CHAPTER 5

COMBINATION OF BIOTRANSFORMATION BY P450 BM3 MUTANTS WITH ON-LINE POST-COLUMN BIOAFFINITY AND MASS SPECTROSCOPIC PROFILING AS A NOVEL STRATEGY TO DIVERSIFY AND CHARACTERIZE P38α KINASE INHIBITORS

Vanina Rea, David Falck, Jeroen Kool, Frans J. de Kanter, Jan N.M. Commandeur, Nico P.E.Vermeulen, Wilfried M.A.Niessen and Maarten Honing

Submitted
Chapter 5  Generation and characterization of modified p38α inhibitors

Abstract
Mutants of bacterial Cytochrome P450 BM3 from Bacillus megaterium have gained increasing interest to support drug metabolism studies by producing human relevant biotransformation products and/or to support drug discovery by producing libraries of new chemical entities based on initial lead compounds. In the present study, a library of 33 P450 BM3 mutants was used to diversify TAK-715, a representative (highly lipophilic) inhibitor of p38α mitogen-activated protein kinase (p38α MAPK or p38α kinase). To simultaneously determine the individual bioaffinity and identity of the different metabolites produced, an analytical high-resolution screening (HRS) approach was used, based on post-column on-line bioaffinity profiling with parallel MS/MS identification. The screening of metabolic mixtures produced by the P450 BM3 mutants demonstrated that introducing mutations in the active site of the P450 BM3 resulted in different profiles of biotransformation products. Several P450 BM3 mutants were mimicking the metabolic profile obtained by human liver microsomes. The major biotransformation products of TAK-715 could be produced in semi-preparative amounts with the most active P450 BM3 mutant, enabling full structure elucidation of metabolites by 1H-NMR and quantification of their p38α kinase affinity.

The present results demonstrate that the combination of catalytically diverse P450 BM3 mutants and the HRS technology for on-line bioaffinity profiling and structure identification of metabolic mixtures might be a valuable platform for the generation of relevant quantities of human relevant drug biotransformation products for pharmacological and toxicological evaluation. Furthermore, linking structural modifications by metabolism to changes in drug target affinity might be efficient tool to construct the pharmacophore model next to the more conventional medicinal chemistry approach of synthesizing structural analogues of lead compounds.
5.1. Introduction

For more than 70% of the top 200 most prescribed drugs hepatic metabolism represents the major mechanism of clearance from the organism (1). The majority of drug metabolism reactions involved are catalyzed by the cytochrome P450 (CYP) enzymes of which CYP1A2, CYP2D6, CYP2C9, CYP2C19 and CYP3A4 in particular are responsible for the bulk of metabolism of known drugs in humans (1). Because it has been established that sometimes drug metabolites can be pharmacologically active towards the same drug target as the parent compound, or towards other targets, leading to undesired off-target pharmacology, it is nowadays required for drug registration to perform safety testing on circulating metabolites if they represent a high proportion of the dose in humans (2). Full pharmacological assessment of biotransformation products usually requires organic synthesis to obtain sufficient amounts of pure compound. As an alternative to organic synthesis, biosynthetic approaches are upcoming methodologies. Biocatalysts (i.e. enzymes) can perform reactions with a high degree of regio- and stereoselectivity that often cannot be achieved by organic synthesis (3, 4). Relatively new in drug discovery is the application of biocatalysts for the generation of new chemical entities or even unique compound libraries (5). When using mono-oxygenase systems, such biotransformation products are particularly interesting in lead optimization as they can show improved physicochemical properties such as solubility and target selectivity without significant loss of their pharmacological activity compared to the parent itself.

Cytochromes P450 (CYPs) are emerging as biocatalysts in biotechnology due to their catalytic versatility and extremely wide substrate diversity (6, 7). In particular, bacterial CYPs are preferred to mammalian CYPs because they are soluble, show much higher turnover rates and can be expressed at high levels in E.coli. A very promising candidate as biocatalyst for the generation of large quantities of drug metabolites is P450 BM3 (CYP102A1) from Bacillus megaterium. This enzyme shows the highest catalytic activity ever recorded for a P450 (8). Over the recent years, many research groups have succeeded in expanding the substrate selectivity of P450 BM3 and improving its catalytic properties by site-directed and/or random mutagenesis, as thoroughly reviewed recently (9). Through rational redesign and directed evolution, P450 BM3 mutants have been obtained that are able to oxidize fatty acids, aromatic hydrocarbons, long-chain and gaseous alkanes, terpenoids, steroids, carboxylic acids and pharmaceuticals.

Although several P450 BM3 mutants have been shown to perform oxygenation reaction with a high degree of regio- and stereoselectivity (10-12), biosynthesis of drug metabolites using engineered P450 BM3s also can lead to the formation of multiple metabolites (5, 13-18). Structural identification and characterization of pharmacological and toxicological properties of individual compounds in mixtures therefore usually depends on elaborate fractionation processes using semi-preparative
separation strategies. Alternatively, characterization of compounds in complex mixtures can be performed using the so-called high-resolution screening (HRS) approach, that combines state-of-the-art analytical separations with post-column online bioaffinity profiling, based on enzyme inhibition or displacement of tracer compounds, and parallel identity assessment with MS. As reviewed recently (19), this approach has been successfully used for the identification of ligands/inhibitors of several drug targets, such as human estrogen receptors α and β, acetylcholinesterase, acetylcholine binding protein, phosphodiesterases, proteases and peptidases, cathepsine B and drug metabolizing enzymes such as cytochromes P450 and glutathione S-transferases.

Recently, a HRS methodology has been developed for the on-line screening of inhibitors of p38α kinase (p38α MAPK or p38α kinase) and simultaneous structure elucidation by high resolution mass spectrometry (HR-MS) (20). p38α kinase is an important drug target in contemporary drug discovery because it plays a central role in inflammatory cellular signaling processes (21). Several companies have reported preliminary human clinical results for p38α kinase inhibitors, but until now no drug has reached the market (22). The HRS methodology for p38α kinase affinity has recently been applied to identify inhibitors in mixtures which were generated by on-line electrochemical conversion of commercially available p38α-inhibitors (23). However, although electrochemistry in some cases has been shown to produce human-relevant oxidation products, it cannot mimic all P450-dependent oxygenation reactions, such as hydroxylation of unactivated sp3-CH-groups, which are synthetically challenging (24).

In the present study, a library of P450 BM3 mutants was used to prepare metabolic mixtures of the p38α kinase inhibitor TAK-715 (N-[4-[2-ethyl-4-(3-methylphenyl)-1,3-thiazol-5-yl]-2-pyridyl]benzamide) which were subsequently characterized using the recently developed HRS platform for p38α kinase inhibitors. The intrinsic high turnover rates of P450 BM3s and the convenient large scale production and purification protocols for these enzymes allow semi-preparative production of human relevant metabolites or new chemical entities for further pharmacological profiling (5). TAK-715 was one of the most potent p38α kinase inhibitor from a series of synthetic 4-phenyl-5-pyridyl-1,3-thiazoles (25), which also induced LPS-stimulated release of TNF-α from human monocytic THP-1 cells (26). TAK-715 was advanced to clinical Phase II trials for rheumatoid arthritis, but was discontinued as it did not satisfy criteria for further development (27). However, recently Verkaar et al. showed that TAK-715 is able to inhibit Wnt-3α-stimulated β-catenin signaling pathway (28) whose aberrations are associated with several malignancies, most notably cancer (29). Therefore, lead diversification of TAK-715 aiming at enhancing its effectiveness, diminish its toxicity, and/or increase its oral absorption is still required for its anti-inflammatory and the chemotherapeutic
indication. In addition, previously reported lead optimization of typical inhibitors was performed using a library encompassing rather lipophilic compounds. Application of these mono-oxygenases is anticipated to improve the physical chemical properties of the lead compounds, while maintaining their bioaffinity.

5.2. Materials and methods

5.2.1. Chemicals
Human recombinant p38α and TAK-715 were a kind gift of MSD Research Laboratories (Oss, the Netherlands). SKF86002 was delivered by Merck KGaA (Darmstadt, Germany). Fused silica tubing (250-μm inner and 375-μm outer diameter) covalently coated with polyethylene glycol (PEG) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Methanol (LC–MS grade) and formic acid (LC–MS grade) were from Biosolve (Valkenswaard, the Netherlands). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Schnelldorf, Germany). Human liver microsomes (HLM) pooled from different individual donors were obtained from BD Gentest TM (San Jose, USA) and contained 20 mg/mL protein (Cat. No. 452161).

5.2.2. Expression and isolation of P450 BM3 mutants
Expression of the BM3 mutants was performed by transforming competent E.Coli BL21 cells with the corresponding pET28+-vectors, as described previously (30). For expression, 600 mL Terrific Broth (TB) medium (24 g/L yeast extract, 12 g/L tryptone, 4 mL/L glycerol) with 30 mg/mL kanamicin 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. Then, protein expression was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The temperature was lowered to 20°C and 0.5 mM of the heme precursor δ-aminolevulinic acid was added. Expression was allowed to proceed overnight. Then, cells were harvested by centrifugation (4600 g, 4°C, 25 min), and the pellet was resuspended in 20 mL KPi-glycerol buffer (100 mM potassium phosphate (KPi) pH=7.4, 10% glycerol, 0.5 mM EDTA, and 0.25 mM dithiothreitol). Cells were disrupted by French press (1000 psi, 3 repeats) and the cytosolic fraction was separated from the membrane fraction by ultracentrifugation of the lysate (120,000 x g, 4°C , 60 min). CYP concentrations were determined using carbon monoxide (CO) difference spectrum assay.

5.2.3. Selection of the P450 BM3 mutant library
In the present study, 33 mutants of P450 BM3 were selected which could be expressed as good levels and which showed catalytic diversity towards a variety of drugs and steroids. Mutants M01 (R47L, F87V, L188Q, E267V, G415S), M02 (R47L, L86I, F87V, L188Q, N319T), M05 (R47L, F81I, F87V, L188Q, E267V, G415S) and M11 (R47L, E64G,
F81I, F87V, E143G, L188Q, Y198C, E267V, H285Y and G415S) were previously constructed by a combination of three site-directed mutations and subsequent random-mutagenesis by error-prone PCR (14). Mutants M01 and M11 where used as templates for additional site-directed mutagenesis of active site residues, guided by available crystal structures of P450 BM3 and by computational modeling of the active site of P450 BM3 (31). Positions which were selected for mutagenesis were residues 72, 74, 82, 87 and 437 which appear to be key active site residues which have profound influence on regio- and stereoselectivity of P450 BM3 mutants. Positions S72 and A74 are both located in the substrate binding channel (32, 33) and have been shown to influence regioselectivity and activity (11, 15). The effect of a negative charged residue (Asp or Glu) in both M01 and M11 in position 72 and 74 is evaluated in this work. Mutation A82W was selected because it strongly influenced regioselectivity of steroid hydroxylation when applied to M01 and M11 (10). Mutations at position 87 have been extensively studied as the amino acid lies very close to the heme, according to available crystal structures of P450 BM3. In our previous studies, we mutated this position in M11 to all possible amino acids, showing that mutation at position 87 has strong influence in the metabolism of alkoxyresorufins, and the regioselectivity of testosterone and clozapine metabolism (30, 34). Mutation of L437 was shown to have strong influence on regio- and stereoselectivity of α-ionone hydroxylation (12) . This position was mutated in M11 to the negative charged residue Glu (L437E) and to three polar residues Asn (L437N), Ser (L437S), and Thr (L437T), which differ in size and hydrogen bonding capabilities.

5.2.4. Metabolism of TAK-715 by P450 BM3 mutants and human liver microsomes. Incubations of TAK-715 were performed in 100 mM KPi buffer pH 7.4 with 250 nM P450 BM3 mutants. The final volume of the incubation was 200 μL, with 100 μM substrate concentration. The reactions were initiated by addition of a NADPH regenerating system with final concentrations of 0.2 mM NADPH, 20 mM glucose-6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase. The reaction was allowed to proceed for 60 min at 25°C and was terminated by the addition of 200 μL of cold methanol. For human liver microsomes (HLM), a final microsomal protein concentration of 5 mg/mL was used and these incubations were performed as described above, at 37°C instead of 25°C. Precipitated protein was removed by centrifugation (15 min, 14000 g), and the supernatant was analyzed by high-resolution UPLC using an Agilent 2000 system using a Zorbax Eclipse XDB-C18 column (1.8 μm, 50 mm x 4.6 mm; Agilent, USA). The gradient used was constructed by mixing the following mobile phases: eluent A (0.8 % methanol, 99 % water, and 0.2 % formic acid); eluent B (99 % methanol, 0.8 % water, and 0.2 % formic acid) with a flow rate of 1
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mL/min. The gradient was programmed as follows: from 0 to 8 minutes linear increase of eluent B from 40% to 100%; from 8 to 9 minutes, isocratic 100% B; from 9 to 9.5 minutes linear decrease from 100% to 40% of eluent B; from 9 to 12 minutes isocratic 40% eluent B. Biotransformation products and substrate were detected at 254 nm.

5.2.5. HRS analysis of metabolites of TAK-715 using the LC-p38α kinase affinity/MS platform

The HRS analysis of the biotransformation products is conducted with an LC–enzyme binding detection/MS platform which has been described previously (20, 23). The platform comprised a LC–MS system from Shimadzu (‘s Hertogenbosch, the Netherlands), including two LC-20AD and two LC-10AD isocratic pumps, an SIL-20AC, a CTO-20AC and a CTO-10AC column oven, an RF-10AXL fluorescence detector, an SPD-AD UV/VIS detector, and a CBM-20A controller coupled to an ion-trap time-of-flight (IT-TOF) hybrid mass spectrometer equipped with an ESI source. In short, the biotransformation product mixtures were separated on a Xbridge C18 column 100×2.1 mm with 3.5 μm particles (Waters, Milford, MA, USA) at 40°C and a flow rate of 113 μL/min. The gradient used was constructed by mixing the following mobile phases: eluent A (1 % methanol, 99 % water, and 0.01 % formic acid); eluent B (99 % methanol, 1 % water, and 0.01 % formic acid). The gradient was programmed as follows: from 0 to 2 minutes isocratic at 20% B; from 2 to 18 minutes, linear increase of eluent B from 20% to 90%; from 18 to 22 minutes, isocratic 90% B; from 22 to 23 minutes linear decrease from 90% to 20% of eluent B; from 23 to 30 minutes re-equilibration isocratic at 20% eluent B. Post-column, the flow was split in a ratio of 1:9 directing 13 μL/min to the bioaffinity detection and 100 μL/min to UV/vis and ESI-HRMS analysis. The bioaffinity detection is based on competition of analytes with a tracer showing fluorescence enhancement. Affinity towards p38α is assessed by mixing the analytes with 45 nM of enzyme and 630 nM of tracer, SKF86002. The readout occurred at excitation 355 nm and emission 425 nm, 15 nm bandwidth each, in the flow-through fluorescence detector. The UV/vis detector was operated in dual wavelength mode at 210 nm and 254 nm.

For ESI the needle voltage was set to 4.5 kV and the source heating block and the curved desolvation line were kept at 200°C. A drying gas pressure of 62 kPa and a nebulizing gas flow-rate of 1.5 L/min assisted the ionization. Full spectra were obtained in the positive-ion mode between m/z 200 and 650. MS² and MS³ spectra were obtained in data-dependent mode between m/z 100 and 650 with an ion accumulation time of 10 ms, a precursor isolation width of 3 Da and a collision energy of 75%. For the analytes in the large scale incubation mixture MS² and MS³ spectra were generated with manual precursor selection and data-dependent analysis, respectively. Structure identification was based on calculating the elemental composition of parent and fragment ions from the accurate mass measurements.
The IC₅₀ determinations were done in flow-injection analysis (FIA) mode, which means the separation column was replaced by a low dead volume union (VICI, Schenkon, Switzerland). The lower dead volume reduces analysis time and minimizes peak broadening compared to a column elution without retention. In these experiments an injection volume of 50 µL was used to achieve adequate final concentrations for full inhibition. Negative peak heights were plotted against the corresponding final concentrations. The latter were calculated as described earlier (20).

5.2.6. Large scale metabolite production and isolation of metabolites by preparative HPLC

The metabolites of TAK-715 were produced on a semi-preparative scale by incubation with the most active P450 BM3 mutant as biocatalyst. A 100 mL reaction volume containing 1 µM P450 BM3, 100 µM TAK-715 and NADPH regenerating system (as described above) was prepared in 100 mM KPi buffer at pH 7.4. The reaction was allowed to continue for 5h at 25°C.

To achieve maximal conversion of TAK-715, the incubation was supplemented every hour with 3 mL of 30 µM P450 BM3 M11 and 5 mL NADPH regenerating system (20 × concentrated). The reaction mixture was extracted three times by 100 mL ethyl acetate. The combined organic layers were collected in a round-bottom flask and evaporated to dryness using a rotary evaporator. The residue was redissolved in 10 mL of 50% MeOH/H₂O and applied by manual injection on a preparative chromatography column Luna 5 µm C18 (250 × 100 mm i.d.) from Phenomenex (Torrance, CA, USA) which was previously equilibrated with 40% eluent B. Flow rate of 2 mL/min and a gradient using the eluent A and B was applied for separation of formed TAK-715 biotransformation products. The gradient was programmed as follows: from 0 to 40 min linear increase of eluent B from 40 to 100%; from 40 to 50 min isocratic 100% B, from 50 to 55 min linear decrease to 40% B, and then re-equilibration was maintained until 65 min. Biotransformation products were detected using UV detection at 254 nm and collected manually. Collected fractions were first analyzed for purity and identity by the analytical UPLC. Fractions containing individual metabolites were evaporated to dryness under nitrogen stream and dissolved in 1 mL deuterium oxide to exchange acidic hydrogen atoms by deuterium atoms. Finally, after drying by a SpeedVac evaporator, the residues were redissolved in 500 µL of methyl alcohol-d₄ and ¹H-NMR spectra were recorded at room temperature. ¹H-NMR-analysis was performed on Bruker Avance 500 (Fallanden, Switzerland) at 500.23 MHz. Afterwards, the samples were dried and redissolved in 30% MeOH and subjected to LC-enzyme binding detection/MS analysis. LogP values were calculated with ChemBioDraw Ultra version 12 from the structures obtained.
5.3. Results

5.3.1. Metabolism of TAK-715 by human liver microsomes and P450 BM3-mutants

As shown in Figure 1 in total 8 metabolites were found after incubation of TAK-715 with HLM, encoded M1 to M8 based on their order of elution.

After 60 minutes of incubation 33 ± 2 % of the substrate was converted by HLM, corresponding to 25.2 nmol product/min/CYP. The main biotransformation product was M7, which represented 29 ± 3% of the total metabolism, respectively. Metabolites M1, M2, M5 and M6 were formed at comparable amounts by HLM, ranging from 14 to 20% of the metabolites, whereas the minor metabolites M3, M4 and M8 were formed at 3 to 5% of total metabolism.

Figure 1. Ultraperformance liquid chromatography (UPLC) chromatograms obtained after incubations of TAK-715 (100 µM) incubated for 60 minutes with 200 nM P450 BM3 M11 (upper line) and 5 mg/mL human liver microsomes (lower line). UV-detection of metabolites was at 254 nm.

TAK-715 was incubated for 60 minutes with pooled human liver microsomes (HLM) and a library of 33 mutants of bacterial P450 BM3, and subsequently analyzed by UPLC, equipped with diode array detector to determine UV absorption maxima of all products formed. The results of these incubations, including the percentage of conversion of substrate and profile of metabolites, are tabulated in Table 1.

As shown in Table 1, the P450 BM3-mutants were able to form almost all human-relevant metabolites although with different activities and with strongly different product ratios. Only metabolites M3 and M4 were not observed in the small scale incubations of P450 BM3-mutants. The most active mutants were M11 and its
mutants at position 74 and 437. Similar to HLM, M11 produced metabolite M7 as major biotransformation product, Figure 1, representing 42 ± 3% of the total metabolism. Metabolite M5 was produced as 22 ± 2% of the total metabolism, whereas metabolites M1, M2, M6 and M8 were formed at 13% or less of the total metabolism. Mutation to a negatively charged amino acid (Asp or Glu) or hydrogen bond donor (Thr) in positions 74 or 437 of M11 has little to no effect on activity but changed the metabolic profile significantly by producing three-fold higher amounts of metabolite M1, making this the major metabolite, Table 1. In contrast, mutant M11 V87I did not produce any metabolite M1, making it more selective to produce M7 (60% of total metabolism).

As shown in Table 1, for 22 out of the 33 P450 BM3-mutants studied, metabolite M5 turned out to be the major metabolite. In particular the mutants showing relatively low activity appeared to be highly selective in the production of this metabolite. The only mutant which showed a significantly different profile was mutant M11 L437N which was the only mutant which produced metabolite M6 as the major metabolite, being 38% of the total of metabolites.

Based on its high activity and its ability to produce most of the metabolites at significant amounts, mutant M11 was selected to further evaluate the affinity of the TAK-715 metabolites towards p38α-kinase in the on-line HRS-affinity assay and to perform a large scale incubation of TAK-715 to obtain sufficient amounts of metabolites for 1H-NMR and affinity assessments with individual metabolites.
Table 1. Percentage of conversion and ratio between the metabolites of TAK-715 incubations with human liver microsomes (HLM) and 33 mutants of P450 BM3<sup>a</sup>

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**a.** All values represent averages of three individual experiments; RSDs were always less than 10%; **b.** [M+H]+-values as determined by exact mass measurements by IT-TOF.
5.3.2. Parallel on-line bioaffinity assay and mass spectrometry of TAK-715 metabolites produced by HLM and P450 BM3 M11

Figure 2 shows the bioaffinity of metabolites of TAK-715 produced by HLM and the extracted ion chromatograms representing the metabolites observed. Retention times differ from those of the UPLC-chromatograms shown in Figure 1 because the HRS/MS system used a conventional reversed phase HPLC system. The order of elution of the metabolites was unchanged.

As shown in Figure 2, next to TAK-715 seven of its metabolites showed affinity toward p38α kinase by showing varying degree of decrease of the fluorescence signal of the tracer SKF86002. TAK-715 eluted at 39.5 minutes showed a strong bioaffinity, as expected and ([M+H]^+ of m/z 400.151. The second strongest decrease in fluorescence was found at 33 minutes, which appeared to closely correspond to the retention times of metabolites M6 (t_{ret} 32.7 min) and M7 (t_{ret} 33.0 min) in this chromatographic systems. The lower resolution of the HRS-system did not allow attributing this bioaffinity signal to any of these metabolites, however. Mass spectrometrical analysis showed that m/z-values of the [M+H]^+ of these metabolites corresponded to 416.146 and 430.123, respectively. M6 therefore corresponds to a monooxygenated metabolite of TAK-715, whereas M7 appears to result from double oxygenation and dehydrogenation. Metabolite M5 (t_{ret} 31.4 min) was a second metabolite with [M+H]^+ of 416.146 and also showed a bioaffinity peak at the same retention time.

Metabolites M1 and M4 which eluted at 23.0 and 27.8 minutes, respectively, both showed bioaffinity peaks and a [M+H]^+ of 432.138, Figure 2, and appear to result from double oxygenation. Metabolite M3 (t_{ret} 24.3 min) showed only a very weak bioaffinity signal and a [M+H]^+ of 432.138. Two of the metabolites formed, M2 (t_{ret} 24.3 min) and M8 (t_{ret} 34.5 min) did not show significant bioaffinity peaks and had [M+H]^+-values of 446.121 and 416.146, respectively.

Next to the eight metabolites previously found by UPLC, two additional metabolites were identified based on small bioaffinity peaks at 8.4 and 17.4 minutes. Metabolite designated M9 (t_{ret} 8.4 min) showed a [M+H]^+ with m/z 312.119 which can be rationalized by hydrolysis of the amide-bond of one of the monooxygenated TAK-715 metabolites. The second biotransformation product M10 showed a [M+H]^+ with m/z 448.138 and apparently results from triple oxygenation of TAK-715. The bioaffinity signal at 4.5 minutes appeared to be unrelated to TAK-715.

Figure 3 shows the p38α bioaffinity traces and extracted ion chromatograms of the metabolic mixture obtained after incubation of TAK-715 with P450 BM3 mutant M11. Three of bioactive peaks were corresponded to M1 (t_{ret} 23.0 min), M5 (t_{ret} 31.4 min) and TAK-715 (t_{ret} 39.5 min). Consistent with the results obtained with metabolites of HLM, a strong bioaffinity peak was observed at the retention times of metabolites M6 and M7.
Figure 2. Correlation between biochemical detection (p38α kinase affinity trace, upper trace) and extracted ion traces from the mass-spectrometric detection (lower trace) of oxidative metabolites of TAK-715 produced in incubations with human liver microsomes (HLM).
Figure 3. Correlation between biochemical detection (p38\(\alpha\) kinase affinity trace, upper trace) and extracted ion traces from the mass-spectrometric detection (lower trace) of oxidative metabolites of TAK-715 produced in incubations with P450 BM3 mutant M11.
5.3.3. Preparative scale biosynthesis of TAK-715 metabolites

To obtain sufficient amounts of TAK-715 metabolites for structural identification and affinity testing of isolated metabolites to p38α kinase, a large volume (100 mL) incubation was performed of TAK-715 with mutant P450 M11 since this appeared to be the most active mutant and was able to produce multiple metabolites of TAK-715. Figure 4 shows the UV-chromatogram (recorded at 254 nm) obtained after separation of the metabolic mixture by preparative HPLC with UV detection at 254 nm. After 5 hours of incubation, with hourly additions of enzyme and cofactors, almost 90% conversion of 100 µM of TAK-715 was obtained.

Figure 4. Preparative HPLC-UV chromatogram of metabolic mixture obtained by large scale incubation of TAK-715 with P450 BM3 mutant M11. UV-Detection of metabolites was at 254 nm.

LC-MS/MS measurements were performed to confirm the identity of isolated metabolites. The main biotransformation product M1 (35.7 min) formed represents 39% of the total metabolism, corresponding to 1.5 mg of product formed. Two monohydroxylated biotransformation products (M5 and M6, 42.6 min and 43.5 min) represent 27% and 12% of the total metabolism, respectively, corresponding to 1 mg and 0.4 mg of product formed. Biotransformation product M7 (43.8 min) represents 15% of the total metabolism, corresponding to 0.6 mg of product formed. Biotransformation product M2 with [M+H]+ with m/z 446.146 (37 min) was formed as less than 5% of the total metabolism. Interestingly, also a small amount of metabolite
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**M4** was found in the large scale incubation. The relative amounts of metabolites obtained by large scale incubation are slightly different than the ratio obtained in the screening experiment because of the longer incubation time (5h vs 1h) and higher enzyme/cofactor concentration. Also the less efficient aeration of large volume incubations might contribute to the qualitative different metabolic profile.

### 5.3.4. Structure elucidation of TAK-715 metabolites

For five of the metabolites of TAK-715 enough material was obtained by preparative HPLC to record a $^1$H-NMR spectrum, which together with the LC-MS/MS data allowed full structure elucidation. By comparison with the spectra and fragmentation patterns of TAK-715, it was possible to assign the structural modifications introduced by drug metabolism for most of the metabolites. Metabolites **M5** and **M6** both showed a $[M+H]^+$ with $m/z$ 416.146, indicative for monoxygenation of TAK-715 at different positions. The integrals and coupling pattern of the $^1$H-NMR spectrum of **M5** showed that both all 12 aromatic hydrogen atoms and the hydrogen atoms of the ethyl moiety were still present. The fact that the singlet of the benzylic methyl-group was not observed anymore, whereas the chemical shifts of the aromatic protons were shifted upfield indicates that **M5** results from hydroxylation of the benzylic methyl-group. In case of metabolite **M6**, the $^1$H-NMR-spectrum indicates that a hydroxylation occurred at the methylene carbon of the ethyl-side chain: the triplet of the terminal methyl-group in TAK-715 was changed to a doublet, whereas the quartet of original methylene-group was shifted upfield and showed half the integral. In case of **M6** also loss of acetaldehyde was observed, consistent with hydroxylation on the methylene-group of the ethyl moiety.

Metabolite **M7**, which was the major metabolite formed by HLM showed a $[M+H]^+$ with $m/z$ 430.123 in LC-MS. The $^1$H-NMR spectrum of **M7** still showed all aromatic hydrogen atoms and an unchanged ethyl side-chain. Similar to metabolite **M5** the signal of the benzylic methyl-group was not observed, whereas the signals of the aromatic ring of the benzylic ring were strongly shifted upfield. This, in combination with the LC-MS/MS data, indicates that the benzylic methyl-group is metabolized to a carboxylate group which can be explained by further oxidation of **M5** by two successive oxidation reactions, Figure 5.

Metabolite **M1**, which according to its $[M+H]^+$ with $m/z$ 432.138 results from two sequential oxygenation reactions, showed a $^1$H-NMR in which both the signals of the benzylic methyl-group and the ethyl-side chain of TAK-715 were strongly affected, suggestion that this metabolite results from hydroxylation of both alkyl side chains. The coupling pattern and integrals of the signals of the ethyl-side chain show that hydroxylation occurred at the methylene-group, consistent with the formation of **M5**.

The $^1$H-NMR spectrum of metabolite **M2**, which has $[M+H]^+$ with $m/z$ 446.138, also showed strong changes in the signals of the benzylic methyl-group and the ethyl-
side chain. The signals of the ethyl-side chain point to hydroxylation at the methylene-position, similar to M1 and M6, whereas the signals of the benzylic group showed the same changes as observed in M7, indicative for formation of a carboxylic acid by sequential oxidations, Figure 5.

For metabolites M3, M4, M8, M9 and M10 no \(^1\)H-NMR data could be obtained since no P450 BM3-mutant was available that can produce these metabolites in sufficient amounts. Structure elucidation for these metabolites was therefore based on the fragmentation patterns of the LC-MS/MS measurements. Metabolite M3 has a [M+H]^+ with \(m/z\) 430.125 and can be rationalized by two sequential hydroxylation reactions, followed by an oxidative dehydrogenation reaction. Inspection of the MS\(^n\) data revealed that M3 showed the same fragmentation as M2 and M7: in all three cases the [M+H]^+ ion showed sequential neutral losses of masses 122.121 (-C\(_7\)H\(_6\)O\(_2\)) and 27.94 (-CO). The first neutral loss might be explained by a combination of a loss of benzaldehyde (C\(_7\)H\(_4\)O) and water, and might point to the presence of an aromatic aldehyde moiety in M3. The loss of acrylonitrile after fragmentation of 280.083 might indicate that the loss of water results from the hydroxylated ethyl-group.

Metabolite M4 has a [M+H]^+ with \(m/z\) 432.141 which can be explained by two oxygenation reactions. Upon secondary fragmentation of this ion both a direct loss of C\(_2\)H\(_4\)O\(_2\) as well as two sequential losses of formaldehyde were observed which might point to hydroxylation of both carbon atoms of the ethyl-side chain.

For metabolites M8 and M10 which result from single and triple oxygenation reactions no fragmentations were found which could assist in structure elucidation.
Figure 5. Proposed structures of TAK-715 biotransformation products by human liver microsomes (HLM) and P450 BM3 mutants.
5.3.5. Quantitative affinity measurements (IC$_{50}$)

Metabolites M1, M2, M5, M6 and M7 were isolated by preparative HPLC. After checking their purity by $^1$H-NMR and analytical HPLC, the concentration of each stock solution was determined by using a standard curve of TAK-715, assuming that the extinction coefficients of each metabolite was equivalent to that of TAK-715. Subsequently, different concentrations of TAK-715 and TAK-715 metabolites were analyzed using the on-line HRS system in flow-injection mode in order to assess the affinity of each compound towards p38$\alpha$-kinase.

Figure 6 shows the concentration-dependence of binding of the individual metabolites and TAK-715 to p38$\alpha$-kinase, after correction for the dilutions intrinsic to the HRS-system used. Calculated IC$_{50}$-values of TAK-715 and its metabolites are presented in Table 2.

![Figure 6. Concentration dependent binding of TAK-715 and purified metabolites M1, M5, M6 and M7 to p38$\alpha$ kinase as determined by flow-injection analysis. Percentage of binding was calculated by the concentration dependent displacement of the tracer SKF86002 from p38$\alpha$ kinase. Error bars represent standard deviations of at least three independent experiments.](image-url)
Table 2. Product identification TAK-715 metabolites

<table>
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<tr>
<th>$t_{ret}^a$ (min)</th>
<th>code</th>
<th>m/z [M+H]$^+$</th>
<th>Structural modification</th>
<th>p38$\alpha$ kinase affinity (nM)</th>
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<td>IC$_{50}$</td>
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<tr>
<td>23</td>
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<td>432.138</td>
<td>+2O</td>
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$a$ $t_{ret} =$ retention time in the HRS analysis (Figures 2 and 3).

n.q., not quantified.

For both TAK-715 and metabolite M1 not a full binding curve could be obtained. In case of TAK-715, peak tailing due to aspecific binding of this highly lipophilic compound to materials of the HRS-system, deformed the Gaussian shape so severely, at concentrations higher than 60 nM, that it was not possible to calculate the local concentration in the assay. Also, the baseline for the following injection could not be recovered in a reasonable amount of time.

In the case of M1, (auto)fluorescence of the metabolite at the assay wavelengths appeared to interfere with the p38$\alpha$ kinase affinity measurement at high concentrations. Due to the different concentration-dependent behavior of both contributions, e.g. increase of M1-(auto)fluorescence and decrease of fluorescence enhancement of the tracer by displacement at increasing M1-concentration, a W-shaped peak was obtained whose area no longer reflects the fraction of binding. Although incomplete binding curves were obtained for TAK-715 and M1, their IC$_{50}^{-}$ values and 95% confidence intervals were estimated by fitting the datapoints by the same fitting procedure as was used for the other compounds, which showed complete binding curves. The IC$_{50}$ obtained for TAK-715, 71 nM is consistent with the value observed previously with this platform (20).

As shown in Figure 6, for three of the metabolites, M1, M5 and M7 the binding curves obtained were shifted to higher concentrations, when compared to that of TAK-715. The largest shift was observed with metabolite M7. This metabolite showed an IC$_{50}^{-}$value of 1500 ± 203 nM which is a 20-fold lower affinity to p38$\alpha$-kinase compared to TAK-715. Metabolites M1 and M5 showed 5 to 7-fold decreased affinity according to their IC$_{50}^{-}$values of 460 ± 165 nM and 310 ± 33 nM, respectively.
Interestingly, metabolite M6 showed high affinity to p38α kinase according to its IC$_{50}$ value of 38 ± 4 nM. However, due to the large standard error of the IC$_{50}$-value of TAK-715, it could not be determined whether the increased affinity of M6 is statistically significant. For metabolite M2 no binding curve was obtained, indicating that this metabolite completely lacked affinity to p38α-kinase.

5.4. Discussion

The aim of this study was to demonstrate the applicability of P450 BM3 mutants to produce significant amounts of metabolites of drugs and to characterize their affinity to p38α kinase. TAK-715 was selected as a model compound for the present study. To determine the affinity of metabolites of TAK-715 to p38α kinase, a recently developed HRS platform was used which was capable of assessing this property for individual compounds in a mixture (23). The approach to use P450 BM3-mutants as biocatalyst for metabolite production in combination with a HRS affinity screening platform to identify active metabolites has been used previously by de Vlieger et al. (35) and Reinen et al. (36) with estrogen receptors as drug targets. De Vlieger et al. applied BM3 mutants for the generation of biotransformation products of the estrogenic compound norethisterone. The metabolic mixture obtained was analyzed by hyphenated screening assay towards the human estrogen receptor ligand binding domain (hER)α and (hER)β integrating target ligand interaction and LC-MS detection. (35). Reinen et al. applied P450 BM3 mutants to generate in vitro human relevant biotransformation products of the mycotoxin zearalenone (36). The hydroxylated metabolites of zearalenone formed did not display an increased ERα bioaffinity.

The results of the present study show that the selected mutants of bacterial P450 BM3 were able to produce significant amounts of metabolites of the p38α-kinase inhibitor TAK-715 allowing both structural characterization by LC-MS/MS and $^1$H-NMR and characterization of affinity of metabolites towards p38α-kinase. By comparison with metabolites formed by HLM it was found that the P450 BM3 only produced human relevant metabolites of TAK-715. Figure 5 shows the metabolic scheme which rationalizes the formation of the metabolites which were identified in the present study. Metabolism of TAK-715 appeared to occur almost exclusively at its alkyl side chains. Metabolites M5 and M7 result from sequential oxygenation reactions on the benzylic methyl-group of TAK-715. The intermediate aldehyde which was expected could not be detected, suggesting that this aldehyde undergoes extensive further oxygenation to M7 and to lesser extent to M3. Although conversion of the alcohol-groups to carboxylates is often catalyzed by sequential alcohol dehydrogenases, aldehyde dehydrogenase and oxidase reactions, conversion of M5 to M7 appears to be fully catalyzed by P450 BM3, since this reaction was also observed when catalyzed by purified P450 BM3 M11 [data not shown]. So far only few examples have been
described on the multistep oxidation of methyl- to carboxylic acid groups by P450 enzymes. Oxidation of a methyl of the t-butyl moiety of terfenadine and ebastine to their corresponding carboxylic acids fexofenadine and carebastine in HLM was previously shown to be catalyzed by CYP3A4 and CYP2J2 (37). More recently as yet unidentified microbial P450s from Streptomyces platensis were also shown to be capable to produce fenofexadine from terfenadine (38). Similar to our P450 BM3 incubations no intermediate aldehyde was found, indicating that the conversion of aldehydes to acids by P450s is highly efficient. The availability of biocatalysts for conversion of methyl groups to carboxylic acids is considered of high value because chemical synthesis is laborious, and because incorporation of carboxylic acids in drug molecules might be beneficial for water solubility, might decrease the risk of hERG inhibition (38) and might restrict passage of blood-brain-barrier of drugs.

When comparing the metabolic profiles of the different P450 BM3 mutants, Table 1, significant differences were observed between the mutants. Considering the fact that M5 and M7 both only involve modification of the benzylic methyl-group, whereas M6 and M4 only consider modification of the ethyl-side chain, it can be concluded that oxygenation of the benzylic methyl-group is by far the major pathway for all mutants studied. The sum of M5 and M7 represents from 50 to 100% of the metabolites, dependent on the mutant. Because metabolites M1, M2 and M3 might be secondary oxidation products of M5 and M7, the total contribution of this pathway might even be underestimated. Because the metabolic profiles appears to result from extensive secondary oxidation of the primary metabolites, and the fact that several metabolites can be formed by different pathways, it was not attempted to rationalize the effects of the mutations on the metabolic profile.

Changes in profile might result from both changes in substrate orientation and/or changes in enzyme activity for each metabolic step. For example, the most selective mutants which almost specifically produce primary metabolite M5, showed only very low enzyme activity, explaining why no secondary metabolism was observed. Only for mutants M11 V87I and M11 V87F the change in metabolic profile might be attributed to changes in substrate orientation, because this is the only mutant which did not show any oxygenation of the ethyl-side chain: the benzylic methyl-oxygenation appeared to represent 90% of metabolism. Both mutants produced a small amount of metabolite M8 which, based on its mass, might result from N-oxygenation or aromatic hydroxylation. Because this minor metabolite did not show affinity to p38α-kinase no attempts were undertaken to identify the structure. Mutant M11 L437N showed the highest amount of metabolite M6, and relative low levels of M5 an M7, which suggests that mutation L437N causes a shift from benzylic methyl-hydroxylation to hydroxylation of the ethyl-side chain.

By using the recently developed hyphenated HRS-system for parallel bioaffinity testing and LC-MS analysis of complex mixtures (20, 23), we were able to quickly determine to
presence of ligands for p38α-kinase in metabolic mixtures of TAK-715, Figures 2 and 3. By isolating the corresponding compounds by preparative HPLC and subsequent analysis by the HRS in flow-injection mode (e.g. in absence of analytical column) the IC₅₀-values of several metabolites could be determined, Table 3. The results of these studies indicate that oxygenation of the benzylic methyl-group appears to be detrimental for affinity to p38α-kinase. The fact that M7 shows a very low affinity, whereas M2 shows no affinity at all, indicate that the carboxylic acid most strongly disturbs binding to p38α-kinase. These results are consistent with these results of Miwatashi et al. which showed that modifying the benzylic methyl-group of TAK-715 by other substituents, or by positioning the methyl-group to the ortho- or para-position, in all cases led to a strong loss in affinity towards p38α-kinase (25). Apparently, the benzylic methyl-group at the meta-position of TAK-715 is essential for high affinity. These is further confirmed by the high affinity of M6 in which only the ethyl-side chain of TAK-715 is hydroxylated. Again this seems consistent with the study of Miwatashi et al. (26) which showed that modification of the thiazole-2-ethyl moiety of TAK-715 to other alkyl-substituents (methyl, propyl) or a large substituent such as 4-methylsulfonylphenyl only showed little effect on p38α-kinase.

In conclusion, the results of the present study shows that the combination of a catalytically diverse set of P450 BM3 mutants as a toolbox to diversify drugs and a HRS-system capable to rapidly screen for affinity to p38α-kinase might be a promising platform to generate potential novel lead compounds which might have improved physico-chemical properties (by improved water solubility) and desired pharmacological properties.

As exemplified by TAK-715 as model compound, the P450 BM3 mutants were able to produce almost all metabolites produced by human liver microsomes at significant levels. Furthermore, profiling the effect of structural modifications introduced by P450 on p38α-kinase affinity gives valuable information on which structural elements are critical and which are non-critical for affinity. This might be a very potent addition to construction of pharmacophore models, next to the more conventional medicinal chemistry approach of synthesizing large series of structural analogues of drug candidates.

Finally, our results should confirm that biocatalysis with CYPs can provide compounds with improved pharmacological and/or physicochemical properties compared to the lead itself which are not easily accessible by classic organic synthesis. This approach can be considered as an extra tool, next to the chemical synthesis in the typical medicinal chemistry approach.
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References


Chapter 5

Generation and characterization of modified p38α inhibitors