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Baeyer–Villiger Monooxygenase FMO5 as Entry Point in Drug Metabolism

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S Supporting Information

ABSTRACT: Flavin-containing monooxygenases (FMOs) are emerging as effective players in oxidative drug metabolism. Until recently, the functions of the five human FMO isoforms were mostly linked to their capability of oxygenating molecules containing soft N- and S-nucleophiles. However, the human FMO isoform 5 was recently shown to feature an atypical activity as Baeyer–Villiger monooxygenase. With the aim of evaluating such an alternative entry point in the metabolism of active pharmaceutical ingredients, we selected and tested drug molecules bearing a carbonyl group on an aliphatic chain. Nabumetone and pentoxifylline, two widely used pharmaceuticals, were thereby demonstrated to be efficiently oxidized *in vitro* by FMO5 to the corresponding acetate esters with high selectivity. The proposed pathways explain the formation of a predominant plasma metabolite of pentoxifylline as well as the crucial transformation of the pro-drug nabumetone into the pharmacologically active compound. Using the recombinant enzyme, the ester derivatives of both drugs were obtained in milligram amounts, purified, and fully characterized. This protocol can potentially be extended to other FMO5 candidate substrates as it represents an effective and robust bench-ready platform applicable to API screening and metabolite synthesis.

Though long considered to be ancillary to the action of cytochrome P450 monooxygenases, the contribution of flavin-containing monooxygenases (FMOs) to human oxidative metabolism was recently shown to be predominant in a number of cases.^{1,2} This has translated into the identification of an increasing number of reactive substrates among pharmaceuticals and dietary-derived compounds along with the awareness that the oxygenating activity of FMOs can produce more polar and readily excretable compounds.^{2,3} Typical FMO substrates include drugs, pesticides, and dietary components and range from rather small molecules, such as trimethylamine and dimethyl sulfoxide to bulkier and pharmacologically active compounds, like benzydamine and moclobemide.^{1,4} Though structurally different, all these molecules share a soft nucleophilic heteroatom (mainly nitrogen and sulfur) as a reactive target of FMO-mediated oxidations.⁴ The corresponding metabolites affect a number of impacting medical conditions in humans (e.g., atherosclerosis,⁵ altered bile and sterol metabolism,⁶ and platelet hyperactivity⁷).

Despite a characteristic FMO-like sequence, human FMO5 (hFMO5) proved to be substantially inactive on nucleophilic

compounds and instead showed a strong catalytic activity directed toward electrophilic carbonyl molecules that are typical substrates of Baeyer–Villiger monooxygenases (BVMOs).^{8,9} Such peculiar behavior for a human FMO parallels the secluded FMO5 gene location, outside the 220 kb cluster on chromosome 1 hosting all other FMO genes.¹⁰ To shed light on this so-far neglected Baeyer–Villiger oxidative branch in drug metabolism, we developed an efficient expression system for the production of recombinant hFMO5, a monotopic membrane-anchored enzyme. Specifically, we have implemented two assays based on *E. coli* membranes and whole cells, which allowed us to obtain substantial amounts of drug metabolites through direct transformation by hFMO5. Finally, analysis by using the stopped-flow technique allowed us to highlight the kinetic properties of hFMO5, which stands out from the general mode of action commonly found in FMOs based on long-lasting stabilization of C4a-(hydro)peroxyflavin

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Figure 1. Conversion of nabumetone and pentoxifylline by purified hFMO5. The picture shows the GC traces obtained from conversion of pentoxifylline (left panel) and nabumetone (right panel) by purified hFMO5 (red line). The comparison with the GC traces obtained from reactions run with purified human FMO3 (blue line) and from the blanks (black line) highlights the two new products produced by hFMO5 (P-M1/P-M2 and N-M1/N-M2).

intermediate.⁴ Overall, excellent overexpression in *E. coli* combined with a high thermal stability and natural immobilization into membranes render this human enzyme a promising biocatalyst for biotechnological applications.

RESULTS

hFMO5 Accepts Nabumetone and Pentoxifylline As Substrates. A few reports recently suggested the participation of hFMO5 in the metabolism of active pharmaceutical ingredients (APIs), such as the anticancer compound E7016¹¹ or the antibacterial agent MRX-1.¹² The proposed catalytic role played by hFMO5 in the oxygenation of the piperidone moiety common to these two compounds was then supported by demonstration of hFMO5 activity on a large set of electrophilic molecules.⁸ Among a series of model substrates, hFMO5 showed prominent and chemoselective activity as BVMO, resulting in oxygen insertion in both aliphatic and cyclic ketones to form the corresponding esters and lactones, respectively. In particular, the excellent reactivity of longer aliphatic unsymmetrical ketones guided us in the selection of suitable candidates among approved drug molecules to be screened for conversion by hFMO5. Nabumetone and pentoxifylline, both ω -substituted 2-alkanones, stood out due to anticipated good accessibility of their carbonyl moiety (Figure 1). Nabumetone, a widely used nonsteroidal anti-inflammatory drug, is prescribed to reduce pain and inflammation in patients suffering from osteoarthritis or rheumatoid arthritis. It is actually a pro-drug because it exerts its pharmacological effects *via* its metabolite 6-methoxy-2-naphthylacetic acid, whose anti-inflammatory properties are ascribed to the inhibition of cyclooxygenase 2.¹³ Pentoxifylline is a phosphodiesterase inhibitor currently used as a hemorheological agent to treat peripheral vascular diseases. Besides increasing red blood cell deformability and decreasing blood viscosity, pentoxifylline has various anti-inflammatory and immunomodulatory properties, which have been inves-

tigated in several diseases characterized by an increased inflammatory or immunological response.^{14,15}

Taking advantage of the availability of a pure and active hFMO5 sample,⁸ the two drug substrates (5 mM) were initially tested through a simple system comprising the purified enzyme (5 μ M) and NADPH (10 mM). In-house produced human FMO3 was utilized as a control to highlight differences in the catalytic selectivity between the two FMO isoforms, which are most expressed in the human liver.¹⁰ After 16 h of reaction time, GC analysis revealed the formation of two additional compounds with about 40% conversion levels for each of the two drugs (N-M1 and N-M2 from nabumetone; P-M1 and P-M2 from pentoxifylline). This transformation was specific for FMO5 and not observed for FMO3 (Figure 1 and Table 1, line A). MS analysis revealed a mass and a fragmentation pattern for each newly formed compound compatible with the insertion of oxygen nearby the carbonyl moiety of the parent compounds (N-M1 and N-M2, MW 244 g·mol⁻¹; P-M1 and P-M2, MW 294 g·mol⁻¹). These results provided the first clues of Baeyer–Villiger oxidation activity by hFMO5 on pharmaceuticals bearing aliphatic carbonyl moieties.

Method Optimization, Scale-up, and Product Identification. The membrane association of metabolizing enzymes is essential for efficient guiding of lipophilic (a property common to many drugs) substrates to their active sites.¹⁶ The employment of purified cell-free membrane proteins may therefore suffer from poor activity due to the absence of a hydrophobic ground. In order to obtain larger quantities of product for full chemical characterization, two alternative methods were devised for increased conversion levels of nabumetone and pentoxifylline by hFMO5. The first strategy was based on lyophilization, which permeabilizes resting cells ensuring efficient transport of the substrate and cofactor (Table 1, line B). The second method, based on *E. coli* membranes isolated by ultracentrifugation following cell breakage, was designed to preserve the intrinsic stability of the enzyme in its physiological environment along with improved substrate and cofactor accessibility, while minimizing the occurrence of

Table 1. Conversion of APIs and Control Substrates by Human FMO5/3 and Comparison among Different Biocatalytic Preparations

		nabumetone^a		pentoxifylline^a		2-heptanone^d	thioanisole^d
		N-M1 ^b	N-M2 ^c	P-M1 ^b	P-M2 ^c	Pentyl acetate	T-M1	T-M2
A	Purified FMO5	40%	<1%	40%	1%	64%	n.t.	n.t.
	Purified FMO3	n.d.	n.d.	n.d.	n.d.	n.t.	88%	n.d.
B	Whole cell-FMO5	32%	<1%	82%	3%	98%	n.t.	n.t.
	Whole cell-FMO3	n.d.	n.d.	n.d.	n.d.	n.t.	73%	n.d.
C	Membranes-FMO5	85%	1%	91% ^e	3% ^e	87%	n.t.	n.t.
	Membranes-FMO3	n.d.	n.d.	n.d.	n.d.	n.t.	93% ^e	7% ^e
D	Controls							
	- substrate +							
	NADPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	- BL21 whole cell							
	- BL21 membranes							

^aConversions based on product calibration. ^bAcetate products. ^cMethyl ester products. ^dConversions based on GC peak areas [P/(S+P)·100]. ^eFull substrate consumption. n.d., not detected; n.t., not tested. ^fThe table reports the conversion levels (%) of nabumetone and pentoxifylline into their ester metabolites (N-M1/N-M2 and P-M1/P-M2, respectively) by human FMO5/3 obtained by different methodologies (line A = purified human FMOs; line B = lyophilized *E. coli* whole cells expressing recombinant human FMOs; line C = *E. coli* membrane fraction obtained after breakage of cells expressing human FMOs; line D = controls performed with native *E. coli* BL21 cells or in the absence of enzyme). 2-Heptanone and thioanisole were employed as controls of specific activities of FMO5 (Baeyer–Villiger transformation to pentyl acetate) and FMO3 (oxidation to T-M1 = methyl phenyl sulfoxide; T-M2 = methyl phenyl sulfone resulting from oxidation of T-M1).

possible side reactions (Table 1, line C). To allow comparison with the initial method employing purified human FMOs, both systems were tested in the presence of NADPH (10 mM) and drug substrates (5 mM). 2-Heptanone and thioanisole were utilized as positive controls for the chemoselective activity of FMO5 and FMO3, respectively. Furthermore, empty *E. coli* BL21 strain was employed as a negative control to exclude the contribution of native bacterial enzymes in the transformation of the two drugs (Table 1, line D). Both lyophilized cells and membrane-embedded hFMO5 entailed a marked increase in the conversion levels of both nabumetone and pentoxifylline, as well as of the control substrates. Overall, the transformation by *E. coli* membranes proved to be most efficient. Remarkably, in the case of nabumetone—the least hydrophilic substrate tested—the membrane-based method led to the doubling of the product amount.

In order to unambiguously determine the chemical structure of the metabolites produced by hFMO5, and to limit the amount of NADPH to be supplied, a whole-cell system was

employed, which exploits the cellular endogenous NADPH-recycling system sustained by the addition of MgCl₂ (10 mM) and trisodium citrate (50 mM).^{3,17} Altogether, starting from 114 mg of nabumetone and 138 mg of pentoxifylline in a total of 10 mL of buffer (corresponding each to 50 mM), only 7 mg of NADP⁺ (1 mM) and 250 mg of lyophilized cells were overall sufficient to transform enough material to recover, respectively, 25 mg and 20 mg of the corresponding oxygenated metabolites after solvent extraction and product purification (20% and 14% isolated yield, respectively). ¹H and ¹³C NMR and HR-MS demonstrated that hFMO5 features a strong regioselectivity for the normal (acetate) Baeyer–Villiger products (Figure 2). The abnormal products (methyl esters) were detected only in trace (yet significant) amounts (<5%).

Proposed Pathways for Nabumetone and Pentoxifylline Metabolism. The highly effective *in vitro* oxidation of nabumetone and pentoxifylline to their corresponding esters raised questions about a possible involvement of the highly expressed hepatic hFMO5 in the metabolism of these two

Figure 2. Schematic representation of BVMO activity of hFMO5 on nabumetone and pentoxifylline. The picture shows the oxidation reactions catalyzed by hFMO5 on nabumetone and pentoxifylline after product characterization by NMR and HR-MS. hFMO5 catalyzes an oxygen insertion on both sides of the carbonyl moiety (85:1 and 30:1 represent the ratio of the two products). Though with a net preference for the formation of the acetate “normal” products (N-M1 and P-M1, inserted oxygen colored red), hFMO5 can also promote the least favored formation of methyl “abnormal” esters (N-M2 and P-M2, inserted oxygen colored blue).

Figure 3. Proposed pathways for the metabolism of nabumetone (left) and pentoxifylline (right) in the human liver following initial Baeyer–Villiger oxidation by hFMO5. The pro-drug nabumetone can be metabolized to the active metabolite 6-methoxy-2-naphthylacetic acid in the human liver (left panel) through four consecutive and distinct catalytic steps: after formation of the ester product N-M1 by hFMO5, hydrolysis leads to formation of an alcohol, which undergoes two rounds of oxidation (by an alcohol dehydrogenase and an aldehyde oxidase, or alternatively aldehyde dehydrogenase). The proposed pathway is also applicable to pentoxifylline metabolite P-M1 produced by hFMO5 (right panel) and would result in formation of pentoxifylline major metabolites known as M-5, the principal human urinary elimination product (right panel).¹⁴

drugs. The hereby discovered transformation of pentoxifylline to an acetate product (P-M1) provides an entry into the oxidative degradation toward carboxylic acid M-5 (Figure 3,

right panel). This pentoxifylline metabolite was indeed shown to be one of the major oxidative metabolites of the drug in humans.^{14,15,18} In the case of nabumetone, the enzymatic steps

Figure 4. Kinetics and mechanistic studies on hFMO5 using pentoxifylline as a model substrate. (a) Overall scheme of FMOs reactions. The NADPH-dependent reduction of the flavin prosthetic group is followed by activation of molecular oxygen as a C4a-(hydro)peroxyflavin. The catalytic cycle is closed either by substrate oxygenation followed by elimination of water (green frame) or by spontaneous elimination of H₂O₂ (uncoupling reaction, red frame). (b) Rapid kinetics studies on hFMO5 catalysis. All traces are the average of three replicates. The left panel shows the spectra observed during the anaerobic hFMO5 reduction using NADPH, in the absence of additional compounds. The inset shows the stopped-flow traces at 455 nm recorded after mixing the enzyme with NADPH in the absence or the presence of pentoxifylline. The measured rate constants are 5- and 2-times lower in the presence of pentoxifylline (0.06 and 0.01 s⁻¹ vs 0.3 and 0.02 s⁻¹ with no substrate), indicating that the substrate tends to slow down the reaction with NADPH. Formation of a small long-wavelength absorption (>500 nm) peak is probably due to a charge-transfer complex between the reduced enzyme and NADP⁺.^{21,22} For comparison, a control assay without NADPH is also shown in the inset. The right panel shows the spectra observed after mixing the anaerobically reduced hFMO5 (15 μM hFMO5 with 30 μM NADPH for 300 s in the aging loop of the stopped-flow instrument) with air-saturated buffer. In both panels, the time (s) is indicated above each corresponding spectrum.

involved in its transformation from pro-drug to the pharmacologically active 6-methoxy-2-naphthylacetic acid metabolite (known to occur in the liver), though traditionally assigned to a cytochrome P450 monooxygenase, have not yet been clearly delineated.^{13,19,20} A P450-based pathway appears unlikely given the proposed occurrence of a particularly difficult C–C bond cleavage step. The so far neglected oxidation of nabumetone to an acetate metabolite (N-M1) by hFMO5 immediately suggests a plausible pathway. Nabumetone is here proposed to be metabolized in four consecutive and distinct catalytic steps: after formation of the N-M1 metabolite by hFMO5 and subsequent facile ester hydrolysis, two final rounds of oxidation can deliver 6-methoxy-2-naphthylacetic acid (Figure 3, left panel). In essence, the involvement of highly expressed human hepatic enzymes with broad substrate specificities (hFMO5, carboxylesterase, alcohol dehydrogenase, and aldehyde oxidase—alternatively aldehyde dehydrogenase) combined with the chemical linearity of the proposed reactions provide a realistic alternative to the current view on the predominant involvement of cytochrome P450s in phase I human metabolism of nabumetone.^{13,19,20}

A Substantial Leak of H₂O₂ during Catalysis. Having identified two drug substrates of hFMO5, we sought to investigate the kinetics of the enzymatic transformation by means of the stopped-flow technique and using pentoxifylline as a reference compound (Figure 4). The rates of anaerobic reduction of hFMO5 by NADPH were best fitted to a double

exponential function, suggesting the presence of two interchangeable conformations with different reactivities toward NADPH, as found in other Baeyer–Villiger monooxygenases.^{21–23} Most importantly, the characteristic spectrum of the C4a-(hydro)peroxyflavin was not detected in the course of the aerobic reoxidation reaction (no absorbance peak around 375 nm; Figure 4B, right panel). Apparently, the enzyme does not afford stabilization of the obligatory peroxyflavin intermediate, which is at the heart of substrate monooxygenation (Figure 4A). This finding raised the hypothesis that recombinant hFMO5 might become catalytically impaired upon extraction from its physiological membrane environment. To address this issue, the efficiency of pentoxifylline Baeyer–Villiger oxidation was evaluated using isolated hFMO5-containing membranes (Figure 5). In the presence of equal amounts of substrate and NADPH, 61% of the product was formed, whereas full conversion (i.e., 100% pentoxifylline consumption) was observed only when NADPH was supplied in double amounts compared to the substrate. In essence, hFMO5 exhibits a substantial degree of uncoupling (the wasteful release of hydrogen peroxide with no generation of oxygenated product), and this feature correlates with a poor stabilization of the flavin-peroxide intermediate. Uncoupling is mitigated only by using a large excess of substrate to become almost negligible with a 5:1 pentoxifylline/NADPH ratio (Figure 5).

Figure 5. Evaluation of substrate–conversion efficiency in hFMO5 embedded in *E. coli* membranes. Conversions of 5 mM pentoxifylline by hFMO5-containing membranes were performed for 16 h at different NADPH concentrations. For each NADPH/substrate molar ratio, the plot shows the percentages of product formed/total substrate (i.e., substrate conversion; blue squares) and of product formed/NADPH consumed (i.e., the so-called coupling; red dots). High coupling is observed only in the presence of a large excess of substrate (~90% coupling at 0.2 NADPH/substrate molar ratio).

DISCUSSION

We were able to demonstrate the strong *in vitro* oxygenation activity of hFMO5 on two approved and widespread drugs bearing a carbonyl moiety on an aliphatic chain, nabumetone and pentoxifylline. This finding unfolds a new pathway by which these two drugs can be metabolized in humans (Figure 3). These results further highlight how the contribution of human FMOs (above all, of hFMO5) to drug metabolism has long been underestimated, assuming that their substrate scope was redundant and/or incidental to the one of cytochrome P450s.

Previous studies with pig liver FMO1 and bacterial FMO showed the remarkable ability of these enzymes to stabilize the peroxyflavin intermediate for at least 30 min in the absence of a suitable substrate^{21,22,24} (Figure 4A). Our stopped-flow analysis demonstrates how this capability is not retained by hFMO5, suggesting that the breakdown of the intermediate is faster than its formation under the assayed conditions. This lack of stabilization of the oxygenating intermediate accounts for a quite inefficient formation of oxygenated products (Figure 5). Although this behavior might be changed when downscaled to *in vivo* ratios of NADPH/substrate, the picture emerges that hFMO5 might be continuously acting as an NADPH oxidase while retaining the ability to perform oxygenations when it comes across a suitable substrate. This property is likely shared with other human FMO isoforms in accordance with the considerable rates of H₂O₂ production, which have been recorded with microsomes overexpressing human FMO1–3,²⁵ and does not deviate from cytochrome P450s, also known to display uncoupling as deflection of their catalytic cycle.²⁶ While a large H₂O₂ byproduct formation might be harmful to the cells, a partial substrate conversion still falls under the general features of detoxification enzymes. Selective pressure may have fostered a “leaky” (nonselective) activity which can be addressed to a large number of potentially harmful xenobiotics rather than to very few specific substrates.

Besides hFMO5 showing one of the highest *T_m* values (48 °C) among known BVMOs,^{8,27,28} a strong catalytic activity on a broad range of carbonyl substrates,⁸ and an excellent expression level as active recombinant protein by *E. coli*, its use as a membrane-bound protein offers the unique advantage of being

“physiologically” immobilized, which might pave the way to the use of *E. coli* membranes as an easy-to-access tool for future biotechnological applications. This methodology, which could be easily coupled/fused with “artificial” NADPH recycling systems,²⁹ avoids the frequent issue of rapid degradation of esters and lactones catalyzed by hydrolytic enzymes from the host. This, in turn, maximizes product accumulation in the reaction medium. While the protocol based on *E. coli* membranes stands out for excellent selectivity and stability properties in analytical investigations, our studies indicate that also whole-cell transformation is feasible, as it granted access to up to 25 mg of pure metabolite product from a relatively small-scale process based merely on 250 mg of lyophilized cells.

In conclusion, our results demonstrate the Baeyer–Villiger transformation of nabumetone and pentoxifylline by human FMO5 and outline the synthetic utility of membrane-associated FMO enzymes. In light of the current and previous data,^{8,11,12} we propose to switch the misleading name of hFMO5 to a more suitable “hBVMO1.”

METHODS

Preparation of Purified Human FMOs. Full-length cDNA encoding for *Homo sapiens* FMO5 (NCBI accession number: Z47553) and *Homo sapiens* FMO3 (NCBI accession number: P31513) were cloned into a modified pET SUMO vector (Invitrogen) to allow insertion of a cleavable N-terminal 8xHis-SUMO tag. Expression, cell disruption, extraction, and purification of both isoforms were performed according to the methods previously described.⁸ FMOs concentrations were calculated on the basis of the following extinction coefficient: hFMO5 $\epsilon_{454} = 12.050 \text{ mM}^{-1} \text{ cm}^{-1}$ and human FMO3 $\epsilon_{448} = 12.750 \text{ mM}^{-1} \text{ cm}^{-1}$, as determined through the release of FAD after denaturation of the protein by the addition of SDS to a final concentration of 0.5% (w/v).³⁰

Cloning and Expression of Native hFMOs for Biotransformations by Resting Cells and Membranes. Full-length cDNA encoding for untagged *Homo sapiens* FMO5 (NCBI accession number: Z47553) and *Homo sapiens* FMO3 (NCBI accession number: P31513) cloned into pMS470 plasmids¹⁷ were kindly provided by Margit Winkler and Martina Geier (ACIB GmbH, Graz, Austria). Plasmids were transformed by heat-shock into *E. coli* BL21 DE3 cells. Cells from one of the resulting colonies were preinoculated into LB broth containing 100 $\mu\text{g/mL}$ ampicillin and grown overnight at 37 °C. The following day, the culture was inoculated into LB broth (1:50) supplemented with ampicillin 100 $\mu\text{g/mL}$. Cultures were grown at 37 °C and 200 rpm until OD $\approx$ 0.7 and then induced by 1 mM isopropyl β -D-1-thiogalactopyranoside. After induction, the temperature was lowered to 20 °C, and growth was allowed to continue for a total time of 24 h. Cells were harvested by centrifugation (5000g, 15 min, 4 °C), washed by resuspension in 50 mM KPi buffer at pH 8.5, and harvested back by centrifugation (5000g, 15 min, 4 °C).

Preparation of Lyophilized Cells Expressing Native hFMOs. Cells were resuspended (1:3) in 50 mM KPi buffer at pH 8.5, flash-frozen in liquid N₂, and lyophilized by a freeze-drier. Lyophilized cells were stored either at –20 °C or –80 °C until further use.

Preparation of Membranes from Cells Expressing Native hFMOs. The isolation of *E. coli* membranes was performed as previously described.⁸ After the final ultracentrifugation step, the membrane fraction was resuspended in 50 mM KPi buffer at pH 8.5 to a total protein concentration of 40 mg mL^{–1}, as assayed by Biuret reagent. Membranes were aliquoted in 1.5 mL tubes, flash-frozen in liquid N₂, and stored at –80 °C until further use.

Conversion Assays. All reactions and controls were performed in duplicate. Substrates were purchased from Sigma (unless otherwise stated) in analytical grade or higher and used without further purification. Nabumetone was purchased from Alfa Aesar. 2-Heptanone and thioanisole stocks (500 mM) were dissolved in EtOH; pentoxifylline stock (500 mM) was dissolved in a 1:1 EtOH/

H₂O solution. Nabumetone stock (500 mM) was dissolved in a 1:1 dioxane/EtOH solution.

Purified hFMOs Assays. Reactions were performed with 5 μ M hFMOs, 5 mM substrate, and 10 mM NADPH in 400 μ L of 50 mM KPi buffer at pH 8.5 containing 0.05% (v/v) Triton X-100 Reduced. Reactions were incubated for 16 h at 30 °C and 120 rpm (shaker incubator). Control experiments were run with all substrates in the absence of enzyme.

Resting Cells Assays. Lyophilized cells expressing native hFMOs were rehydrated for 30 min at room temperature in 50 mM KPi buffer at pH 8.5 supplemented with 50 μ M FAD. Reactions were performed with 25 mg of lyophilized cells, 5 mM substrate, and 10 mM NADPH in 1 mL of 50 mM KPi buffer at pH 8.5. Reactions were incubated for 16 h at 30 °C and 750 rpm (Eppendorf ThermoMixer). Control experiments were run with all substrates with lyophilized native BL21 DE3 cells.

Membrane Assays. Reactions were performed with 125 μ L of 40 mg mL⁻¹ (total protein concentration) resuspended membranes, 5 mM substrate, and 10 mM NADPH in 1 mL of 50 mM KPi buffer at pH 8.5. Reactions were incubated for 16 h at 30 °C and 750 rpm. Control experiments were run with all substrates using the membrane fraction isolated from native BL21 DE3 cells.

For GC sample preparation, reaction mixtures were extracted with ethyl acetate (2 $\times$ 200 μ L for purified hFMOs assays; 2 $\times$ 500 μ L for resting cells and membranes assays) in the presence of 10 mM 1-decanol as an internal standard. The combined organic phases were dried over anhydrous Na₂SO₄ and analyzed by GC-FID (see below). With 2-heptanone and thioanisole, products were identified using commercial or authentic reference standards. Calibration curves for quantification were obtained from duplicates of three product concentrations (2.5 mM, 5 mM, 10 mM) in the presence of the internal standard.

Preparative-Scale Biotransformation. Multimilligram conversions were performed by means of multiple small scale transformations to ensure the best reproducibility from an optimized protocol, using a total of 250 mg of lyophilized cells and 114 mg of nabumetone or 138 mg of pentoxifylline. Each transformation contained 25 mg of lyophilized cells, 50 mM substrate, 1 mM NADP⁺, 10 mM MgCl₂, 50 mM trisodiumcitrate, and 50 μ M FAD in 1 mL of 50 mM KPi buffer at pH 8.5. Reactions were incubated in a 24-well plate sealed with oxygen permeable foils for 16 h at 30 °C and 750 rpm (Eppendorf ThermoMixer). The reaction mixture was extracted with ethyl acetate (2 $\times$ 500 μ L), and the combined organic phases from 10 replicates were dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude extract obtained from nabumetone biotransformation (65 mg) was purified *via* flash chromatography on silica gel by using hexane/ethyl acetate (95:5, v/v) yielding 25 mg of N-M1 (20% isolated yield). The crude extract obtained from pentoxifylline biotransformation (80 mg) was purified *via* flash chromatography on silica gel by using ethyl acetate/triethylamine (99:1, v/v) yielding 20 mg of P-M1 contaminated with 3% of P-M2 (14% isolated yield). The corresponding product fractions were combined, the solvent was removed under reduced pressure and the resulting product characterized by ¹H NMR and HR-MS.

Efficiency Evaluation in hFMO5 Attached to *E. coli* Membranes. Reactions were performed in duplicate as previously described (see *Membrane Assays* section) with a fixed pentoxifylline concentration (5 mM) in the presence of different NADPH concentrations (NADPH/substrate molar ratios = 0.2, 0.4, 0.6, 0.8, 1, 2). After 16 h, the reactions were extracted with ethyl acetate to quantify the amount of product formed *via* GC analysis.

Analytical Methods for Determination of Conversion Levels and Product Quantification. All compounds were identified by comparison with commercially available reference materials or using purified products characterized by NMR and analyzed *via* GC-MS and GC. GC-MS measurements of pentoxifylline and nabumetone were carried out on a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), equipped with a 5975C mass selective detector and an HP-5MS column (5% phenylmethylsiloxane, 30 m $\times$ 0.20 mm $\times$

0.25 μ m, J&W Scientific, Agilent Technologies) using He as a carrier gas. Temperature injector, 300 °C; temperature program; 200 °C; hold for 0.5 min; 5 °C/min to 300 °C; hold for 2 min. Conversions were obtained from measurements on an Agilent Technologies 7890 A GC system equipped with a FID detector and a 7693 autosampler by using H₂ as carrier gas and a 14% cyanopropylphenyl–86% dimethylpolysiloxane capillary column (J&W DB-1701, 30 m, 0.25 mm ID, 0.25 μ m film) for pentoxifylline, nabumetone, and thioanisole or a 5% phenylmethylpolysiloxane capillary column (J&W HP-5, 30 m, 0.32 mm ID, 0.25 μ m film) for 2-heptanone. 1-Decanol was used as an internal standard (10 mM in ethyl acetate). Samples were injected with a split ratio of 50:1. Conversions of all substrates were determined by GC-FID.

Nabumetone and Pentoxifylline. Temperature injector, 250 °C; temperature detector, 250 °C; temperature program, 180 °C; hold for 1 min; 5 °C/min to 280 °C; hold for 10 min; retention times, pentoxifylline 24.29 min, P-M1 23.13 min, P-M2 23.60 min, nabumetone 15.48 min, N-M1 14.51 min, N-M2 14.88 min.

Thioanisole. Temperature injector, 250 °C; temperature detector, 250 °C; temperature program, 80 °C; hold for 4 min; 15 °C/min to 280 °C; hold for 3 min; retention times, thioanisole 8.14 min, TM-1 12.51 min, TM-2 13.51 min.

2-Heptanone. Temperature injector, 250 °C; temperature detector, 250 °C; temperature program, 60 °C; hold for 0.5 min; 10 °C/min to 200 °C; hold for 0 min; 20 °C/min to 280 °C; hold for 2 min; retention times, 2-heptanone 1.23 min, pentyl acetate 1.37 min.

Analytical Methods for Product Characterization (N-M1 and P-M1). ¹H NMR and ¹³C NMR. A total of 10 mg of purified products was used for NMR. NMR spectra were measured on a Bruker Avance III 300 MHz NMR spectrometer. Chemical shifts are reported relative to TMS (δ = 0.00 ppm); coupling constants *J* are given in Hz.

High-Resolution MS. Samples were dissolved to approximately 0.1 mM in acetonitrile containing 0.1% formic acid. A total of 25 μ L of sample was injected into a 100 μ L/min flow of 0.1% FA acetonitrile for measurement on a Q-Exactive MS instrument (Thermo Fisher) equipped with a heated electrospray ion source. A full scan mode (*m/z*: 150–500) was employed; fwhm resolution was set to 70,000. Source conditions: spray voltage 3300 V; transfer capillary temperature 250 °C; sheath and sweep gas flow rate 35 and 10 instrument units; gas temperature 200 °C.

N-M1. ¹H NMR (300 MHz, CDCl₃): δ 2.06 (s, 3H), 3.09 (t, *J* = 7.1 Hz, 2H), 3.94 (s, 3H), 4.38 (t, *J* = 7.1 Hz, 2H), 7.14–7.73 (m, 6H). ¹³C NMR: δ 171.1 (CO); 157.4 (Ar); 133.3 (Ar); 132.9 (Ar); 129.0 (Ar); 129.0 (Ar); 127.8 (Ar); 127.1 (Ar); 126.9 (Ar); 118.9 (Ar); 105.6 (Ar); 65.0 (CH₂OCO); 55.3 (OMe); 35.0 (CH₂Ar); 21.0 (COCH₃). Calcd for C₁₅H₁₆O₃H⁺: *m/z* 245.11722. Found: 245.11705.

P-M1. ¹H NMR (300 MHz, CDCl₃): δ 1.69–1.75 (m, 4H), 2.05 (s, 3H), 3.59 (s, 3H), 4.03 (s, 3H), 4.06–4.13 (m, 4H), 7.52 (s, 1H). Characteristic of methyl ester (~3% P-M2 present): 2.38 (m, 2H), 3.67 (s, 3H). ¹³C NMR: δ 171.2 (CO); 155.3 (CO); 151.5 (CO); 148.8 (CO); 141.4 (CH=N); 107.6 (C=C); 64.2 (OCH₂); 40.9 (CH₂N); 35.6 (NCH₃); 29.7 (NCH₃); 26.2 (CH₂); 24.7 (CH₂); 21.0 (CH₃CO). Calcd for C₁₃H₁₈O₄N₄H⁺: *m/z* 295.14008. Found: 295.1400.

Stopped-Flow Assays. The catalytic cycle of hFMO5 was investigated using a SX20 stopped-flow spectrometer (Applied Photophysics, Surrey, UK). The single- and double-mixing modes were used to investigate enzyme reduction and reoxidation, respectively. A xenon lamp and a photodiode array detector were used. All experiments were carried out at 25 °C in 50 mM Tris-HCl at pH 8.5 and 5 mM KCl. All assays were run in duplicate or triplicate by mixing equal volumes of reactants. The enzyme reduction was performed in the absence of dioxygen. To make the stopped-flow spectrometer anaerobic, the flow-circuit of this apparatus was repeatedly washed with anaerobic buffer. Nitrogen was bubbled through the solutions for 10 min to make them anaerobic. Only in the case of the enzyme solution, nitrogen was blown on the surface of the solution for 10 min. *Aspergillus niger* glucose oxidase and glucose were added to the solutions to remove any residual traces of dioxygen (final concentrations 0.5 μ M and 2 mM, respectively). Stopped-flow traces

at 455 nm were fit to the corresponding exponential function to determine the observed rates using the software Pro-Data (Applied Photophysics, Surrey, UK). The anaerobic reaction of hFMO5 with NADPH was studied by monitoring the loss of the 455 nm absorbance peak. The stopped-flow traces were best fitted to a double exponential function with measured rate constants $k_{\text{obs}1}$ and $k_{\text{obs}2}$. To study the oxidative half-reaction, the double-mixing mode of the stopped-flow instrument was employed. hFMO5 was anaerobically reduced with NADPH and subsequently mixed with an equal volume of air-saturated buffer immediately before starting data acquisition. The reoxidation of hFMO5 was studied by monitoring the increase in the absorbance peak at 455 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.7b00470.

Fragmentation patterns and ^1H NMR and ^{13}C NMR of nabumetone and pentoxifylline products are reported (PDF)

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E.R. and F.F. carried out stopped-flow experiments, which were designed together with M.W.F. and A.M. F.F. carried out all the other experiments, which were designed and analyzed together with M.H., K.F., and A.M. The project was supervised by A.M. and M.H. F.F., M.H., and A.M. wrote the manuscript with comments from all authors.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

FMO, flavin-containing monooxygenase; BVMO, Baeyer–Villiger monooxygenase; hFMO5, human FMO5; API, active pharmaceutical ingredient

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