazole and fenbendazole in sheep and cattle. *Drug Metab Dispos* **32**:536–544.

Wu RF, Liao CX, Tomita S, Ichikawa Y, and Terada LS (2004) Porcine FAD-containing monooxygenase metabolizes lidocaine, bupivacaine and propranolol in vitro. *Life Sci* **75**:1011–1019.

Ziegler DM (1990) Flavin-containing monooxygenases: enzymes adapted for multisubstrate specificity. *Trends Pharmacol Sci* **11**:321–324.

Ziegler DM (1993) Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. *Annu Rev Pharmacol Toxicol* **33**:179–199.

Address correspondence to: Dr. Marco W. Fraaije, Molecular Enzymology Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747AG Groningen, the Netherlands. E-mail: m.w.fraaije@rug.nl