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Efficient Screening of Cytochrome P450 BM3 Mutants for Their Metabolic Activity and Diversity toward a Wide Set of Drug-Like Molecules in Chemical Space[S]

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ABSTRACT:

In the present study, the diversity of a library of drug-metabolizing bacterial cytochrome P450 (P450) BM3 mutants was evaluated by a liquid chromatography-mass spectrometry (LC-MS)-based screening method. A strategy was designed to identify a minimal set of BM3 mutants that displays differences in regio- and stereoselectivities and is suitable to metabolize a large fraction of drug chemistry space. We first screened the activities of six structurally diverse BM3 mutants toward a library of 43 marketed drugs (encompassing a wide range of human P450 phenotypes, cLogP values, charges, and molecular weights) using a rapid LC-MS method with an automated method development and data-processing system. Significant differences in metabolic activity were found for the mutants tested and based on this drug library screen; nine structurally diverse probe drugs were selected that were subsequently used to study the metabolism of a library of 14 BM3 mutants in more detail. Using this alternative screening strategy, we were able to select a minimal set of BM3 mutants with high metabolic activities and diversity with respect to substrate specificity and regioselectivity that could produce both human relevant and BM3 unique drug metabolites. This panel of four mutants (M02, MT35, MT38, and MT43) was capable of producing P450-mediated metabolites for 41 of the 43 drugs tested while metabolizing 77% of the drugs by more than 20%. We observed this as the first step in our approach to use of bacterial P450 enzymes as general reagents for lead diversification in the drug development process and the biosynthesis of drug(-like) metabolites.

Introduction

Cytochromes P450 (P450s) represent the most important class of enzymes among other drug-metabolizing enzymes. Because it has been shown that drug metabolites produced by P450s may have improved pharmacological activities and are often responsible for the toxicity or unwanted pharmacological side effects, there is increasing interest in systems enabling the facile (bio)synthesis of sufficient quantities of metabolites for structural elucidation and pharmacological and toxicological evaluation (Chefson and Auclair, 2006). Biocatalytic metabolite production can be performed by large-scale incubations with recombinant human P450s. However, because of their relatively low activities and instability (Guengerich et al., 1996), the yield of the metabolites often is very low and the costs high. Attempts to increase the specific activity of the human P450s toward drugs by genetic engineering so far has had limited success resulting in less than 10-fold increases in specific activity (Kumar and Halpert, 2005).

Compared with their human counterparts, microbial P450s generally show much higher stability, and their specific activity toward their natural substrates can be more than 100- to 1000-fold higher compared with the highest activities observed with human P450s (Bernhardt, 2006). One of the most promising microbial P450s as biocatalyst for metabolite production is P450 BM3 (CYP102A) from *Bacillus megaterium* because it is the most active P450 so far identified and is a highly stable soluble enzyme (Narhi and Fulco, 1986; Munro et al., 2002). As a natural fusion protein, BM3 contains the heme domain and reductase on a single polypeptide chain, which makes the enzyme catalytically self-sufficient. These characteristics, in combination with the availability of crystal structures and large-scale production and purification protocols, make BM3 an ideal candidate for protein engineering and application in biocatalytic processes. Rational design and directed evolution have already been successfully used, both separately and in tandem, to generate BM3 mutants that are capable of metabolizing both native and exogenous substrates with increased activities and altered regio- and stereoselectivities. These substrates include aromatics (Carmichael and Wong, 2001; Li et al., 2001a; Whitehouse et al., 2008), alkanes (Appel et al., 2001; Peters et al., 2003; Kubo et al., 2006), hydrocarbons (Seifert et al., 2009), and carboxylic acids (Munzer et al., 2005). Moreover, van

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ABBREVIATIONS: P450, cytochrome P450; LC-MS, liquid chromatography-mass spectrometry; ACN, acetonitrile; MS, mass spectrometry; HLMs, human liver microsomes; CV, coefficient of variation.

Vugt-Lussenburg et al. (2007) and others (Landwehr et al., 2006; Otey et al., 2006; Kim et al., 2008, 2009, 2011; Rentmeister et al., 2009; Sawayama et al., 2009) have shown that P450 BM3 mutants can also be used for the production of both human relevant and BM3 unique drug metabolites. A study recently published by Sawayama et al. (2009) describes the drug-metabolizing potential of a panel of 120 BM3 mutants and demonstrated that this panel could produce both human relevant and BM3 unique metabolites for the two marketed drugs, verapamil and astemizole (Sawayama et al., 2009). However, screening libraries of compounds against such large panels of BM3 mutants can be time-consuming, cumbersome, and expensive. Therefore, ideally a limited number of BM3 mutants with maximal diversity would be more suitable for drug library diversification.

In the present study, we designed a strategy that enabled the identification of a panel of BM3 mutants that display differences in regio- and stereoselectivities and are suitable to metabolizing a large fraction of drug chemistry space. First, a set of six structurally diverse BM3 mutants was screened against a total library of 43 drugs, which were selected to represent a large diversity in drug chemistry space. Both substrate depletion and metabolite formation were measured in this drug library screen using a rapid LC-MS method with an automated method development and data-processing system. The second step was to select a smaller set of drugs, for which significant differences in activities and metabolite profiles between mutants were observed in the drug library screen. For this set of nine probe drugs, the metabolic activity and the metabolite distribution by a library of in total fourteen BM3 mutants were investigated to gather information on the effect of the different mutations introduced and the usefulness of the selected probe drugs as tools to screen BM3 libraries for diversity. Based on the results of the drug library and the mutant library screens, it was possible to select four stable BM3 mutants with high metabolic activities and, more importantly, altered substrate specificities and regiospecificities that can be used for drug lead diversification and the biosynthesis of metabolites of drug(-like) compounds.

Materials and Methods

Enzymes and Plasmids. The bacterial P450 BM3 mutants M01, M02, M05, and M11 in the pET28a$^+$ vector were described previously (van Vugt-Lussenburg et al., 2007). The other 10 mutants were selected from an already existing library (J. S. van Leeuwen and E. Stjernschantz, unpublished results) and were constructed as follows. Three different site-directed mutants of BM3 M01 were constructed containing the additional mutations A74E (MT41), S72D (MT43), and S72E (MT44). Seven different site-directed mutants of BM3 M11 were constructed as follows: L437E (MT32), L437N (MT33), L437S (MT35), L437T (MT36), A74E (MT34), A74D (MT37), and S72D (MT38). The mutations were introduced into the corresponding templates in the pBSIIKS$^+$-BM3 vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following oligonucleotides (and their reverse complements) were used for the mutations (the codon for the amino acid substitution is underlined): S72D/E 5′-CGTACAAATTTAAGCGCT-TGSTCTAAGTTTTTATCA-3′, A74D/E 5′-GATAAAAACTTAAGT-CAAGAWCTTAAATTTGTACGT-3′, and L437 5′-CTACGAGCTC-GATATTAAAGAGACTNNSACGTTAAAACCTGAAGGCTTTGTGG-3′. After mutagenesis, the presence of the desired mutations was confirmed by DNA sequencing (Service XS, Leiden, The Netherlands). The genes of these 10 site-directed mutants were cloned from the pBSIIKS$^+$-BM3 system where they reside between the BamHI/EcoRI restriction sites into the pET28a$^+$ vector. The resulting His-tagged P450 BM3 mutants MT32, MT33, MT34, MT35, MT36, MT37, MT38, MT41, MT43, and MT44 were used in this study.

Expression. The M01, M02, M05, and M11 mutant proteins were expressed as described previously (Damsten et al., 2008b). The His-tagged pET28a$^+$ constructs of MT32, MT33, MT34, MT35, MT36, MT37, MT38, MT41, MT43, and MT44 were transformed into *Escherichia coli* BL21 (DE3) cells using standard procedures. For expression, 600 ml of Terrific Broth (Tartof and Hobbs, 1988) medium (24 g/l yeast extract, 12 g/l tryptone, 4 ml/l glycerol) with 30 μg/ml kanamycin was inoculated with 15 ml of an overnight culture. The cells were grown at 175 rpm and 37°C until the OD_{600} reached 0.6. Protein expression then was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyrasonide. The temperature was lowered to 20°C, and a 0.5 mM concentration of the heme precursor δ-aminolevulinic acid was added. Expression was allowed to proceed for 18 h. Cells were harvested by centrifugation (4600*g*, 4°C, 25 min), and the pellet was resuspended in 20 ml of potassium phosphate-glycerol buffer (100 mM potassium phosphate, pH 7.4, 10% glycerol, 0.5 mM EDTA, and 0.25 mM dithiothreitol). Cells were disrupted using a French press (1000 psi, 3 repeats), and the cytosolic fraction was separated from the membrane fraction by ultracentrifugation of the lysate (120,000*g*, 4°C, 60 min).

Screening of the 43 Drug Library. The degree of metabolism of 43 marketed drugs by the BM3 mutants M01, M02, M11, MT35, MT38, and MT43 was investigated. The concentration of the 43 reference compounds was 40 μM, the final dimethyl sulfoxide concentration in the incubation was set at 4%, and all mutants were incubated at a 500 nM enzyme concentration. The reactions were initiated by addition of 50 μl of an NADPH-regenerating system resulting in final concentrations of 0.5 mM NADPH, 0.38 mM glucose 6-phosphate, and 0.5 units/ml glucose-6-phosphate dehydrogenase. The final volume of the incubations was 250 μl. The reaction was allowed to proceed for 90 min at 24°C and terminated by the addition of 500 μl of ice-cold acetonitrile (ACN). After centrifugation at 14,000 rpm for 10 min, 120 μl of supernatant was mixed with 480 μl of a mixture of water and ACN (50:50).

To compare the activity and the metabolic profile of the BM3 mutants with those formed by human P450s, incubations were also performed with pooled human liver microsomes (HLMs) from BD Gentest (Woburn, MA). A final microsomal protein concentration of 1 mg/ml was used, and these incubations were performed as described above at 37°C instead of 24°C.

Metabolites and parent compounds were analyzed using reversed-phase chromatography using a Synergi Polar-RP column (4μ, 30 × 2.00 mm i.d.; Phenomenex, Torrance, CA), with an elution gradient composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH). The gradient applied was constant at 2% B for 0.5 min, linear from 2% B to 95% B in 1.5 min, constant for 0.9 min, and back to 2% B in 0.1 min, constant at 2% B for 0.5 min at a total flow rate of 0.5 ml/min. Auto-tuning (tuning solution concentration was 50 μM) and auto-method generation were performed using an automaton on a PE/Sciex API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Waltham, MA) with Ionics EP10+ upgrade equipped with an electron spray ionization interface. Mass spectra were recorded in the positive mode with multiple reaction monitoring using the turbo ionspray interface. Automated peak detection and integration for analytical batches were performed using Auto-Quan software. Raw data were analyzed with PE/Sciex software Analyst 1.4.1, and data were qualitatively controlled and reviewed using an in-house developed expert decision support system and Analyst 1.4.1.

Screening of the BM3 Mutant Library. The nine drug molecules selected for the initial mutant characterization were amitriptyline, buspirone, cilostazol, citalopram, diltiazem, irbesartan, ondansetron, propafenone, and repaglinide. The metabolic incubations were performed in 100 mM KPO_4 buffer, pH 7.4, with the cytosolic fraction containing 1 μM of the different BM3 mutants and a 40 μM substrate concentration. The reactions were initiated by the addition of 50 μl of an NADPH-regenerating system resulting in final concentrations of 0.5 mM NADPH, 0.38 mM glucose 6-phosphate, and 0.5 units/ml glucose-6-phosphate dehydrogenase. The final volume of the incubations was 250 μl. The reaction was allowed to proceed for 90 min at 24°C and terminated by the addition of 500 μl of ice-cold ACN. After centrifugation at 14,000 rpm for 10 min, 400 μl of supernatant was mixed with 800 μl of water, and the sample was analyzed by LC-MS. All reactions were performed in triplicate.

Metabolites and parent compounds were separated by reversed-phase chromatography using a C8(2)-column (Luna 3μ, 50 × 3.00 mm i.d.; Phenomenex), which was eluted by a binary gradient, composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH). Different gradients were used for each compound but the general setup of the gradients applied was as follows: constant at 10% MeOH for 1 min, linear from 10% MeOH to a compound specific percentage of MeOH in 4 min, constant at this

percentage of MeOH for 0.5 min, linear increase to 100% MeOH in 0.01 min, constant for 1.5 min, linear decrease to 10% MeOH in 0.25 min, constant for 0.75 min. The compound-specific percentages of MeOH are as follows: 80% for amitriptyline, 60% for buspirone, 95% for cilostazol, 65% for citalopram, 70% for diltiazem, 95% for irbesartan, 40% for ondansetron, and 70% for propafenone. The gradient applied to analyze the metabolism of repaglinide started at 40% MeOH and increased to 85% MeOH while decreasing back to 40% MeOH at the end of the gradient. For identification of parent compounds and metabolites, a Finnigan LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was used with positive electron spray ionization. N_2 was used as a sheath gas (40 psi) and auxiliary gas (20 psi), the needle voltage was 5000 V, and the heated capillary was at 275°C. LC-MS data of the metabolites were processed with Xcalibur/Qual Browser version 1.2 (Thermo Fisher Scientific). Standard curves of the parent compounds were linear between 0.5 and 200 μM.

Results

Selection of the P450 BM3 Mutant Library. The four P450 BM3 mutants, M01, M02, M05, and M11 (see Supplemental Table S1), have previously been demonstrated to display good expression and activity toward drug-like compounds (van Vugt-Lussenburg et al., 2007; Damsten et al., 2008a,b; Stjernschantz et al., 2008) and were therefore included in the mutant library. Ten novel mutants (see Supplemental Table 1) using both M01 and M11 as template were additionally selected from an already existing library (J. S. van Leeuwen and E. Stjernschantz, unpublished results). In these novel mutants, the residues targeted for mutagenesis were located across the active site of P450 BM3 (see Fig. 1). Four mutants of the Leu437 residue, which together with Thr438 forms a tight turn that is involved in substrate contacts (Li and Poulos, 1997) and has previously been demonstrated to influence NADPH-coupling efficiency (Carmichael and Wong, 2001), were selected. Mutant MT32 contained an extra negative charge in the active site at the 437 position since the nonpolar uncharged hydrophobic leucine was replaced by the polar acidic glutamic acid (MT32; L437E). The other three mutants contained an extra polar group at the same position in the active site because the nonpolar leucine was replaced by either the polar asparagine (MT33; L437N), serine (MT35; L437S), or threonine (MT36; L437T), which differ in size and hydrogen-bonding capabilities.

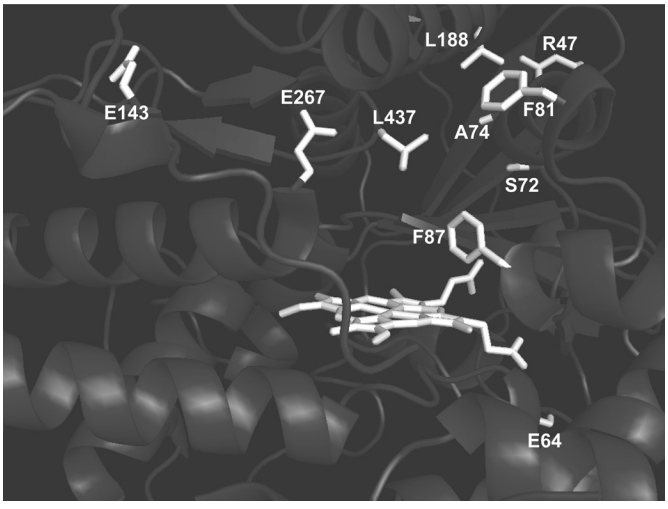

FIG. 1. Crystal structure of the active site of P450 BM3 (Protein Data Bank code 1BU7). The residues that were altered in mutant M11 and the additional residues, which have been altered in this study to obtain the different novel BM3 mutants, are labeled and depicted in white. The heme is also displayed in white.

Six mutants of the uncharged Ser72 and Ala74 residues that contained a negatively charged amino acid at these respective positions were selected. The Ser72 and Ala74 residues are located around the substrate binding channel (Li et al., 2001b; Otey et al., 2006; Dietrich et al., 2009) of the protein and have been shown to influence regioselectivity (Otey et al., 2006; Dietrich et al., 2009) and metabolic efficiency (Li et al., 2000, 2001b; Fasan et al., 2007) in previous studies. Two M01 mutants that contained mutations at the Ser72 position, MT43 (S72D) and MT44 (S72E), and one M01 mutant that contained a mutation at the Ala74 position (MT41; A74E) were selected. Furthermore, one M11 mutant (MT38; S72D) that targeted the Ser72 position and two M11 mutants that targeted the Ala74 position, MT34 (A74E) and MT37 (A74D), were selected. The expression levels and stability of the novel mutants were determined by measuring the intensity of the characteristic Soret band at 450 nm upon reduction by dithionite and the addition of CO.

Screening of the 43 Drug Library. To probe the metabolic properties of the BM3 mutants, a diverse set of 43 commercial drugs was selected to encompass a wide range of human P450 phenotype, cLogP value (0.4–7.6), molecular weight (162.2–670.9), and charge (as listed in Supplemental Table 2 and displayed in Fig. 2). The human P450s that are known to be involved in the metabolism of the majority of drugs (3A4, 2C9, 2D6, 2C19, 1A2, and 2C8) were all represented in this selection (Zanger et al., 2008). Many of the selected drugs were positively charged (Supplemental Table 2; Fig. 2), which is in agreement with the observation that a high proportion of drugs are weak bases (Manallack, 2008). Three of the six BM3 mutants (M01, M02, and M11) that were chosen for the drug library screening have previously been demonstrated to display different metabolic activities (van Vugt-Lussenburg et al., 2007; Stjernschantz et al., 2008). The other three mutants (MT35, MT38, and MT43) were selected because they contained mutations at different residues in the active site, which was expected to result in changes in metabolic activity and diversity (Carmichael and Wong, 2001; Li et al., 2001b; Dietrich et al., 2009).

The results of the drug library screen for the six selected mutant and HLM incubations are listed in Table 1. The overall precision [coefficient of variation (CV)] of the screening assay was less than 5% of the 43 drugs screened, and 34 drugs were metabolized by 20% or more by at least one of the six BM3 mutants after 90 min of incubation. MT35 metabolized most of the compounds for more than 20% (65% success rate). The drugs aripiprazole, cinacalcet, rosiglitazone, and thioridazine were metabolized to a high extent by all six of the tested mutants. The drugs diclofenac, diltiazem, glipizide, irbesartan, midazolam, repaglinide, saquinavir, and verapamil were mainly metabolized by MT35. For three other drugs, cilostazol, nelfinavir, and terfenadine, it was found that they were mainly metabolized by MT38 and MT43 (which both share the S72D mutation), whereas paroxetine was mainly metabolized by M11. For nine drugs (alprazolam, aprepitant, carbamazepine, indomethacin, lacosamide, meloxicam, minaprine, phenacetine, and *R*-warfarin), none of the mutants tested displayed conversion above 20%. However, seven of these nine drugs were metabolized by at least one of the mutants because small amounts of P450-mediated metabolites could be identified by LC-MS. Only indomethacin and meloxicam failed to be metabolized by any of the mutants.

The results of the drug library screen were used to make a partial correlation matrix, which is shown in Table 2. From this table, it can be seen that significant differences exist between the mutants. The lowest correlation based on the metabolic activity (as measured by substrate depletion) can be found between MT35 and MT43, which indicates that these mutants display a high substrate diversity. MT43 and MT35 also display a low correlation with M11 and M02, respec-

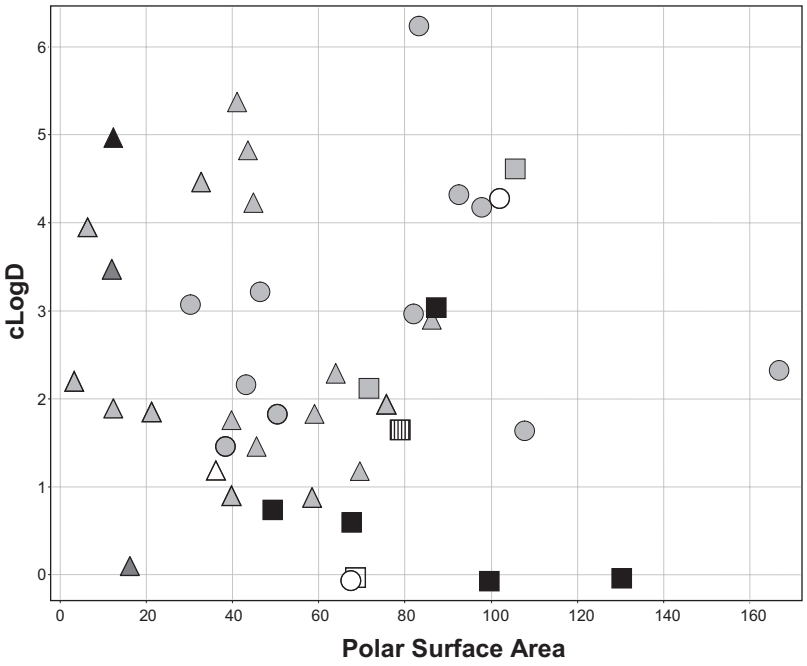

FIG. 2. Representation of the variation in chemistry space of the 43 drug screening set. The polar surface area (PSA) of the different drugs has been plotted against the cLogP value at pH 7.4 (cLogD). Negatively charged, positively charged, and neutral compounds are shown as squares, triangles, or circles, respectively, whereas the major human P450s involved in the metabolism of the drug are represented by color (1A2 dark gray, black encircled; 2C19 white, black encircled; 2C8 black and white striped, black encircled; 2C9 black; 2D6 light gray, black encircled; 3A4 light gray).

tively. A high similarity in the metabolic activity was found for M01 with M11 and for MT38 with MT43.

The applied LC-MS detection method allowed the possibility to follow multiple selected MS traces simultaneously. Because it was expected that the major P450-mediated metabolites were either mono- or dihydroxylated or mono- or didemethylated products, the M+16, M+32, M-14, and M-28 traces were monitored. In addition, the M+2 (hydroxylation in combination with demethylation) and the M-2 (dehydrogenation or hydroxylation followed by dehydration) traces were monitored. Using this setup, it was possible to detect many of the generated metabolites and explain most of the substrate depletion results. For the drugs for which a high depletion was observed but no major metabolites were detected with the standard assay, in most cases reanalysis of samples proved that other P450-mediated products were formed. In these cases, the metabolites were not the products expected from any of the reactions described above but were formed through other reactions, as was the case for the drugs astemizole, aripiprazole, carvedilol, and terfenadine.

The Spotfire Decision Site software package (version 8.2; Tibco Software, Somerville, MA) was used to perform multiple regression analysis to assess correlations of metabolism by the six BM3 mutants with the different properties of the 43 drugs. The degree of metabolism of the drugs poorly correlated with molecular weight, cLogP, cLogD, polar surface area, and human P450 phenotype characteristics. However, it was apparent that the mutants MT38 and MT43 displayed an increased activity toward neutral drugs compared with both MT35 and M11 (see Fig. 3). Negatively charged drugs, on the other hand, demonstrated an increased activity toward MT35 compared with the other mutants, whereas M11 predominantly metabolized positively charged drugs.

Screening of the BM3 Mutant Library. Of the set of 43 drugs, nine drugs (see Table 3) were selected based on the drug library screen for the purpose of being used as molecular probes to evaluate the BM3 library of fourteen mutants for its metabolic characteristics while still covering a wide range of cLogP values (2.2–6.0), molecular sizes (between 271.4 and 452.6), and human P450 isoforms (see Supplemental Table 2). Amitriptyline, buspirone, citalopram, ondansetron and propafenone were selected because they were metabolized to different extents in the drug library screen by the various mutants tested. Diltiazem, irbesartan, and repaglinide were mainly metabolized by MT35 in the drug library screen, and we wanted to investigate whether a similar trend could be observed for the other Leu437 mutants. Cilostazol was selected to further investigate the influence of the Ser72 position because this drug was mainly metabolized by MT38 and MT43. Although the selection of nine drugs contained one neutral and two negatively charged drugs, the majority of the drugs were positively charged, thus also allowing the investigation of the effect of the introduction of negatively charged residues in the active site of some of the BM3 mutants.

The results of the metabolism of the nine drugs by the 14 BM3 mutants are displayed in Table 3. To determine the metabolic activity of the mutants, the percentage substrate depletion was calculated and the profile of the metabolites was characterized by LC-MS. By comparing HLM metabolism data for the nine selected drugs, it was possible to distinguish between human relevant and BM3 unique metabolites. For example, amitriptyline is converted by the mutants tested into a total of eight metabolites, the masses of which are M+16 (four peaks), M+32 (two peaks), M-14, and M-28 with turnover ranging between 48 and 93%. From Table 3, it can be concluded that the nine probe drugs were all metabolized by the selected BM3 mutants and typically generated at least two metabolites. The major metabolites were either mono- or dihydroxylated (M+16 and M+32) or mono- or di-demethylated (M-14 and M-28) products of the corresponding parent compound, which is consistent with P450-mediated oxidation. Using the obtained MS/MS spectra it was possible to determine the types of reactions involved for the majority of M+16 metabolites formed (see Table 3). In addition, for a single drug significant differences in substrate depletion and metabolite distribution were observed between the mutants and the mutants produced both human relevant and BM3 unique metabolites. For the drugs buspirone, cilostazol, and propafenone, the selected BM3 panel was capable of forming all observed human metabolites.

In Table 3 (and in more detail in Supplemental Table 3), the effect of the novel mutations on substrate depletion is displayed. It can be seen that for M11 the L437E (MT32), L437S (MT35), and S72D (MT38) mutations significantly increased the level of substrate deple-

TABLE 1

Substrate depletion results for the metabolism of 43 drugs by six BM3 mutants

Compound	M01	M02	M11	MT35	MT38	MT43	HLMs
				500 pmol/ml[a]			*1 mg/ml*[a]
Alprazolam	<5[b]	<5[b]	<5[b]	10	<5[b]	<5[b]	<5[b]
Amitriptyline	36	34	74	96	84	25	18
Aprepitant	10	<5[b]	10	<5[b]	9	17	45
Aripiprazole	99	99	99	93	99	99	93
Astemizole	95	8	94	68	<5[b]	10	22
Buspirone	46	75	83	67	9	29	17
Carbamazepine	<5[b]	<5[b]	<5[c]	11	<5[b]	10	<5[b]
Carvedilol	86	9	78	66	51	32	8
Cilostazol	<5[b]	25	17	37	82	93	38
Cinacalcet	96	89	97	87	67	96	46
Citalopram	29	7	56	78	27	10	<5[b]
Dextromethorphan	68	23	95	97	80	50	27
Diclofenac	18	<5[b]	13	50	<5[b]	7	45
Diltiazem	6	<5[b]	<5[b]	55	11	10	28
Duloxetine	18	<5[b]	39	51	53	20	<5[b]
Glipizide	<5[b]	<5[b]	7	35	<5[b]	9	44
Imatinib	51	80	77	30	13	<5[b]	<5[b]
Indomethacin	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[b]
Irbesartan	12	<5[b]	7	80	23	6	<5[b]
Lacosamide	<5[b]	<5[b]	<5[b]	<5[b]	7	<5[b]	<5[b]
Meloxicam	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[b]
Midazolam	<5[c]	<5[c]	<5[c]	40	<5[c]	<5[c]	36
Minaprine	11	6	<5[b]	19	7	9	<5[b]
Nelfinavir	7	<5[b]	<5[b]	<5[b]	24	37	<5[b]
Nicotine	36	8	<5[b]	43	37	21	48
Nifedipine	63	32	<5[b]	<5[b]	58	67	<5[b]
Nilotinib	9	23	<5[b]	21	10	10	24
Ondansetron	30	6	11	9	18	30	6
Paroxetine	10	6	41	17	17	6	<5[b]
Phenacetine	<5[b]	<5[b]	<5[b]	<5[b]	<5[b]	6	9
Pimozide	16	38	<5[b]	26	37	11	<5[b]
Propafenone	10	<5[b]	21	23	22	21	<5[b]
Quinidine	<5[b]	17	9	23	<5[b]	<5[b]	<5[b]
Repaglinide	<5[b]	<5[b]	13	55	<5[b]	<5[b]	33
Rosiglitazone	99	99	95	82	76	99	<5[b]
R-warfarin	6	<5[b]	<5[b]	<5[b]	<5[b]	<5[b]	<5[b]
Saquinavir	<5[b]	<5[b]	<5[b]	35	<5[b]	8	52
Sorafenib	<5[c]	<5[b]	36	<5[b]	38	36	71
Tamoxifen	62	68	36	28	63	73	8
Terfenadine	10	21	7	7	62	54	<5[b]
Thioridazine	99	91	96	97	97	83	7
Tipranavir	23	<5[c]	<5[b]	63	63	69	58
Verapamil	<5[b]	<5[b]	<5[b]	27	<5[b]	<5[b]	30

[a] The substrate depletion is calculated by using the average peak area of the parent at 90 min and at time zero. Values are expressed in percentages of the average peak area of the parent at time zero. Measurements were performed in duplicate.
[b] The calculated substrate depletion was below the overall precision (CV) of the screening assay but minor amounts of P450-mediated metabolites were detected by LC-MS.
[c] The calculated substrate depletion was below the overall precision (CV) of the screening assay and no P450-mediated metabolites could be detected by LC-MS.

tion (by more than 20%) for the majority of drugs tested. For the M01 mutants (MT41, MT43, and MT44), the mutations introduced at the positions Ala74 and Ser72 were beneficial for the activity toward cilostazol, citalopram, and propafenone. However, these mutations affected the activity toward buspirone in a negative manner. The MT35 mutant displayed the highest activity toward the drugs amitriptyline, diltiazem, and repaglinide. Citalopram and propafenone are metabolized to the highest extent by MT38, whereas MT43 displayed the highest substrate depletion for cilostazol and ondansetron. Buspirone is most efficiently metabolized by mutant M02.

When looking at the metabolite distribution profiles of the nine probe drugs generated during the mutant library screen, it can be observed from Table 3 that for some drugs the profiles were very similar, whereas for other drugs significant differences were found. For cilostazol, citalopram, diltiazem, and irbesartan, all mutants produced one identical major metabolite, and only small differences were observed between the distributions of the minor metabolites. For amitriptyline, larger differences were observed in the metabolite distribution profiles, although all mutants still produced one identical major metabolite. For buspirone, ondansetron, propafenone, and repaglinide, it was observed that significant differences existed between the metabolite distribution profiles generated by the mutant library. The profile of buspirone by M02 differed significantly from those produced by M01 and M11. In addition, for buspirone, it was shown that the novel mutations at positions Ser72 and Ala74 in M01 significantly altered the regiospecificity. Mutations at the same position in M11 also led to a change in the metabolite distribution. For ondan-

TABLE 2

Partial correlation matrix based on the drug library screen results

	M01	M02	M11	MT35	MT38	MT43	HLMs
M01	1	0.5595	0.7397	0.4382	0.4081	0.4394	0.0386
M02		1	0.5082	0.2476	0.3664	0.4829	0.0529
M11			1	0.5540	0.3311	0.2600	0.0518
MT35				1	0.3345	0.1829	0.0705
MT38					1	0.7549	0.0668
MT43						1	0.0994
HLM							1

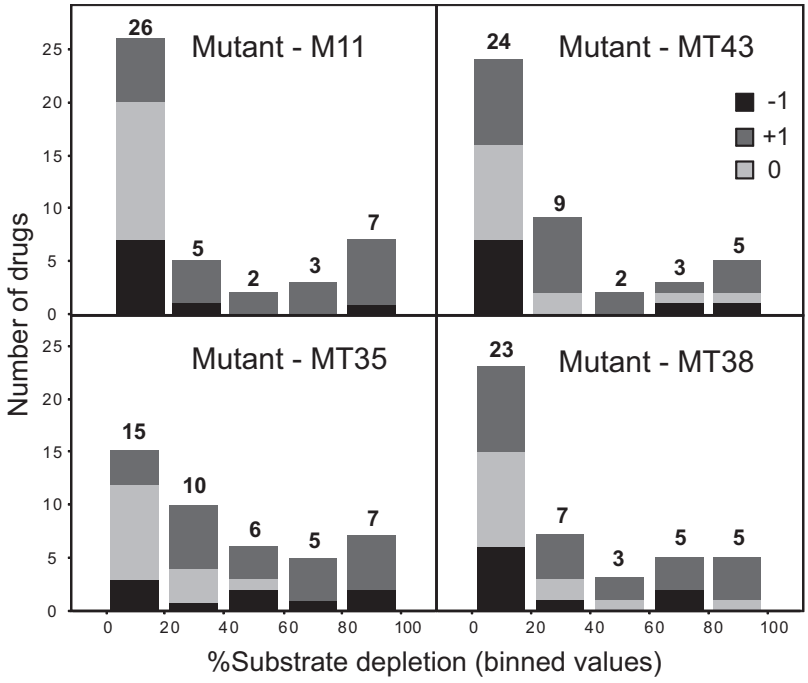

FIG. 3. Assessment of mutant activity based on drug charge. The substrate depletion data (binned values) has been plotted against the number of drugs, and the charge distribution has been included. Positively charged (+) drugs are displayed in dark gray, negatively charged (−) drugs are shown in black, and neutral drugs (0) are shown in light gray. The numbers of drugs that are represented per value bin are also displayed in the figure.

setron, 13 metabolites were formed in total by the different mutants, and the novel mutations at the Ser72 and Ala74 positions in both M01 and M11 resulted in altered metabolite distributions. For propafenone and repaglinide, the novel mutations also resulted in changes in the metabolite distribution profiles and the metabolic activities. It is interesting to see that M11 and MT33 produce only the N-depropylated metabolite (M-42) for propafenone, whereas the other mutants also produce additional monohydroxylated metabolites that are human relevant.

Discussion

In the present study, we aimed to design a strategy to efficiently screen BM3 mutants against a diverse set of drugs, which cover a wide range of chemistry space to identify a panel of BM3 mutants with high metabolic activity and altered substrate specificities and regiospecificities that can be used for drug lead diversification and the biosynthesis of metabolites of drug(-like) compounds. Lussenburg et al. (2005) constructed a triple mutant (R47L/F87V/L188Q) of BM3 that could metabolize several drug-like molecules (van Vugt-Lussenburg et al., 2006) and used random mutagenesis to improve the activity of this triple mutant, which resulted in the identification of the highly active drug-metabolizing BM3 mutants M01, M02, M01, and M11 (van Vugt-Lussenburg et al., 2007). To further investigate the drug-metabolizing potential of these four mutants, they were included in the BM3 library of 14 mutants (Supplemental Table 1), which further consisted of novel mutants of M01 (MT41, MT43, and MT44) and M11 (MT32, MT33, MT34, MT35, MT36, MT37, and MT38) (J. S. van Leeuwen and E. Stjernschantz, unpublished results). The novel mutations introduced in these mutants targeted the active site residues Ser72, Aal74, or Leu437. The first step of our strategy was to screen a small selection of structurally diverse BM3 mutants against the total set of 43 drugs using an LC-MS method with an automated method development and data-processing system to get information on enzyme activity and insight into the types of metabolites being formed. In the second step of our strategy, we wanted to investigate whether the drug metabolism by the different BM3 mutants, besides the differences in metabolic activities, which were found in the drug library screen, would also lead to altered metabolite profiles. In addition, we wanted to investigate the usefulness of nine drugs as tools to screen BM3 libraries for diversity.

When the effects of the different mutations are compared with the activity and regioselectivity of the corresponding mutant templates used (as listed in Table 3 and Supplemental Table 3), it can be concluded that performing mutations at the Leu437 and Ser72 positions improved the metabolic activity of the M11 mutant while also significantly altering the metabolite distribution. For the M01 mutant, the mutations at the Ala74 and Ser72 position improved activity toward most drugs tested, and these amino acid substitutions also altered the metabolite distribution. This is especially the case for buspirone where M01 predominantly performs an aromatic hydroxylation, whereas the MT41 (A74E), MT43 (S72D), and MT44 (S72E) form all five hydroxylated metabolites. Mutations at the same positions using the M11 template (A74E–MT34, A74D–MT37, and S72D–MT38) also change the metabolic profile from almost exclusively aromatic to the formation of at least two additional monohydroxylated metabolites resulting from an aliphatic hydroxylation. When evaluating the usefulness of the nine selected probe drugs as tools to screen BM3 mutants for diversity, it was observed that significantly altered metabolic profiles for buspirone, ondansetron, propafenone, and repaglinide were generated by the different mutants. It was interesting that the trends for these differences corresponded relatively well with the correlation data from Table 2. This was especially the case for buspirone and ondansetron where high similarities were found for M01 and M11 whereas significant differences were observed between MT35 and MT43, M11 and MT43, and M02 and MT35. For the other six probe drugs evaluated, the differences in metabolite distribution profiles were less significant, which makes these drugs less suitable to screen this BM3 library for diversity.

The results of the drug library and BM3 mutant library screens clearly demonstrated that the P450 BM3 mutants used in this study have very promising properties for drug library diversification and the production of human relevant drug metabolites. The most interesting BM3 mutants analyzed in this study are M02, MT35, MT38, and MT43. The M02 and MT43 mutants displayed an increased activity

TABLE 3

Metabolic activity and diversity of nine probe drugs by 14 different BM3 mutants

Drug	M01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D)	MT38 (S72D)	HLM[a]
Amitriptyline (%)[b]	**59**	**59**	**48**	**54**	**53**	**89**	**70**	**80**	**65**	**93**	**72**	**81**	**73**	**92**	
M+16_1 (*[c])	5	13	13	5	9	23	11	15	10	19	15	14	12	14	√
M+16_2 (*)	1	2	2	1	6	2	1	1	2	3	3	1	1	1	√
M+16_3 (**)	16	10	13	16	14	9	10	7	6	4	6	11	12	16	√
M+32_1		10	3	3				1	2		3				**N**
M+32_2	1	1	1		1	1	1	2		4	1	1	1	1	**N**
M-14	65	53	58	64	57	50	69	60	71	58	63	65	66	55	√
M-28	12	11	10	11	13	15	8	14	9	12	9	8	8	13	**N**
Buspirone (%)[b]	**56**	**39**	**56**	**33**	**91**	**62**	**25**	**47**	**3**	**33**	**18**	**1**	**19**	**13**	
M+16_1 (*)	2	16	25	26	8	1		4		2	2	5	37	35	√
M+16_2 (*)		8	15	14	8										√
M+16_3 (*)		35	25	6	65							3			√
M+16_4 (***)	71	16	16	22	10	85	79	90	94	91	88	54	39	45	√
M+16_5 (**)	16	9	14	16	3	10	14	2		3	5	21	24	20	√
M+32					6										√
M-26	11	16	5	16		4	7	4	6	4	5	17			√
Cilostazol (%)[b]	**7**	**36**	**87**	**75**	**27**	**24**	**5**	**62**	**7**	**34**	**29**	**19**	**4**	**75**	
M+16_1 (*)	69	88	88	91	65	86	85	88	78	86	84	94	100	97	√
M+16_2 (*)	6	4	4	4	9	7	7	3	5	5	6	6		3	√
M+16_3 (*)	25	8	3	5	26	7	8	9	17	9	10				√
M+32			5												**N**
Citalopram (%)[b]	**2**	**28**	**20**	**24**	**1**	**30**	**7**	**47**	**12**	**46**	**31**	**6**	**11**	**51**	
M+16 (**)	100	3	5	4	12	2	2	2	2	2	2	5	4	4	√
M-14		95	93	93	88	95	97	88	94	91	92	95	96	88	√
M-28		2	2	3		3	1	10	4	7	6			8	**N**
Diltiazem (%)[b]	**1**	**13**	**11**	**16**	**1**	**1**	**2**	**24**	**31**	**43**	**4**	**1**	**8**	**19**	
M-14_1	8	2	3	2		9	9	3	2	2	4			4	√
M-14_2	92	96	97	97	100	91	91	93	94	87	96	100	100	92	√
M-28_1								1		1				1	√
M-28_2		2		1				4	4	10				3	√
Irbesartan (%)[b]	**16**	**52**	**9**	**21**	**1**	**37**	**15**	**72**	**32**	**69**	**46**	**15**	**15**	**38**	
M+16 (*)	99	98	99	99	94	99	97	99	99	99	99	98	95	99	√
M+32	1	2	1	1	6	1	3	1	1	1	1	2	5	1	**N**
Ondansetron (%)[b]	**11**	**7**	**37**	**14**	**12**	**1**	**4**	**6**	**12**	**3**	**2**	**8**	**1**	**16**	
M+16_1 (?)	1		1		1	3	3	3		4	3			1	√
M+16_2 (*)	3		3		2	5	5	4		6	4			1	√
M+16_3 (?)	10	2			2	7	8	4	10	11	14	2	2	2	√
M+16_4 (*)	31	20	6	7	22	20	21	11	20	19	25	13	21	15	√
M+16_5 (*)	28	60	50	44	28	18	18	9	12	9	8	22	49	26	√
M+16_6 (*)				16								15		14	√
M+16_7 (*)	3	5	8	9	12	4	4	13	8	8	4	14	6	12	√
M+32_1			6	3	1									2	**N**
M+32_2			2	1											√
M-14_1	12	4	11	4	10	22	21	25	29	20	22	9	12	5	√
M-14_2	8	2			7	16	13	11		11	13			2	**N**
M+38	4	6	8	10	15	5	6	18	11	11	6	19	9	13	√
M+34		1	5	6			1	2	10	1	1	6	1	7	**N**
Propafenone (%)[b]	**2**	**19**	**22**	**24**	**3**	**4**	**10**	**7**	**5**	**12**	**5**	**12**	**25**	**26**	
M+16_1 (*)	19	60	49	32	57	22		12		9	14	28	65	56	√
M+16_2 (*)	41	31	43	46	20	24		22		17	19	48	22	30	√
M+16_3 (?)		2	2	5		10		6		7	9	3	2	2	√
M-42	40	7	6	17	23	44	100	60	100	67	58	21	11	12	√
Repaglinide (%)[b]	**1**	**7**	**2**	**1**	**6**	**8**	**11**	**12**	**11**	**36**	**5**	**5**	**1**	**4**	
M+16_1 (*)	6	100			8	16	16	10	10	8	10		100		√
M+16_2 (?)	20		20		2	1			2	2					√
M+16_3 (*)	58		8	25	68	45	42	66	56	72	37	100		46	√
M+16_4 (*)								4	7	7	38				√
M-2	16		72	75	22	38	42	20	25	11	15			54	**N**

[a] Indicates whether the metabolite in question is detected in HLM incubations (√) or BM3 unique (**N**).

[b] The substrate depletion (in boldface) is calculated by using the average peak area of the parent at 90 min and at time zero. Values represent the mean of three replicates and are expressed in percentages of the average peak area of the parent at time zero.

[c] Indicates whether the reaction involved was an aliphatic hydroxylation (*), heteroatom oxygenation (**), or aromatic hydroxylation (***) or whether the type remained unknown (?).

and altered regiospecificity compared with both M11 and MT35 and, in the case of buspirone, were capable to produce novel metabolites that are not formed by the other two mutants. It has been suggested previously that the Leu437 residue might be a slight bottleneck in the substrate binding channel and that replacement of this residue by the smaller serine residue could promote substrate entry and binding (Carmichael and Wong, 2001) and introduce hydrogen-bonding potential. This is in agreement with our observation that the replacement of leucine at position 437 by the smaller serine residue (MT35) introduces an increased activity toward the majority of drugs tested and, in particular, negatively charged compounds (see Fig. 3) compared with M11. Replacing the polar uncharged serine residue of residue 72 in the active site by the polar negatively charged aspartic acid amino acid in both MT43 and MT38 results in an increased activity toward neutral drugs (see Fig. 3) while also affecting the regioselectivity. This is in agreement with the findings of Dietrich et

al., 2009 who have reported that mutation of the 72 position alters the regioselectivity of lauric acid metabolism. Based on these findings, it would be very interesting to further rationalize the influence of the introduced mutations by looking into the metabolism of a small set of compounds in more detail. Another very interesting option is to use the information obtained in this study to design new site-directed mutants to improve metabolic efficiency and substrate diversity of the existing mutants.

The panel of BM3 mutants presented in this study can be useful in the drug-development process as general reagents for lead diversification. More importantly, these mutants can also be used for the identification and rapid production of relevant quantities of human relevant drug(-like) metabolites for pharmacological and toxicological evaluation. An early proactive screening for metabolites will be beneficial because active metabolites will be identified as soon as possible (Schroer et al., 2010). This has become very important in toxicology screening since a regulatory guidance for industry was issued in 2008 by the U.S. Food and Drug Administration, which defines that drug metabolites present as >10% (formerly >25%) of the parent drug in circulation are important as metabolites in safety testing (Smith and Obach, 2009). The BM3 mutants presented here overcome the limitations of most human P450 isoforms, such as low expression levels, generally low specific activity, and low uptake rate of some biotransformation substrates, and therefore are ideal candidates for such studies. For example, when the analytical scale incubation with MT35 (500 nM) and irbesartan (40 μM) used during the drug library screen would be upscaled to 1 liter of batch culture (~500 nmol of P450), approximately 14 mg of the major M+16 metabolite can be produced in 90 min using 50 μmol of NADPH, 2.5 mmol of glucose 6-phosphate, and 500 units of glucose 6-phosphate dehydrogenase. Taking into account the currently available sophisticated analytical techniques and high-throughput screening technologies, such amounts of metabolite in general should be sufficient for early structural elucidation studies and pharmacological and toxicological evaluation.

The present study demonstrates that it is possible to screen a selected set of BM3 mutants quantitatively and also qualitatively for their activities toward a large set of drug-like compounds to find promising mutants with improved metabolic characteristics. We designed a strategy that enabled the identification of a panel of BM3 mutants, which displayed differences in regio- and stereoselectivities and was suitable to metabolize a large fraction of drug chemistry space. This panel of four mutants (M02, MT35, MT38, and MT43) was capable of producing P450-mediated metabolites for 41 of the 43 drugs tested while metabolizing 77% of the drug library by more than 20%. In addition, it was shown that the drugs buspirone and ondansetron are valuable tools to screen BM3 mutant libraries for diversity. The methods and experiments described in this article are useful tools for future research to find better mutants for a selected structurally diverse compound library, and the mutants described in this study could be used as a starting point for further random or site-directed mutagenesis to further improve metabolic efficiency and alter substrate diversity and regioselectivity. Future research to further rationalize the effect of the introduced mutations by looking at the metabolism in more detail would be very helpful in this process.

Authorship contributions

Participated in research design: Reinen, Grootenhuis, Decker, Saunders, and Commandeur.

Conducted experiments: Reinen and van Leeuwen.

Contributed new reagents or analytical tools: Reinen, Li, and Sun.

Performed data analysis: Reinen and Li.

Wrote or contributed to the writing of the manuscript: Reinen, van Leeuwen, Grootenhuis, Decker, Saunders, Vermeulen, and Commandeur.

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