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SPECIAL
ISSUE

Exploring PTDH–P450BM3 Variants for the Synthesis of Drug Metabolites

Nina Beyer,^[a] Justyna K. Kulig,^[b, c] Marco W. Fraaije,^[a] Martin A. Hayes,^[b] and
Dick B. Janssen^{*[a]}

The conversion of a series of pharmaceutical compounds was examined with three variants of cytochrome P450BM3 fused to phosphite dehydrogenase (PTDH) to enable cofactor recycling. Conditions for enzyme production were optimized, and the purified PTDH–P450BM3 variants were tested against 32 commercial drugs by using rapid UPLC–MS analysis. The sets of mutations (R47L/F87V/L188Q and R47L/F87V/L188Q/E267V/G415S) improved conversion for all compounds, and a variety

of products were detected. Product analysis showed that reaction types included C-hydroxylation, N-oxidation, demethylation, and aromatization. Interestingly, enzymatic aromatization could occur independent of the addition of reducing coenzyme. These results identified new conversions catalyzed by P450BM3 variants and showed that a small set of mutations in the oxygenase domain could broaden both substrate range and reaction type.

Introduction

The in vivo mammalian metabolism of drug compounds comprises a wide variety of biotransformations catalyzed by diverse enzymes localized mainly in the liver.^[1] A drug is often metabolized by multiple (iso)enzymes, leading to a complex mixture of products that can influence drug efficacy and safety.^[2] The metabolites of newly developed drugs therefore need to be identified and tested according to regulatory guidelines.^[3,4]

Most primary drug metabolites (≈80%) formed in the human liver are produced by isoforms of cytochrome P450 monooxygenases (P450s).^[5,6] These heme-containing enzymes catalyze important biotransformations in primary and secondary metabolism, for example in steroid biosynthesis, and also take part in many detoxification pathways.^[7,8] The versatility of P450s is reflected in the large range of reactions they catalyze: hydroxylations, epoxidations, dealkylations, sulfoxidations, deaminations, and dehalogenations.^[9–11] In the case of hydroxylation reactions, two electrons and dioxygen are used to form water and an iron(IV)–oxo radical species (compound I) that initiates substrate oxidation by abstraction of a hydrogen atom,

followed by recombination of the substrate radical with the hydroxy group bound to the heme iron.^[12,13] To feed electrons into the reaction cycle, the cofactor NAD(P)H and auxiliary proteins transferring electrons from NAD(P)H to the heme component are required. These electron-transfer proteins either occur in transient assemblies with the respective P450 or may be naturally fused to the monooxygenase.^[14]

The diversity, specificity, and complexity of the performed biooxidations make P450s attractive for use in preparative conversions. Often, the chemical synthesis of drug metabolites is challenging and product formation in sufficient quantity and purity is not cost effective.^[15] Both liver microsomes and heterologously expressed human P450s have been explored for the preparation of drug metabolites. The engineering of mammalian P450s for biotechnological applications was reviewed by Lamb et al. and Gillam.^[16,17] The Guengerich group investigated important human class II P450s (i.e., 1A2, 2A6, 2C8, 2C9, 2D6, and 3A4). These are attached to the endoplasmic reticulum, depend on NADPH-P450 reductase for electron transfer and have diverse functions in vivo.^[18–22] Though crystal structures have been solved for CYP1A2,^[23] 3A4,^[24] and other mammalian isoforms, they do not give a consistent explanation for many of the observed catalytic features.^[16] For selectivity engineering, yeast or bacterial hosts are used for enzyme production, and colorimetric activity screening with surrogate substrates is used for activity profiling. For example, semirandom mutagenesis of the six substrate recognition sequences of P450 1A2 followed by screening of activity with 3,5-dimethylimidazo[4,5-f]quinolone resulted in a 3- to 4-fold improvement in the activity and a broadening of the substrate range, whereas sequential rounds of error-prone polymerase chain reaction (epPCR) and screening of approximately 12 000 mutants per generation led to an 12-fold increase in activity.^[25,26] For P450 2A6, both mutagenesis approaches were combined to target the conver-

[a] N. Beyer, Prof. M. W. Fraaije, Prof. D. B. Janssen
Biotransformation and Biocatalysis, University of Groningen
Nijenborgh 4, 9747AG Groningen (The Netherlands)
E-mail: d.b.janssen@rug.nl

[b] Dr. J. K. Kulig, Dr. M. A. Hayes
Cardiovascular and Metabolic Diseases, DMPK
Innovative Medicines and Early Development
AstraZeneca R&D Gothenburg
Pepparedsleden 1, 43150 Mölndal (Sweden)

[c] Dr. J. K. Kulig
Crop Science Division, Bayer AG
Alfred-Nobel-Strasse 50, 40789 Monheim am Rhein (Germany)

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sion of indole and 5-hydroxybenzylindole.^[27] Sieber et al. investigated chimeras between the human P450 1A2 and the bacterial P450BM3 that showed improved functional expression and characteristic 1A2 activity.^[28] Recurrent bottlenecks are the highly variable expression levels of mammalian P450s, as well as their low catalytic activity and poor stability.^[29–33]

Bacterial P450s with human-like activities are an attractive alternative for studies on P450-catalyzed conversions of drug compounds because of their better stability and elevated activities, which can be 100–1000 times higher than those reported for human P450s.^[9] One of the most active P450s discovered so far is P450BM3 (CYP102A1, EC 1.14.14.1) from *Bacillus megaterium*. The enzyme has been proposed as a suitable substitute for mammalian class II P450s. As a natural fusion enzyme, it consists of a flavin adenine dinucleotide (FAD)- and a flavin mononucleotide (FMN)-containing NADPH-cytochrome P450 *B. megaterium* reductase (BMR) domain and a heme-containing oxygenase domain (class VIII P450).^[34] It can be produced in good yields in an *Escherichia coli* expression system (> 150 mg L⁻¹), and the structures of the separate domains have been solved.^[35–37] With NADPH as an electron donor, the wild-type enzyme naturally converts medium-to-long-chain fatty acids.^[38] Its broad substrate range allows many more reactions, especially if the fatty-acid recognition residues and other groups surrounding the active site are mutated.^[3,39]

The conversion of pharmaceuticals by P450BM3 and variants thereof has also been studied. Whitehouse et al. reviewed research on P450BM3, listing a great variety of mutants that mediate the conversion of bulky substrates (several of them drug compounds).^[39] Progress on the use of P450BM3 for the production of drug metabolites was reviewed by Di Nardo and Gilardi.^[3] Regarding the conversion of pharmaceutically relevant compounds, the mutants R47L/F87V/L188Q (LVQ) and R47L/F87V/L188Q/E267V/G415S (LVQVS, or M01 elsewhere) were repeatedly described (Table S1 in the Supporting Information).^[40–44] Several compounds converted by P450BM3 LVQ and LVQVS are also substrates for human P450s. Residue Arg47 of P450BM3 is located on the surface of the enzyme, more than 22 Å from the active-site heme iron. Together with Tyr51, it acts as a gate keeper: Arg47 interacts with fatty acids by formation of a bidentate ion pair, while Tyr51 that also flanks the substrate access channel, provides additional stabilization.^[45–48] One of the most mutated residues in P450BM3 is Phe87, which is close to the heme iron, and mutations at this position increase space in the active site. The mutation F87V has a large impact on activity and specificity and is, therefore, frequently applied for the production of drug metabolites.^[49] However, it also enhances uncoupling, as evident from increased production of hydrogen peroxide.^[50,51] A mutant combining R47L, F87V, and L188Q (all three mutated in the LVQ and LVQVS variants) showed 90-fold higher activity towards dextromethorphan than the human P450 2D6.^[41] Residue Glu267 (mutated to Val in LVQVS) is located in the I helix in proximity to the heme iron and is part of a conserved acid–alcohol pair (Glu267–Thr268). The E267V mutation enhances the coupling efficiency in certain conversions compared to the wild-type (WT) enzyme and is possibly related to reduced water binding

in the active site. In a random mutagenesis study with the LVQ triple mutant as template, a mutant with the additional substitutions E267V and G415S showed increased activity towards 3,4-methylenedioxymethamphetamine (MDMA) and dextromethorphan.^[52] However, Gly415 is located far away from the catalytic heme and known substrate recognition sites, and only E267V contributed to the increased conversion.^[39,52]

In this work we sought to broaden the range of reactions catalyzed by PTDH–P450BM3 by introducing the mutations discussed above and examining them in the conversion of 32 important drugs, 21 of which, to the best of our knowledge, have not been described before as being converted by a P450BM3 variant. We used a previously described fusion of phosphite dehydrogenase (PTDH) to P450BM3 (PTDH–P450BM3).^[37] The fusion enzyme accepts electrons from phosphite to reduce NADP⁺, thus recycling NADPH, and has slightly higher activity than the nonfused enzyme. The drug library was broadly chemically diverse and included compounds with molecular weights (200–735 Da) greater than that of the natural substrate. Substrate depletion and product formation were determined by LC–MS, rates of the most important conversion were determined, and exemplary conversions were performed by regenerating NADPH from phosphite. The results show that the mutated fusion enzymes are versatile biocatalysts for the transformation of pharmaceutical compounds (27 drugs converted), predominantly catalyzing C-hydroxylations but also N-oxidation and aromatization, the latter proceeding with amlodipine, independent of the presence of the reduced nicotinamide cofactor.

Results and Discussion

Production of PTDH–P450BM3 variants

To broaden the range of cytochrome P450BM3-catalyzed conversions of pharmaceuticals, we used the PTDH-fused wild-type P450BM3 and the mutant enzymes R47L/F87V/L188Q (LVQ) and R47L/F87V/L188Q/E267V/G415S (LVQVS, elsewhere called M01).^[40–44] The mutations in these P450BM3 variants allow conversion of drugs in screening assays for human-P450-like activity. To produce the enzymes as bifunctional catalysts fused with phosphite dehydrogenase for cofactor recycling, the mutations were introduced into the template pCre2–P450BM3 by site-directed mutagenesis, which resulted in pCre2–P450BM3 LVQ and pCre2–BM3 LVQVS.^[37] The enzymes PTDH–P450BM3 WT, PTDH–P450BM3 LVQ, and PTDH–P450BM3 LVQVS were heterologously expressed in *E. coli* TOP10.

Initial expression experiments showed that all variants were well expressed, albeit with the mutant enzymes at slightly lower levels than the WT though the cultures reached the same optical densities at 600 nm [≈ 66 mg L⁻¹ (mg of active PTDH–P450BM3 WT per liter of culture) compared to 28 mg L⁻¹ for PTDH–P450BM3 LVQ]. Furthermore, higher losses were observed during the purification procedure, which consisted of His-tag purification followed by desalting with a 50 kDa Centri-con filter. To improve the expression of active enzyme that could withstand the purification procedure, variations in culti-

vation conditions were tested with the mutant LVQ enzyme: the use of baffled versus non-baffled flasks and the addition of 0.4% glycerol (v/v) to the culture. The amount of active enzyme obtained following purification was determined after expression under three different conditions: A) growth in Erlenmeyer flasks with baffles and medium without glycerol; B) in flasks without baffles and medium without glycerol; and C) in flasks without baffles and with 0.4% glycerol added to the medium. The use of flasks without baffles improved the yield of active P450 by a factor of almost 8 (from 6.4 mg L⁻¹ of isolated active P450 under conditions A to 52 mg L⁻¹ with conditions B). The addition of glycerol to the medium improved enzyme production by another 64% (85 mg L⁻¹ of active P450 after purification; Table S2). Although these conditions were also beneficial for the production of PTDH-P450BM3 WT, the effects were less strong—an increase of 33% was observed.

The lower yields found with the mutant P450s and the higher sensitivity to the expression conditions indicated that the incorporation of mutations that broaden the substrate scope had a negative effect on the expression of functional enzyme. The mutant enzymes are deleteriously affected by the combined influence of a higher aeration rate in baffled flasks and the inactivation by NADPH in the absence of a suitable substrate.^[53–55] We also observed that uncoupling, defined as substrate-independent NADPH conversion, was increased in the mutants (see section Activity profiling, Table 1). Cowart et al. described a similar effect from mutations at positions R47 and F87: upon mutation the production of hydrogen peroxide in conversions of lauric acid was significantly increased.^[51]

Activity profiling

A selection of 32 drug compounds with varying physicochemical properties (molecular weight, polarity, lipophilicity) were tested for conversion by the PTDH-fused P450BM3 variants. Initial screening was performed without activating the PTDH domain by adding NADPH in excess and no phosphite to the potential substrates (10 μM). The depletion of substrate and the formation of products were determined by UPLC–MS as end-point measurements after 18 h incubation. Conversion was observed for 27 of the 32 drug compounds, thus illustrating the broad acceptance of the P450BM3 enzyme and its mutants. Among the tested compounds were 11 drugs described before as substrates for the chosen P450BM3 variants.^[41,43,44] Those were confirmed by product formation at levels similar or higher than those reported (Table S1). Rosiglitazone was the best substrate. Of the 21 drugs, which to our knowledge have not been described as substrates for P450BM3 before, 16 were converted by P450BM3, whereas olmesartan, phenytoin, tienilic acid, sulfamethoxazole, and valsartan were not. With the exception of phenytoin, the unconverted compounds were also not substrates for human P450 3A4 (Table S3).

For 16 of the converted drugs, the level of substrate depletion by three variants of PTDH-fused P450BM3 was plotted against the number of drugs converted (Figure 1), and this showed that the introduction of the mutations increased the capacity of PTDH-P450BM3 to metabolize drugs in terms of

Figure 1. Substrate acceptance of PTDH-P450BM3 WT, LVQ, and LVQVS. Substrate depletion is plotted against the number of drugs that were converted by the specific variants.

number of compounds converted and degree of conversion. Compounds only accepted by the mutant enzymes included celecoxib, clopidogrel, donepezil, erythromycin, fluoxetine, levofloxacin, metoprolol, nevirapine, and tacrine. This is in agreement with the findings of van Vugt-Lussenburg et al. and Reinen et al. and confirmed that as few as three mutations could be sufficient to broaden the substrate scope significantly.^[41,43]

Eleven drug substrates were converted by more than 10% of the starting material by at least one of the enzyme variants, of which nine drugs gave metabolites that could be clearly detected (more than 10% of at least one metabolite). Further work focused on amlodipine (AML), celecoxib (CEL), clopidogrel (CLO), donepezil (DON), imipramine (IMI), nevirapine (NEV), pioglitazone (PIO), ticlopidine (TIC), and zafirlukast (ZAF). Among them were four low-molecular-weight drugs (<350 Da; CLO, IMI, NEV, and TIC) but even the bulky compound zafirlukast (>500 Da) was metabolized. Whereas the LVQ and LVQVS variants mainly accepted the same substrates, they often showed different rates of conversion (Table 1). For substrates that were accepted by the wild-type variant, the LVQ mutant of PTDH-P450BM3 had the highest conversion [amlodipine (54%) and pioglitazone (>99%)]. Clopidogrel was also converted more efficiently by this variant. This suggested that the mutations R47L/F87V/L188Q mainly influenced the rate of conversion rather than substrate acceptance. The other drugs were more challenging for the WT and were converted best by LVQVS, with the exception of ticlopidine, for which the conversion by the two mutants did not differ significantly. Residue E267 is located close to the kink region in the I helix hovering over the heme. Mutation to the sterically less hindering valine can, therefore, explain why mutant LVQVS can accept a bulkier substrate than variant LVQ. Almost full substrate depletion was detected for pioglitazone by all variants, for clopidogrel by LVQ, and for donepezil by variant LVQVS. Compounds particularly well converted by only the mutants included CEL, CLO, DON, IMI, NEV, TIC, and ZAF, of which CEL, DON, and NEV were not accepted by the WT enzyme at all.

Table 1. Conversion of pharmaceuticals by variants of PTDH-P450BM3.^[a]

Compound	Conversion [%]			NADPH oxidation rate [s ⁻¹]	
	WT	LVQ	LVQVS	LVQ	LVQVS
AML	43	69	58	0.11 ± 0.01	0.11 ± 0.01
CEL	0	10	49	0.15 ± 0.03	1.05 ± 0.30
CLO	2	>99	73	5.83 ± 0.33	1.89 ± 0.16
DON	0	15	96	0.58 ± 0.05	1.53 ± 0.04
IMI	3	49	76	0.53 ± 0.07	0.58 ± 0.03
NEV	0	3	23	0.46 ± 0.02	1.39 ± 0.10
PIO	92	96	91	4.77 ± 0.41	3.17 ± 0.19
TIC	32	>99	99	10.35 ± 0.84	1.47 ± 0.05
ZAF	4	17	45	0.62 ± 0.04	0.59 ± 0.04
MeOH/EtOH				0.52 ± 0.08	0.70 ± 0.07
DMSO				0.56 ± 0.08	0.58 ± 0.04

[a] Conversion is defined as depletion of 10 μM substrate as analyzed by UPLC–MS after 18 h reaction time. Controls were reactions without enzyme. NADPH oxidation rates (μmol NADPH converted per μmol enzyme per s) were determined by following NADPH depletion (150 μM) at $\lambda = 340$ nm in the presence of 100 μM substrate or without substrate but with cosolvent. Cosolvents were a 1:1 mixture of MeOH/EtOH or DMSO (pioglitazone and zafirlukast). Enzyme variants tested were PTDH-P450BM3 WT (WT), PTDH-P450BM3 R47L/F87V/L188Q (LVQ), and PTDH-P450BM3 R47L/F87V/L188Q/E267V/G415S (LVQVS).

Specific activities (μmol NADPH converted per mol enzyme per second) towards these substrates were estimated by following NADPH oxidation, both in the presence and absence of substrate, to assess substrate-independent conversion of NADPH by the reductase domain. Compared to PTDH-P450BM3 WT, substrate-independent NADPH oxidation increased by a factor of 2–3 for both mutants (WT: 0.22 s^{-1} with 10% MeOH/EtOH).^[37] In general, the observed rates of NADPH oxidation are in good agreement with the results for substrate depletion (Table 1). Compounds for which the conversion exceeded 50% showed NADPH consumption rates that exceeded 1 s^{-1} and were at least twice as high as NADPH consumption rates observed for reactions without a substrate. A mutant that showed a higher conversion of a specific drug also exhibited a higher specific activity towards that drug. Considering a k_{cat} of 8 s^{-1} for the conversion of lauric acid by PTDH-P450BM3 WT^[37] and an incomplete conversion at 10 μM for the tested pharmaceuticals over several hours, the NADPH oxidation rates were unexpectedly high. The conversions of CLO, PIO, and TIC by LVQ and those of DON, PIO, and TIC by LVQVS were driven almost to completion. The reaction might therefore occur more quickly than that indicated by the end-point measurements. For CEL and NEV, the rate of NADPH conversion by variant LVQVS was considerably higher than by the residual amount of substrate after 18 h of biotransformation would suggest. No catalase was added to the reactions, so enzyme inactivation by increased uncoupling with an unnatural substrate might occur (H_2O_2 formation). The PTDH-fused WT enzyme shows reduced uncoupling if a suitable substrate is easily accessible,^[37] and the lower substrate concentration in the screen (most likely below the K_m) might contribute to this effect. Exceptions were AML, IMI, and ZAF, for which the observed rates of NADPH oxidation were slightly lower or equal to those determined for the reactions without substrate. Though inhibi-

tion mechanisms can occur, a reaction rate below the substrate-independent NADPH oxidation rate seems likely, considering that full conversion of substrate (10 μM) was not reached within the 18 h reaction time. Substrate-specific effects on the coupling of the NADPH oxidation to the monooxygenase reaction, which were ignored here, could decrease the uncoupling and thereby decrease the NADPH oxidation rate.

In general, the NADPH oxidation rates for LVQVS were lower than those for LVQ, though the conversions of some specific compounds were higher. Stjernschantz et al. described higher couplings (defined as the ratio of product formed and NADPH consumed at restricted NADPH concentrations) for the conversions of dextromethorphan and MDMA by P450BM3 variants, including the mutation E267V, and suggested that the coupling efficiency was related to substrate-induced conformational changes of the heme propionates in the mutants, which in turn influenced the heme reduction potential.^[52] This effect might explain the lower NADPH depletion but higher or equal product formation by the mutant LVQVS.

Reaction types

To identify metabolites and to establish the types of reactions catalyzed by wild-type and mutant PTDH-P450BM3 variants, we used MetaboLynx 4.1 software (Waters) to examine the UPLC–MS data and to assign product signals (Table 2). With most drugs, more than one metabolite was formed, but identification focused on products with a yield of more than 10%. Variations in the product yield compared to the substrate depletion could therefore be explained by minor metabolites formed.

From the antihypertensive amlodipine (m/z 409.151 $[\text{M}+\text{H}]^+$), a single metabolite was formed with all enzyme variants with a yield >40%, which was slightly increased for the mutants. On the basis of the fragmentation data, aromatization of the dihydropyridine moiety took place to form the corresponding pyridine metabolite (AML M1, m/z 407.135 $[\text{M}+\text{H}]^+$). Dehydroamlodipine is one of the primary human metabolites formed from amlodipine,^[56] and Zhu et al. demonstrated that in human liver microsomes P450 3A4 was the major catalyst.^[57]

The anti-inflammatory drug celecoxib (m/z 382.082 $[\text{M}+\text{H}]^+$) was not converted by PTDH-P450BM3 WT, only slightly by LVQ, and well converted by LVQVS. A monohydroxylation was evident from a mass shift from the parent compound of 15.994 Da (CEL M1). Celecoxib metabolism in human is initiated by P450 2C9 and 3A4, which produce hydroxycelcoxib by hydroxylation of the methyl group.^[58,59] This is also very likely the metabolite CEL M1 formed by PTDH-P450BM3.

Clopidogrel, a platelet aggregation inhibitor (m/z 322.065 $[\text{M}+\text{H}]^+$), was metabolized extensively by both mutants, each of which formed three major metabolites. A dihydroxylated metabolite (CLO M2, m/z 354.055 $[\text{M}+\text{H}]^+$) and a demethylated and aromatized metabolite (CLO M3, m/z 304.019 $[\text{M}+\text{H}]^+$) were produced by both variants, whereas only transformations with LVQ resulted in a hydroxylated, aromatized, and demethylated metabolite (CLO M4, m/z 322.031 $[\text{M}+\text{H}]^+$). LVQVS gave a monohydroxylated product (CLO M1, m/z 338.062 $[\text{M}+\text{H}]^+$). A

Table 2. Metabolites formed from target drugs by PTDH–P450BM3 variants.^[a]

Drug	Metabolites Reaction type	Proposed structure	WT [%]	LVQ [%]	LVQVS [%]
amlodipine	AML M1: –2 Da aromatization		43	68	58
celecoxib	CEL M1: + 16 Da hydroxylation			10	51
clopidogrel	CLO M1: + 16 Da hydroxylation				10
	CLO M2: + 32 Da 2 hydroxylations			26	9
	CLO M3: –18 Da aromatization, ester hydrolysis			49	26
	CLO M4: + 0.4 Da hydroxylation, desaturation, ester hydrolysis			12	
	*CLO M5: –12 Da hydroxylation, aromatization
donepezil	DON M1: + 16 Da N-oxidation			14	78

Table 2. (Continued)					
Drug	Metabolites Reaction type	Proposed structure	WT [%]	LVQ [%]	LVQVS [%]
imipramine	IMI M1: –14 Da demethylation		1	38	37
	IMI M2: –28 Da didemethylation			2	15
nevirapine	NEV M1: +16 Da hydroxylation			3	15
pioglitazone	PIO M1: +16 Da hydroxylation		84	76	62
	PIO M2: +14 Da hydroxylation, oxidation			21	16
ticlopidine	TIC M1: –2 Da desaturation		32	33	30
	TIC M2: –4 Da aromatization			47	54
	TIC M3: +12 Da aromatization, hydroxylation			16	7
	*TIC M4: +16 Da hydroxylation

Table 2. (Continued)

Drug	Metabolites Reaction type	Proposed structure	WT [%]	LVQ [%]	LVQVS [%]
zafirlukast	ZAF M1: + 16 Da hydroxylation		3	14	
	ZAF M2: -2 Da desaturation		2	10	
[a] Metabolites produced by at least one enzyme variant at a level of $\geq 10\%$ of the total related peak area after detection by UPLC-MS. Data represent conversion after 18 h reaction time. WT: PTDH-P450BM3 WT, LVQ: PTDH-P450BM3 R47L/F87V/L188Q, LVQVS: PTDH-P450BM3 R47L/F87V/L188Q/E267V/G415S. Product formation is corrected for metabolite formation in the buffer control ($< 1\%$). Metabolites that were not observed in the initial screens but were detected in phosphite-driven reactions are marked with an asterisk (*).					

few minor metabolites ($< 10\%$, data not shown) were formed with both enzyme variants. In general, LVQ gave better yields than LVQVS. Clopidogrel is a prodrug that is activated in the human liver by two P450-catalyzed steps: first, a thiolactone is formed (2-oxoclopidogrel) that is then hydrolyzed to form the pharmacologically active thiol acid (Scheme S1). Metabolites formed by LVQ and LVQVS therefore differ from the human P450 metabolites. Whereas CLO M1 formed by LVQVS might be the thiolactone, the thiol acid was not formed.

A metabolite with a hydrolyzed carbomethoxy group, which is formed by carboxylesterase 1 in human metabolism,^[60–64] on the other hand was produced by PTDH-P450BM3: the carboxylic acid derivative was formed as a minor metabolite by LVQVS (6%) and subsequent metabolites not described in man occurred with both mutants. For tetracyclic donepezil (m/z 380.220 $[M+H]^+$), the production of an *N*-oxide as the major metabolite was observed (m/z 396.21 $[M+H]^+$) in good yield by the LVQVS enzyme. Several minor metabolites were also formed from this cholinesterase inhibitor. Ten human metabolites are described to be formed, particularly by P450 2D6, 3A4, and 1A2. The main metabolic pathways are O-dealkylation, hydrolysis, and N-oxidation.^[65–67]

Secondary reactions after an initial biotransformation step were observed for imipramine (m/z 281.201 $[M+H]^+$), a dibenzazepine with antidepressive effects. Demethylation occurred once (IMI M1, m/z 267.166 $[M+H]^+$) or twice (IMI M2, m/z 253.170 $[M+H]^+$), especially with variant LVQVS, indicating faster turnover of this substrate. Many minor metabolites were formed by LVQVS as well. In the liver, imipramine is converted by several P450 isoenzymes (e.g., 1A2, 2D6, 3A4, and 2C9) into

the active metabolites desipramine (IMI M1) and 2-hydroxydesipramine.^[68–70]

The reverse transcriptase inhibitor nevirapine (m/z 267.124 $[M+H]^+$) was only converted ($> 3\%$) by the mutant LVQVS, and hydroxylation of the alkyl chain occurred to form 12-hydroxynevirapine (m/z 283.12 $[M+H]^+$; NEV M1) on the basis of the fragmentation pattern. This is one of five main products in human metabolism and is formed by P450 3A4 and 2D6.^[71] If further sulfated, this can lead to the formation of toxic compounds.^[72] All three enzymes converted the antidiabetic drug pioglitazone well (m/z 357.126 $[M+H]^+$; $> 80\%$). Major metabolites were formed by hydroxylation of the alkyl chain (PIO M1, m/z 373.120 $[M+H]^+$) and subsequent oxidation of the integrated hydroxy group (PIO M2, m/z 371.120 $[M+H]^+$). By comparing the elution pattern of the potential metabolites with hydroxylation on the alkyl chain, the exact position of hydroxylation for PIO M1 could be solved. With a yield of 76% for metabolite PIO M1 and 21% for PIO M2, variant LVQ had higher specificity for the production of the main metabolites than mutant LVQVS, whereas variant LVQVS formed more minor metabolites. Both metabolites present the main biologically active metabolites of pioglitazone in humans, which are formed mainly by P450 2C8 and 3A4.^[73,74]

Ticlopidine (antiplatelet, m/z 264.045 $[M+H]^+$) conversions resulted in three major metabolites. Metabolites TIC M1 and M2 were formed by the consecutive aromatization of the piperidine moiety, which resulted in the formation of dihydrothienopyridinium and thienopyridinium (m/z 262.045 and 260.026 $[M+H]^+$). PTDH-P450BM3 only formed TIC M1; this indicated that the reaction rate was slower than that with the

mutants. Both mutated variants produced similar amounts of TIC M1, but LVQVS gave a slightly higher yield of TIC M2. LVQ, on the other hand, had a higher yield of a third metabolite (TIC M3, m/z 276.024 $[M+H]^+$), which was further hydroxylated. Whereas the principal routes of human metabolism involve N-dealkylation, N-oxidation, and oxidation of the thiophene ring, two- or four-electron oxidation of the tetrahydropyridine ring also occurs.^[75,76]

Significant amounts of two potential metabolites occurred with zafirlukast (m/z 576.216 $[M+H]^+$) in samples taken directly after enzyme addition and in samples without enzyme: a gain of 31.99 Da and a loss of 68.06 Da. The potential metabolites were regarded as impurities and were, therefore, omitted from the list of expected biocatalytic products in P450-catalyzed conversions. Both mutants yielded two enzymatically formed metabolites, but LVQVS was the only catalyst with a product yield > 10%. Zafirlukast was either hydroxylated (ZAF M1, m/z 592.210 $[M+H]^+$) or desaturated (ZAF M2, m/z 574.202 $[M+H]^+$). In dogs, rats, and mice, the primary metabolism involves hydrolysis, N-acetylation, and N-desmethylation of the indole group, and several monohydroxylated metabolites were also identified.^[77] Kassahun et al. described P450-catalyzed formation of an unsaturated iminium metabolite that inactivated P450 3A4 irreversibly.^[78] Cyclic tertiary amine groups were a prominent position for reactivity, as CLO and TIC also underwent two- or four-electron oxidation and an N-oxide formed at this position with donepezil.

From this product analysis, the main reaction types observed were C-hydroxylation, N-oxidation, demethylation, and (partial) aromatization, which reflects the great versatility of P450BM3 and shows that the chosen variants are good candidates to screen for formation of mammalian metabolites from drugs.^[9,10] Reinen et al. described various mutants (with manifold amino-acid substitutions) for the conversion of drug compounds.^[43] Integration of these or similar mutations in combi-

nation with substitutions that lead to increased stability of the enzyme might also be beneficial for future development.^[79]

Phosphite-driven conversions

Phosphite-driven turnover by either PTDH-P450BM3 WT or both mutants was examined in biotransformations over a shorter reaction time (4 h) with five compounds: amlodipine, ticlopidine, clopidogrel, nevirapine, and pioglitazone. Reactions driven on NADPH or $NADP^+$ and phosphite were compared to controls without enzyme and without cofactor, as well as samples taken directly after enzyme addition.

Aromatizations were the main reactions discovered for AML and TIC. The mutants formed multiple metabolites from TIC that were not exclusively aromatization/desaturation products (Table 2); therefore, aromatization reactions were analyzed in more detail for PTDH-P40BM3 WT. The same metabolite as that observed in the initial screen was formed from AML (AML M1): aromatization of the dihydropyridine moiety took place. The yields of the reactions with phosphite and NADPH as the initial electron source were very similar. The shorter reaction time relative to that for the initial screen led to lower product yields. Surprisingly, amlodipine was also aromatized if NADPH (and $NADP^+$ and phosphite) was omitted, even in a higher yield than that observed for samples containing NADPH or $NADP^+$ and phosphite (14.4 vs. 9.0 and 7.0%; Figure 2A). Zhu et al. observed that the production of dehydroamlodipine was NADPH dependent in human liver microsomes, which suggests that a different aromatization mechanism occurs in P450BM3.^[57]

Ticlopidine conversions resulted in the identification of two major metabolites, one of which was TIC M1: dehydroticlopidine. The other metabolite, hydroxyticlopidine (TIC M4, m/z 280.06 $[M+H]^+$), did not form in the previous screen but might be a potential precursor to TIC M3. In reactions driven by phosphite, complete aromatization also occurred in small

Figure 2. Conversions of A) amlodipine and B) ticlopidine by PTDH-P450BM3 variants. Substrate (10 μ M) in a total volume of 100 μ L (Tris buffer, 50 mM, pH 7.5). Reactions were performed at 30 $^{\circ}$ C; reaction progress was measured after 4 h in comparison to samples by UPLC-MS. Phosphite-driven reactions contained phosphite and $NADP^+$, whereas NADPH-driven reactions contained NADPH and phosphite. The yields of product and remaining substrate were determined as percentages of the respective peak area to the total related peak area. Reactions were performed in duplicate (AML = amlodipine, AML M1 = dihydroamlodipine, TIC = ticlopidine, TIC M1 = dihydroticlopidine, TIC M4 = monohydroxylated ticlopidine).

amounts (TIC M2 < 5% data not shown), indicating that the phosphite-driven conversions were slightly more efficient. Oxidation to dihydrothienopyridinium (TIC M1) occurred without added cofactor but not without enzyme. However, the yield was increased in reactions with NADPH/NADP⁺ and phosphite (12 vs. 25 and 31% for the reaction driven on NADPH and NADP⁺ and phosphite, respectively). The hydroxylated metabolite (TIC M4) was formed only in reactions with an added electron source (NADPH: 8%; phosphite: 6%; Figure 2B). The total turnover of TIC was 33% in NADPH-driven conversions, which is comparable to the yield obtained previously in the screen (32%), and was slightly increased in conversions driven on phosphite and NADP⁺ (40%). Dalvie and O'Connell demonstrated that the formation of dihydrothienopyridinium (TIC M1) was also catalyzed by horseradish peroxidase, whereas thienopyridinium was produced in substantial amounts in reactions with human P450s or myeloperoxidase.^[76] Although the pyridine group in amlodipine is a secondary amine, amlodipine and ticlopidine share some structural similarity in the converted moiety. In both cases, cofactor-independent turnover was enzyme dependent and exceeded the amount of enzyme added to the reaction. Phosphite was not added to the control reactions without NADPH/NADP⁺; therefore, catalysis by the standard reaction mechanism fueled by electrons from NADPH/NADP⁺ co-purified with the enzyme could be excluded. The aromatization occurred on the dihydropyridine moiety, which resembles the redox-active dihydropyridine ring of NADPH. NADPH is accepted as an electron donor in the naturally fused FAD-dependent reductase domain of P450BM3. For other FAD-dependent oxidoreductases, the substitution of NADPH by cheaper compounds was reported by Knaus et al.^[80] We hypothesize that the reductase domain of P450BM3 can act as an "ene" reductase-like enzyme on this compound and that TIC and AML themselves act as the reducing agents. To probe whether AML and TIC could possibly be accepted by the heme and reductase domains of P450BM3, we performed preliminary docking simulations (PDB ID: 1JPZ the for heme domain, 4DQL for the reductase FAD/NADPH domain; YASARA autodock, global docking).^[81] Amlodipine never bound into the heme domain in 25 attempted dockings, but in the case of the reductase domain, 17/25 dockings placed AML in proximity to FAD close to the position of NADPH in the crystal structure. For ticlopidine, 8/25 times the ligand was located close to the heme in the P450 domain and 17/25 times in the active site of the reductase domain, matching the fact that for TIC, cofactor and cofactor-independent conversions were observed. Whereas the docking results suggest that AML and TIC are oxidized by electron transfer to the reductase domain of P450BM3, trials to run the conversion of lauric acid on AML instead of NADPH failed (data not shown). If AML is accepted as a substrate by the FAD domain, it would concomitantly inhibit electron transfer to the heme domain for the conversion of natural substrates. A mechanism similar to that for peroxidases, as suggested by Dalvie and O'Connell, could also explain our observation.^[76]

The conversions of clopidogrel, nevirapine, and pioglitazone were highest with the variants LVQ and LVQVS. The mutants

were therefore chosen as catalysts in conversions by comparing NADPH-driven conversion to conversions with NADP⁺ and phosphite over 4 h. In contrast to the conversions of AML and TIC, all reactions occurred only with the addition of the cofactors.

The conversion of clopidogrel was, as in the previous screen (18 h in an NADPH-driven reaction), almost complete. Again, the metabolites CLO M1, M2, and M3 (monohydroxylated, dihydroxylated, and demethylated and aromatized metabolites) were formed. An aromatized and hydroxylated metabolite (CLO M5, *m/z* 334.03 [*M*+H]⁺) was produced instead of CLO M4 [Table 2, marked with an asterisk (*)] only by variant LVQ. While aromatization and hydroxylation are found both in CLO M4 and M5, CLO M4 is also demethylated, suggesting that CLO M5 was a precursor of M4. A few minor metabolites (< 10%, data not shown) were formed with both enzyme variants. The mutant LVQVS had a stronger preference for hydroxylation reactions than LVQ, and the production of the dihydroxylated metabolite M2 was approximately double that caused by LVQ. On the other hand, a combination of hydroxylation and aromatization was only detected for mutant LVQ; the ratio of aromatized metabolites to nonaromatized metabolites was equal. Interestingly, the LVQ mutant did not produce the monohydroxylated metabolite if the reaction was driven on NADPH (Figure 3). The amount of the other metabolites was not changed on the basis of the initial electron donor. The effect was not observed for reactions catalyzed by LVQVS, and the mechanism is still unclear.

Figure 3. Conversions of clopidogrel by PTDH-P450BM3 LVQ and LVQVS. Substrate (10 μ M) in a total volume of 100 μ L. Reactions were performed at 30 °C; reaction progress was measured after 4 h in comparison to 0 h samples by UPLC-MS. Phosphite-driven reactions contained phosphite and NADP⁺, whereas NADPH-driven reactions contained NADPH and phosphite. The yields of product and remaining substrate were determined as percentages of the respective peak area to the total related peak area. Reactions were performed in duplicate.

For nevirapine, the same metabolite as that discovered in section Reaction types was formed. In reactions with both potential electron sources, hydroxylation of the alkyl chain was catalyzed. The yield in both cases was approximately 25%.

For pioglitazone, the metabolites from section Reaction types were also confirmed. For both mutants, the conversion was almost complete after a 4 h reaction time. With a yield of

76% for metabolite PIO M1 and 21% for PIO M2, variant LVQ had higher specificity for the production of the main metabolites than the mutant LVQVS (62 and 16%). Variant LVQVS formed more minor metabolites. Reactions driven on NADPH instead of phosphite for the regeneration of NADPH showed no significant differences.

Conclusion

Variants of bacterial P450BM3 that mimic the catalytic activity of human cytochrome P450 monooxygenases (P450s) have potential for use in the production of drug metabolites that can be difficult to synthesize chemically. The native enzyme was shown to have a strong preference for fatty-acid substrates, but mutations at positions R47, F87, and E267 modified enzyme–substrate interactions and thus could yield variants with complementary and more useful substrate profiles, as described for the R47L/F87V/L188Q (LVQ) and R47L/F87V/L188Q/E267V/G415S (LVQVS) enzymes used in this work.^[39,41,42] We investigated the conversions of established drugs that were tested for in vitro conversion by P450 monooxygenases. For these experiments, we selected fusion proteins, in which variants of P450BM3 were linked to phosphite dehydrogenase, which accepts phosphite as the source of electrons for NADPH regeneration. The introduced mutations broadened the selectivity, allowing for the identification of several new drugs as P450BM3 substrates. Besides the typical P450-mediated hydroxylations and N-oxidations, we observed diverse reaction types, including cofactor-independent oxidation of amlodipine and ticlopidine to form aromatic products. Most drugs were converted with surprisingly high rates, and phosphite-dependent turnover could be verified for selected compounds, which makes the investigated system cost efficient in high throughput or for larger scale applications.

This study showed that the bifunctional fusion enzyme phosphite dehydrogenase–P450BM3 could be an efficient system for the discovery of both known and novel metabolites from pharmaceutical compounds. The efficient conversion of several drugs and the phosphite-driven turnover make the investigated system interesting for high-throughput and large-scale applications.

Experimental Section

Materials: Unless stated otherwise, all chemicals and enzymes were obtained from Sigma–Aldrich, Oriental Yeast Co. (Tokyo, Japan), New England Biolabs, and Agilent and were used without further purification. Oligonucleotide primers were obtained from Sigma Genosys. DNA sequencing was performed at GATC Biotech AG (Konstanz, Germany). The investigated drug compounds were accessed from Compound Management, Discovery Sciences at AstraZeneca R&D (Göteborg, Sweden).

Bacterial strains, plasmids, and cloning: *E. coli* TOP10 (Invitrogen) was used for genetic manipulations and expression. The vector pCre2–P450BM3 described previously was used to integrate the desired mutations into P450BM3 fused to PTDH.^[37] Site-directed mutagenesis was used to generate the mutants R47L/F87V/L188Q (LVQ) and R47L/F87V/L188Q/E267V/G415S (LVQVS; position related

to the start of P450BM3 not the N-terminal PTDH). The QuikChange Primer design tool by Agilent was used to design the primers (Table S2); mutations were introduced individually or two simultaneously (R47L and L188Q) into the prior template by utilizing the PHusion polymerase kit according to the manufacturers recommendations (New England Biolabs) with a twofold or fourfold increased amount of deoxynucleotides (dNTPs).

Enzyme expression: Expression of PTDH–P450BM3 and variants thereof, bearing mutations in the P450BM3 domain, was performed in *E. coli* TOP10 from the vector pCre2–P450BM3.^[37] Cells of *E. coli* Top10 harboring pBAD vectors encoding the respective variants were pregrown overnight (37 °C) in lysogeny broth (5 mL) supplemented with carbenicillin (50 mg mL^{−1}). Terrific Broth (TB) medium (carbenicillin supplemented, with or without 0.4% glycerol) in baffled plastic flasks (2 L, 500 mL medium) or nonbaffled glass shaking flasks (2 L, 400 mL medium) with cotton as a plug was inoculated to an OD₆₀₀ of 0.08, and the cultures were incubated at 37 °C and 180 rpm. Expression was induced at an OD₆₀₀ of 0.7–0.8 by the addition of arabinose (0.02% w/v), and 5-aminolevulinic acid (0.5 mM) was added. Overexpression took place at 17 °C over 40 h with constant stirring at 180 rpm. Cells were harvested by centrifugation and were washed once with ice-cold sodium phosphate buffer (50 mM, pH 7.5). The resulting cell pellets were stored frozen until further use (−20 °C).

Enzyme purification: Frozen cell pellets were resuspended in Tris–HCl buffer (50 mM, 500 mM NaCl, 12.5 mM imidazole, pH 7.5 supplemented with DNase, RT) and disrupted by sonication (4 °C, 7 intervals of 30 s at 40% power with 1 min break). The cell-free extract (CFE) was obtained by centrifugation (4 °C, 8873 g, 45 min) and diluted to a protein concentration of ≈20 mg mL^{−1}. The CFE was then purified by the N-terminal His-tag by affinity chromatography on a 5 mL HF HisTrap column (GE-Healthcare) with an ÄKTA purifier (GE-Healthcare). The target enzyme was eluted with 250 mM imidazole, which was removed by desalting in a 50 kDa Centricon or ammonium sulfate (AS) precipitation with 65% AS (favored for larger volumes of CFE). The resulting AS precipitation pellet was resuspended in ice-cold Tris–HCl (50 mM pH 7.5). Remaining AS was removed by ion-exchange chromatography on gravity Econo-Pac 10DG columns (Bio-RAD). The columns were equilibrated in Tris–HCl with 20 mg mL^{−1} sucrose for storage stabilization of the enzyme.^[82] For storage, the enzyme was shock frozen in liquid nitrogen and stored at −80 °C. The concentration of PTDH–P450BM3 variants was determined by measuring CO difference spectra in triplicate.^[83] To record a baseline spectrum from λ = 400 to 500 nm, a spatula tip of sodium dithionite was added to 1 mL enzyme solution (5 min incubation). Samples were bubbled with carbon monoxide (≈60 s, 10 min incubation) before the spectrum from λ = 400 to 500 nm was recorded. The difference spectrum was calculated by subtraction of the baseline spectrum, and the amount of P450 was calculated on the basis of the maximum absorbance at λ = 450 nm subtracted by the absorption at λ = 490 nm ($\epsilon_{450} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$).

Conversion of drug compounds: In total, 32 drug compounds (10 μM) were incubated separately with either PTDH–P450BM3 WT, LVQ, or LVQVS. Reaction mixtures consisted of NADPH (0.15 mM) in Tris–HCl buffer (50 mM, pH 7.5) in 96-well plates with round bottoms in a final volume of 100 μL. Reactions were initiated by the addition of enzyme (0.5 μM) and incubated at 30 °C and 500 rpm. Reactions were stopped by adding 50 μL samples to 100 μL 100% acetonitrile (conical 96-well plate). After centrifugation, the supernatant was diluted 1:4 in 40% acetonitrile in Milli-Q water (final

volume 200 μL) and stored at -20°C (96-well Nunc plate) until UPLC analysis. All reactions were done in duplicate.

P450 activity coupled to the phosphite-driven NADPH regeneration was analyzed for selected drug compounds as described above but with either 0.15 mM NADP^+ and 1.5 mM phosphite or 0.15 mM NADPH. Controls were reactions without enzyme and without NADPH/ NADP^+ and phosphite.

Rates of NADPH oxidation were determined spectrophotometrically by following the absorption at $\lambda = 340\text{ nm}$ and 30°C over 2 min ($\epsilon_{340} = 6.2 \times 10^3\text{ M}^{-1}\text{ cm}^{-1}$) in quartz cuvettes. Reaction mixtures contained the respective substrate (100 μM), NADPH (150 μM), and PTDH-P450BM3 (100 nM) in a final volume of 500 μL Tris-HCl buffer (50 mM, pH 7.5). Without addition of phosphite, the PTDH domain remained inactive. Stock solutions of the substrates were either prepared in ethanol/methanol (1:1, amlodipine, celecoxib, clopidogrel, donepezil, imipramine, nevirapine, ticlopidine) or DMSO (pioglitazone, zafirlukast). The final concentration of cosolvent was 1%. Reactions were started by the addition of enzyme and were performed in triplicate.

Analytical methods: Analysis was performed as described by Spandolf et al.^[84] with a Waters ACQUITY UPLC liquid chromatography system coupled to a Waters Synapt HDMS mass spectrometer equipped with an electrospray (ESI) ionization source. Samples (5–10 μL) were injected for chromatographic separation on an Acquity UPLC BEH C18 column (130 Å, $1.7\text{ }\mu\text{m} \times 2.1\text{ mm} \times 100\text{ mm}$; Waters) by applying a flow rate of 0.5 mL min^{-1} at a column temperature of 45°C . The mobile phase consisted of ultrapure water with formic acid (0.1% v/v, phase A) and pure acetonitrile (phase B). The gradient applied for separation was 0.0–6.0 min (10–70% phase B), followed by a return to the initial mobile phase composition over 0.01 min. MS analysis was performed with positive ESI in V-mode. A generic method with two scan functions was used as follows: m/z 80–1000, cone voltage 20 V, and 0.1 s scan time; the collision energy (CE) in function 1 was 20 V, and in function 2 an energy ramp of 15–45 V was applied; the transfer cell CE was 12 V. Data was collected in centroid mode. Leucine-enkephalin was used as lock mass (m/z 556.2771) for internal calibration at a concentration of 250 pg mL^{-1} and a flow rate of 0.04 mL min^{-1} .

The MS data were processed in MetaboLynx 4.1 (Waters) by using both the mass defect filter (MDF) and the dealkylation tool.^[84] The list of proposed metabolites was reviewed manually. A semiquantitative substrate conversion and metabolite formation were assessed by calculation of the fraction of the substrate peak area or a product peak area in comparison to the total related peak area detected.

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