Electrochemical and enzymatic synthesis of oxidative drug metabolites for metabolism studies
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Microbial flavoprotein monooxygenases as mimics of mammalian flavin-containing monooxygenases for the enantioselective preparation of drug metabolites

Mammalian flavin-containing monooxygenases are difficult to obtain and study while they play a major role in detoxifying various xenobiotics. In order to provide alternative biocatalytic tools to generate FMO-derived drug metabolites, a collection of microbial flavoprotein monooxygenases, sequence-related to human flavin-containing monooxygenases (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 LC-MS/MS analyses of the conversions of 3-(methylthio)aniline, albendazole and fenbendazole revealed that the respective sulfoxides are formed in good to excellent enantiomeric excess 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 an e.e. of 95%, while 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.

5.1. 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 (CYP450s) [1]. However, apart from CYP450s, recent studies have shown that the so-called flavin-containing monooxygenases (FMOs) also 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 5 isoforms, FMO1-FMO5, all of which have their typical tissue-dependent expression patterns and roles in metabolism [2]. FMOs have been shown to be involved in the oxygenation of heteroatom-containing compounds, such as amines and sulfides [2-4]. Different from CYP450s, which contain a heme cofactor, FMOs utilize a flavin cofactor for oxidations which also translates into a different oxidative mechanism. Furthermore, to discriminate between metabolism by human FMOs or CYP450s, often differences in stability and specific inhibitors can be used [5]. FMO enzymes require NADPH for reducing the 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 [1,2,4,6-9] for mechanistic details).

While it has been established that human FMOs are essential in oxidizing a variety of xenobiotics, biochemical and metabolic studies on these enzymes are hampered by their poor availability. Human FMOs (hFMOs) and their mammalian orthologs are typically membrane associated and often thermolabile which appear to be the major reasons for their problematic isolation from tissue [10,11] and inefficient recombinant production. While human FMOs can be studied using microsomal preparations and some human FMOs were expressed as functional enzymes in heterologous hosts [12-15], these enzyme preparations involve costly and cumbersome isolation procedures, and often suffer from low activity and stability [2,16]. Sequence comparison studies have revealed that FMOs are part of a large family of monooxygenases, the so-called Class B flavoprotein monooxygenases [17]. Intriguingly, many bacteria and fungi contain sequence-related Class B flavin-containing monooxygenases [18] that are typically involved in catalyzing Baeyer-Villiger oxidations forming a subfamily of Baeyer-Villiger monooxygenases (BVMOs). Biocatalytic studies on these microbial monooxygenases confirmed that they employ the same catalytic mechanism as FMOs [19] and, interestingly, are also able to catalyze oxygenations of heteroatom containing compounds. In
contrast to hFMOs, many microbial BVMOs are soluble enzymes and can be easily produced in recombinant form [20].

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. By choosing the proper monoxygenase, all tested xenobiotics (nicotine, lidocaine, 3-(methylthio)aniline, albendazole, and fenbendazole, see Figure 1) could be converted. Chiral LC-MS/MS analysis showed that sulfides were converted to the corresponding sulfoxides with excellent and complementary enantioselectivities. This 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.

![Figure 1. Substrates used in microbial monooxygenase-catalyzed conversions.](image)

5.2. Materials and Methods

5.2.1. Materials

3-(Methylthio)aniline, albendazole, ricobendazole (racemic albendazole sulfoxide), fenbendazole, lidocaine, nicotine, 1,4-dioxane and Tris were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Acetaminophen was purchased from Fluka, ultra-pure HPLC grade acetonitrile and HPLC grade methanol were purchased from Biosolve (Valkenswaard, The Netherlands). Catalase was purchased from Fluka while phosphite dehydrogenase was prepared using an established protocol [21]. Ultrapure water was obtained from a Milli-Q Advantage A10 Water
Purification system (Millipore Corp., Billerica, MA, USA). Oasis HLB 30 mg solid phase extraction (SPE) cartridges were purchased from Waters (Manchester, UK).

5.2.2. Recombinant expression of BVMOs and preparation of cell extracts

The enzymes were overexpressed in *Escherichia coli* using previously established conditions and protocols. CHMOAc, PAMOM446G and BVMORj24 were expressed using the pCRE2 expression vector [22], yielding the enzyme fused to His-tagged phosphite dehydrogenase which facilitates cofactor regeneration. His-tagged PAMO and Strep-tagged FMOjE were expressed as described previously [23] while for expressing BVMOMt1, a pET_SUMO vector was used. Precultures were grown overnight at 37 °C with shaking (180 rpm) in lysogeny broth (LB) medium containing ampicillin (50 μg/mL). The exception was BVMOMt1 for which cells were grown in the presence of kanamycin (100 μg/mL). Flasks containing 200 mL TB medium with the respective antibiotic were inoculated 1:100 (v/v) using the preculture and grown for another 4 h at 30 °C. After that, each flask was supplemented with inducer: 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) for BVMOMt1, and 0.02% arabinose for PAMO, PAMOM446G, and CHMOAc, and 0.002 % arabinose for the remaining enzymes. After 48 h of growth at 24 °C with shaking (130 rpm) cells were harvested by centrifugation at 4 °C, 17,000 g and resuspended in 50 mM Tris buffer pH 8. Cells were diluted to an OD600 of 212 for TB samples and an OD600 of 98 for LB samples. Subsequently, the cells were disrupted by sonication for 90 s using 2 s sonication pulses and 2 s 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 negative control *E. coli* cells were grown without expression plasmid and used for the preparation of cell extract as described above. Overexpression of the enzymes was confirmed with SDS-PAGE by analyzing OD-normalized samples from bacterial cultures.

5.2.3. Monooxygenase-catalyzed conversions

For conversions, cell extracts (100 μL) were supplemented with 1.0 mM substrates (except for 3-(methylthio)aniline: 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. In order 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 min of incubation at room temperature, a 100 μL sample was taken and proteins were precipitated by
adding 300 μL acetonitrile containing 0.2 % formic acid. Samples were vortexed for 30 s and centrifuged at 13000 rpm for 6.5 min. After centrifugation, 200 μL of the supernatants was evaporated to dryness under nitrogen prior to solid phase extraction (SPE). SPE was performed on Oasis HLB 30 mg cartridges that were wetted with acetonitrile and equilibrated with H2O/acetonitrile (95:5). Dried samples were dissolved in 200 μL water and loaded onto the cartridge. Water (3 x 250 μL) was used to wash the cartridges and the final elution was performed with acetonitrile (4 x 250 μL). For the LC-MS/MS analysis, samples were 10x diluted in water containing 10 μM acetaminophen, as an internal standard for LC-MS/MS signal normalization.

5.2.4. Chiral LC-MS/MS in the Selected Reaction Monitoring (SRM) mode

LC-MS/MS analyses in the SRM mode were carried out on an HPLC system with an Accela Autosampler and a Surveyor Pump coupled to a TSQ Quantum AM triple quadrupole mass spectrometer (Thermo Finnigan, San José, CA) with an ESI interface in the positive mode (see Tables 1 and 2 for details). 3-(Methylthio)aniline, albendazole, fenbendazole and their chiral sulfoxide products were separated with an amylose tris(3-chlorophenyl carbamate)-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 LC separation of lidocaine and its products was performed with a C18 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 (HILIC) column (Xbridge amide, 3.5 μm particle size, 2.1x150 mm; Waters, Milford, MA, USA) at a flow rate of 250 μL/min. The following set of solvents was used for the separations: solvent A (H2O with 0.1% formic acid), solvent B (acetonitrile with 0.1% formic acid), solvent C (H2O with 20 mM ammonium bicarbonate (pH 9, adjusted with NH3)), solvent D (acetonitrile) and solvent E (H2O 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 min) 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 min) and fenbendazole sulfoxides were separated with 40% solvent C / 60% solvent D at 200 μL/min (15 min), 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 min which was held for 1 min. Solvent B was decreased rapidly to 5% in 20 s and the column re-equilibrated at 5% solvent B.
for 4 min. The LC-MS/MS analysis of nicotine and its N-oxide was performed by HILIC applying a linear gradient starting from 10% to 90% solvent E in solvent D (acetonitrile) over 8 min which was held for 2 min. Solvent E was decreased rapidly to 10% in 20 s and finally the column re-equilibrated at 10% solvent E for 3 min. Acetaminophen was used as internal standard to normalize the peak areas across LC-MS/MS runs.

### 5.2.5. Circular Dichroism

In order to assign the absolute configuration of the products, samples were analyzed by 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 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.

Table 1. Parameters for the MS analysis of the different substrates and their conversion products. Scan time, Q1 peak width and skimmer offset were set to 1 s, 0.70 amu FWHM and 0, respectively.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Spray Voltage V</th>
<th>Sheath gas</th>
<th>Auxiliary gas</th>
<th>Capillary Temp. °C</th>
<th>Tube lens offset</th>
<th>Scan Range m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(methylthio)aniline</td>
<td>2750</td>
<td>60</td>
<td>30</td>
<td>270</td>
<td>74</td>
<td>50-300</td>
</tr>
<tr>
<td>Albendazole</td>
<td>3000</td>
<td>30</td>
<td>15</td>
<td>300</td>
<td>90</td>
<td>50-400</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>3000</td>
<td>40</td>
<td>15</td>
<td>350</td>
<td>90</td>
<td>50-500</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>3500</td>
<td>40</td>
<td>20</td>
<td>350</td>
<td>90</td>
<td>50-300</td>
</tr>
<tr>
<td>Nicotine</td>
<td>2500</td>
<td>60</td>
<td>15</td>
<td>350</td>
<td>80</td>
<td>50-250</td>
</tr>
</tbody>
</table>
Table 2. SRM transitions and corresponding collision energies for the substrates and their oxidation products. All SRM measurements were performed with a dwell time of 100 ms and Q1 and Q3 peak widths of 0.70 amu FWHM. 1 mTorr collision gas pressure was used for all compounds except for nicotine and its N-oxide products (1.5 mTorr).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>SRM Transition m/z</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(methylthio)aniline</td>
<td>140/93</td>
<td>20</td>
</tr>
<tr>
<td>3-(methylthio)aniline sulfoxide</td>
<td>156/93</td>
<td>20</td>
</tr>
<tr>
<td>albendazole</td>
<td>266/234</td>
<td>20</td>
</tr>
<tr>
<td>albendazole sulfoxide (ricobendazole)</td>
<td>282/240</td>
<td>20</td>
</tr>
<tr>
<td>fenbendazole</td>
<td>300/268</td>
<td>20</td>
</tr>
<tr>
<td>fenbendazole sulfoxide</td>
<td>316/191</td>
<td>21</td>
</tr>
<tr>
<td>lidocaine</td>
<td>235/86</td>
<td>30</td>
</tr>
<tr>
<td>lidocaine N-oxide</td>
<td>251/86</td>
<td>30</td>
</tr>
<tr>
<td>nicotine</td>
<td>163/132</td>
<td>20</td>
</tr>
<tr>
<td>nicotine-1`N-oxide</td>
<td>179/117</td>
<td>15</td>
</tr>
<tr>
<td>nicotine-1-N-oxide</td>
<td>179/148</td>
<td>15</td>
</tr>
<tr>
<td>acetaminophen (IS)</td>
<td>152/110</td>
<td>20</td>
</tr>
</tbody>
</table>

5.3. Results

5.3.1. Conversion of the sulfide 3-methyl(thio)aniline

We selected seven microbial flavoprotein monooxygenases originating from three different microorganisms. Besides three well-studied BVMOs, phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* [23], the Met446Gly PAMO mutant (PAMO<sub>M446G</sub>) [24] and cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (CHMO<sub>Ac</sub>) [22], two recently discovered BVMOs from *Rhodococcus jostii* (BVMORj4 and BVMORj24) [25] and a BVMO from the fungus *Myceliophthora thermophila* (BVMOMt1) were included in this study. Furthermore, we also included a representative of a newly discovered distinct subfamily of microbial monooxygenases, the so-called Type II FMOs, that share characteristics of both BVMOs and FMOs: FMO<sub>RjE</sub> from *R. jostii* [25]. All studied monooxygenases belong to the Class B flavoprotein monooxygenases and, hence, are distantly related to mammalian
FMOs as evidenced by significant sequence identities (20-40 %) and highly conserved sequence motifs [4]. Also the obtained crystal structures of a bacterial FMO and several BVMOs have confirmed that Class B flavoprotein monoxygenases share structural and mechanistic features [8,26]. CHMO<sub>A</sub> and PAMO display complementary and broad substrate acceptance profiles [19], while the other monoxygenases have been hardly explored for their substrate scope. Therefore, we anticipated that by studying such a large panel of different microbial monoxygenases, several targeted compounds could be converted by one or more monoxygenases.

All monoxygenases were produced in *E. coli* as 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 monoxygenase 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 monoxygenases, 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 [27]. Human FMOs are also known for their ability to perform sulfoxidations of aromatic sulfides or thioureas [2,28]. Depending on the type of sulfide and FMO isoform, various enantioselectivities by mammalian FMOs have been described [29-31]. Conversion of this relatively simple aromatic thioether was probed with all 7 studied monoxygenases. 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 min 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 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 monoxygenases produced significant amounts of sulfoxides but with markedly different enantioselectivity (Figure 2). CD analysis of the 3-(methylthio)aniline sulfoxides formed in the BVMO<sub>Rj4</sub> and BVMO<sub>Mt1</sub> samples gave $[\alpha]_D^{25}$ values of +20.8 and -16.0, respectively (Figure 3). Based on comparison with literature data for (R)-3-(methylthio)aniline we assign the first eluting enantiomer (at 8.4 min) to (+)-(R)-3-(methylthio)aniline sulfoxide and the second eluting enantiomer (at 11.2 min) to (-)-(S)-3-(methylthio)aniline sulfoxide [32,33].
Figure 2. Chiral LC-MS/MS in the SRM mode of 3-(methylthio)aniline sulfoxide (3-MTA-SO) (SRM transition: 156/93). 3-(methylthio)aniline was incubated for 135 min in the presence of the following microbial monooxygenases: a) PAMO\textsubscript{M446G}, b) CHMO\textsubscript{Ac}, c) PAMO, d) FMO\textsubscript{RjE}, e) BVMO\textsubscript{Rj4}, f) BVMO\textsubscript{Rj24} and g) BVMO\textsubscript{Mt1}. Based on CD analysis and data reported in the literature, the first eluting enantiomer at 8.4 min was assigned to (R)-3-methylthioaniline sulfoxide and the second eluting enantiomer at 11.5 min to (S)-3-methylthioaniline sulfoxide [32]. The enantiomeric excess is given as e.e.

Most monooxygenases have a preference for forming the (R)-3-(methylthio)aniline sulfoxide (e.e. values 66-88%). However, BVMO\textsubscript{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\textsubscript{Rj4} and BVMO\textsubscript{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 min incubation. The conversion reached 72% for BVMO\textsubscript{Rj24} and 97% for BVMO\textsubscript{Mt1} (Figure 7). This indicates that with the current approach 4-5 mg of enantiopure sulfoxide metabolite is produced in 1 h using a cell extract from a 1 L culture.
Figure 3. Top panel: CD spectra of 3-methylthioaniline sulfoxide enantiomers. Bottom panel: assignment of the first eluting enantiomer to (+)-(R)-3-(methylthio)aniline sulfoxide and the second eluting enantiomer to (-)-(S)-3-(methylthio)aniline sulfoxide based on the CD spectra and reported $[\alpha]_{D}^{25}$ value in literature data [32,33].

5.3.2. 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 [31]. Testing the panel of microbial monooxygenases revealed that three monooxygenases (CHMO$_{Ac}$, BVMO$_{Rj4}$, and BVMO$_{Mt1}$) converted albendazole, yielding sulfoxides in significant enantiomeric excess (Figure 4). Only BVMO$_{Mt1}$ was able to catalyze the sulfoxidation of fenbendazole. Determination of the $[\alpha]_{D}^{25}$ 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. [34], allowing us to assign the first eluting enantiomers to (R)-albendazole sulfoxide (3.1 min) and (R)-fenbendazole sulfoxide (3.5 min), respectively, and the second eluting enantiomers to (S)-albendazole sulfoxide (5.5 min) and (S)-fenbendazole sulfoxide (7.1
min), respectively. Chiral LC-MS/MS in the SRM mode showed that CHMOAc and BVMOMt1 formed the same product as mammalian FMOs, (R)-albendazole sulfoxide (32 and 55\% e.e., respectively), whereas BVMO\textsubscript{R24} enzyme produced (S)-albendazole sulfoxide with an enantiomeric excess of 95\%. The yields of albendazole sulfoxide were determined with standard addition and reached 55\% for BVMO\textsubscript{R24} and 25\% for BVMO\textsubscript{Mt1} (Figure 8). This corresponds to 1-2 mg/h and per L of bacterial culture. Less than 1\% of the sulfone product was formed by BVMO\textsubscript{R24}, BVMO\textsubscript{Mt1} and CHMOAc upon conversion. No other side products were detected. Chiral LC-MS/MS in the SRM mode showed that conversion of fenbendazole by BVMO\textsubscript{Mt1} yields (S)-fenbendazole sulfoxide in 96\% e.e. (Figure 5).

Figure 4. Chiral LC-MS/MS in the SRM mode of albendazole sulfoxide (ABZ-SO) (SRM transition: 282/240). Albendazole was incubated for 135 min in the presence of the following monooxygenases: a) CHMO\textsubscript{Ac}, b) BVMO\textsubscript{R24}, c) BVMO\textsubscript{Mt1}. The first eluting enantiomer at 3.1 min was assigned to (R)-albendazole sulfoxide and the second eluting enantiomer at 5.5 min to (S)-albendazole sulfoxide based on literature data [34].
Figure 5. Chiral LC-MS/MS in the SRM mode of fenbendazole sulfoxide (FBZ-SO) (SRM transition: 316/284). Fenbendazole was incubated for 135 min in the presence of the studied monoxygenases of which only BVMOMt1 showed activity. The first eluting enantiomer at 3.5 min was assigned to (R)-fenbendazole sulfoxide and the second eluting enantiomer at 7.1 min to (S)-fenbendazole sulfoxide based on literature data [34].

5.3.3. 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 while nicotine is a plant alkaloid which 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 BVMORj24, while nicotine is converted by CHMOAc (Figure 6). With the applied conditions, the degree of conversion for both substrates was rather low, below 10%.
Figure 6. Relative yields of the N-oxide products of lidocaine (top) and nicotine (bottom) in the presence of different bacterial monoxygenases as determined by LC-MS/MS in the SRM mode. Experiments were performed in duplicate.
For lidocaine no other products (specifically the Cytochrome 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) [35,36]. 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.

5.3.4. Quantification of product yields with standard addition method

5.3.4.1. 3-methyl(thio)aniline

Since a 3-(methylthio)aniline sulfoxide standard was not available, we measured the concentration of 3-(methylthio)aniline remaining after the 135 min conversion reaction. During the conversion reaction 3 mM 3-(methylthio)aniline was used. In the extraction step, the sample was diluted 4 times (to a nominal concentration of 750 \(\mu\)M) and in the SPE purification step it was diluted a further 20 times. For the LC-MS/MS quantification, control (extract with no monooxygenase expressed), BVMORj24 and BVMO Mt1 samples were diluted 10 times more, and used as unknowns in the standard addition method (see Table 3). All the quantification experiments were duplicated.

The control sample was used to determine the 3-(methylthio)aniline concentration after extraction and SPE cleanup, but without conversion. Samples were analyzed with LC-MS/MS and peak areas were plotted (Figure 7). According to the standard addition curve the concentration of unknown (control) was 3.2 \(\mu\)M. The remaining 3-(methylthio)aniline concentrations after 135 min incubation in BVMO Rj24 and BVMO C14 samples were found to be 0.9 \(\mu\)M and 0.1 \(\mu\)M (Figure 7). Based on these concentrations and that of the control, the consumption of 3-(methylthio)aniline after 135 min incubation was calculated to be roughly 72% for BVMO Rj24 and 97% for BVMO Mt1, without correcting for recovery or sample instability during storage.
Table 3. Standard addition method used for quantification of 3-(methylthio)aniline, albendazole and albendazole sulfoxide in enzymatic conversion. All dilution series had a total volume of 1 mL.

<table>
<thead>
<tr>
<th>Unknown (µL)</th>
<th>Acetaminophen (IS) (µL)</th>
<th>Reference std (µL)</th>
<th>Concentration of reference std (µM)</th>
<th>Water (µL)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>700</td>
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<td>100</td>
<td>200 (1 µM stock)</td>
<td>0.2</td>
<td>600</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>400 (1 µM stock)</td>
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</tr>
<tr>
<td>100</td>
<td>100</td>
<td>80 (10 µM stock)</td>
<td>0.8</td>
<td>720</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>160 (10 µM stock)</td>
<td>1.6</td>
<td>640</td>
</tr>
</tbody>
</table>

5.3.4.2. Albendazole sulfoxide

BVMO_{B24} and BVMO_{M1} samples, which showed the highest of albendazole sulfoxide peaks in LC-MS/MS were used for quantification. The same extraction, SPE purification and dilution steps were followed for albendazole as stated for 3-(methylthio)aniline (see Table 3).

To a control extract 1 mM of ricobendazole (racemic albendazole sulfoxide) was added and used as a control (Figure 8) to correct for the loss during the extraction and SPE purification steps. The concentration determined to be 2.0 µM. The albendazole sulfoxide product concentrations were calculated to be 1.1 µM for BVMO_{B24} and 0.5 µM for BVMO_{M1}. The estimated product yield of albendazole sulfoxide is therefore 55% for BVMO_{B24} and 25% for BVMO_{M1} (Figure 8).
Figure 7. Standard addition curve of 3-(methylthio)aniline. The concentration of 3-(methylthio)aniline in: a) control sample was found to be 3.2 $\mu$M, b) BVMORj24 was found to be 0.9 $\mu$M, and c) BVMOMt1 was found to be 0.1 $\mu$M.
Figure 8. Standard addition curves of albendazole sulfoxide. The concentration of albendazole sulfoxide in: a) control sample was found to be 2 µM, b) BVMORj24 was found to be 1.1 µM, and c) BVMOt1 was found to be 0.5 µM.
5.4. Discussion

Human FMOs and other mammalian FMOs play a crucial role in degrading a wide range of xenobiotics, including many drugs. While 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 [4]. 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 [37]. 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 that is key to catalyze substrate oxygenation [19,38]. 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 determines the substrate specificity and the enantio- and regioselectivity of each monooxygenase. As a consequence and different from many other flavoprotein monooxygenases and CYP450, 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, that 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 [23,39]. 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 monoxygenases. Chiral LC-MS/MS in the SRM mode was instrumental in establishing activity and selectivity of each enzyme towards 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 (CHMOAc and BVMO Mt1 for the (R)-sulfoxide and BVMO R24 for the (S)-sulfoxide). Previously, it has been shown that mammalian FMOs have a preference for forming (R)-albendazole sulfoxide from albendazole [30,31]. For fenbendazole, only one active enzyme (BVMO Mt1) was identified which preferentially forms the (S)-sulfoxide. Fenbendazole has been shown to be converted into the (R)-sulfoxide by mammalian FMOs with significant enantiomeric excess [30]. 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 making 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 monoxygenases are chemo-, regio- and enantioselective. Monoxygenases that form the N-oxides of nicotine (CHMOAc) and lidocaine (BVMO R24) were also identified. In the literature it was reported that human FMO3 is responsible for selective formation of nicotine-1`-N-oxide [16], which was also the major N-oxide formed by CHMOAc. This shows that the microbial monoxygenases can also be used for chemo- and regioselective N-oxidations of xenobiotics.

Our study illustrates that sequence-related microbial monoxygenases can be used for the production of FMO-related metabolites. As for mammalian FMOs [11,16], 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.
5.5. References


