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Combination of biotransformation by P450 BM3 mutants with on-line post-column bioaffinity and mass spectrometric profiling as a novel strategy to diversify and characterize p38α kinase inhibitors

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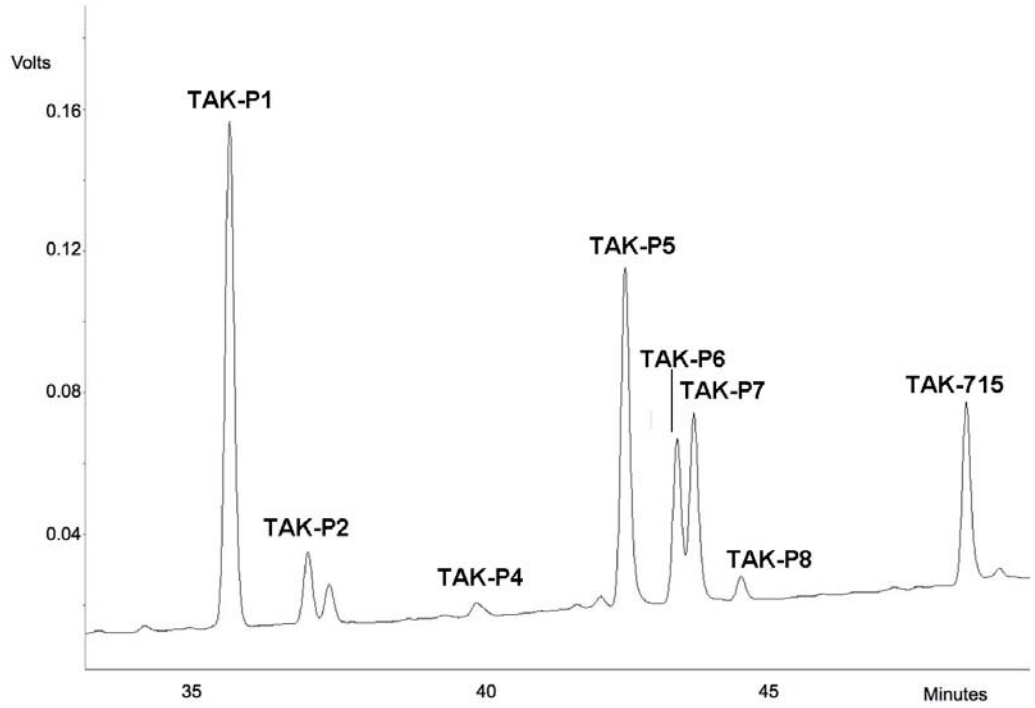


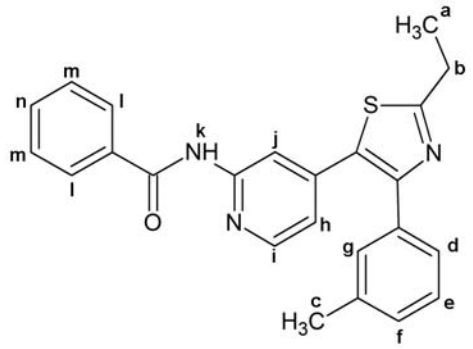
Fig. S1:
Preparative HPLC-UV (254 nm) chromatogram of mixture of biotransformation products obtained by large-scale incubation of TAK-715 with P450 BM3 mutant M11.

Table S1: Percentage of conversion and ratio between the biotransformation products of TAK-715 incubations with human liver microsomes (HLM) and 33 mutants of P450 BM3a.

Enzyme	% Conv	**TAK-P1**	**TAK-P2**	**TAK-P3**	**TAK-P4**	**TAK-P5**	**TAK-P6**	**TAK-P7**	**TAK-P8**
m/z of $[M+H]^+$) [b]	)[a]	432.138	446.121	430.123	432.138	416.146	416.146	430.123	416.146
HLM	33	19	14	5	3	17	10	28	4
M11	38	11	6	0	0	22	3	42	13
M11 L437E	36	35	9	0	0	23	15	16	2
M11 A74E	36	35	8	0	0	18	13	24	2
M11 A74D	33	34	7	0	0	20	14	24	3
M11 L437S	31	20	7	0	0	25	15	27	6
M11 L437T	30	33	5	0	0	23	15	20	3
M11 V87I	25	0	0	0	0	32	0	60	8
M01 L437E	20	18	0	0	0	42	26	9	5
M11 V87F L437S	20	21	0	0	0	44	20	9	6
M11 A82W	19	3	2	0	0	23	4	57	11
M05	19	6	0	0	0	34	6	40	14
M01 S72D	18	11	2	0	0	38	8	28	13
M11 V87F L437N	18	23	0	0	0	31	23	13	10
M01 A82W	18	5	0	0	0	38	5	48	4
M01 S72E	17	14	0	0	0	48	26	7	5
M01 A74D	17	13	0	0	0	48	26	7	6
M11 V87I L437N	16	4	0	0	0	47	6	28	15
M11 A82W V87F	15	0	0	0	0	78	0	0	14
M11 V87F	15	0	0	0	0	82	5	6	7
M11 L437N	15	9	0	0	0	29	38	18	6
M11 S72D	12	12	6	0	0	52	17	6	7
M11 V87A	10	10	0	0	0	53	8	25	4
M11 V87I L437S	9	0	0	0	0	70	1	6	23
M01	7	0	0	0	0	60	14	6	20
M11 V87Y	7	0	0	0	0	76	6	6	12
M02	7	0	0	0	0	75	10	6	9
M11 V87L	6	0	0	0	0	79	5	3	13
M11 A82Y L437S	4	0	0	0	0	100	0	0	0
M11 A82Y V87I	4	0	0	0	0	85	15	0	0
M11 A82Y V87F	4	0	0	0	0	100	0	0	0
M11 V87Q	3	0	0	0	0	85	15	0	0
M01 A82C	3	0	0	0	0	100	0	0	0
M11 A82W L437S	2	0	0	0	0	100	0	0	0

a. All values represent averages of three individual experiments; RSDs were always less than 10%;
b. $[M+H]^+$-values as determined by accurate-mass measurements by HR-MS.

Table S2: ^{1}H-NMR-spectra of TAK-715 and major biotransformation products formed by P450 BM3 M11.



	TAK-715	**TAK-P 1**	**TAK-P 2**	**TAK-P 5**	**TAK-P 6**	**TAK-P 7**
H^{a}	1.45-1.48 (3H,t)	1.61-1.63 (3H,d)	1.64-1.65 (3H,d)	1.45-1.48 (3H,t)	1.62-1.64 (3H,d)	1.44-1.49 (3H,t)
H^{b}	3.09-3.13 (2H,q)	5.08-5.12 (1H,q)	5.08-5.12 (1H,q)	3.09-3.14 (2H,q)	5.08-5.12 (1H,q)	3.08-3.14 (2H,q)
H^{c}	2.33 (3H s)	~ 4.9* (s)	-	~ 4.9* (s)	2.32 (3H,s)	-
H^{d}	7.19-7.26 (3H,m)	7.19-7.26 (3H,m)	7.64-7.66 (1H,d)	7.35-7.39 (3H,m)	7.18-7.25 (3H,m)	7.58-7.62 (1H,d)
H^{e}	"	"	7.43-7.46 (1H,t)	"	"	7.40-7.43 (1H,t)
H^{f}	"	"	7.99-8.01 (1H,d)	"	"	7.98-8.00 (1H,d)
H^{g}	7.34 (1H,s)	7.51-7.54 (3H,m)	8.18 (1H,s)	7.51-7.54 (3H,m)	7.34 (1H,s)	8.14 (1H,s)
H^{h}	6.95-6.96 (1H,d of d)	7.00-7.01 (1H,d of d)	7.00 (1H,s)	6.98-6.99 (1H,d of d)	6.97-6.98 (1H,d of d)	6.95-6.96 (1H,d of d)
H^{i}	8.21-8.22 (1H,d)	8.23-8.24 (1H,d)	8.25-8.26 (1H,d)	8.22-8.23 (1H,d)	8.22-8.23 (1H,d)	8.21-8.22 (1H,d)
H^{j}	8.31 (1H,d)	8.30 (1H,d)	8.33 (1H,d)	8.29 (1H,d)	8.31 (1H,d)	8.31 (1H,d)
H^{k}	-	-	-	-	-	-
H^{l}	7.94-7.96 (2H,d)	7.94-7.95 (2H,d)	7.94-7.95 (2H,d)	7.94-7.95 (2H,d)	7.94-7.96 (2H,d)	7.94-7.95 (2H,d)
H^{m}	7.51-7.54 (2H,d of d)	7.51-7.55 (3H,m)	7.51-7.54 (2H,d of d)	7.51-7.54 (3H,m)	7.51-7.54 (2H,d of d)	7.51-7.54 (2H,d of d)
H^{n}	7.59-7.62 (1H,t)	7.59-7.62 (1H,t)	7.58-7.60 (1H,t)	7.59-7.62 (1H,t)	7.59-7.62 (1H,t)	7.58-7.62 (1H,t)

* Chemical shift and integral of benzylic protons could not be determined accurately due to interference with solvent signal.

Materials and methods

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).

Expression and isolation of P450 BM3 mutants

Expression of the CYP 102A1 mutants was performed by transforming competent *E.Coli* BL21 cells with the corresponding pET28+-vectors, as described previously[1]. 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. Afterwards, 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 × g, 4°C, 60 min). CYP concentrations were determined using a carbon monoxide (CO) difference spectrum assay.

Selection of the P450 BM3 mutant library

In the present study, 33 mutants of P450 BM3 were selected which could be expressed at 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, E267V, G415S) were previously constructed by a combination of three site-directed mutations and subsequent random-mutagenesis by error-prone PCR[2]. 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[3]. 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.

Position S72 and A74 are both located in the substrate binding channel[4,5] and have been shown to influence regioselectivity and activity[6,7]. The effect of a negatively 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[8].

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[1,9].

Mutation of L437 was shown to have strong influence of regio- and stereoselectivity of α-ionone hydroxylation[10]. This position was mutated in M11 to the negatively charged residue Glu

(L437E) and to three polar residues Asn (L437N), Ser (L437S), and Thr (L437T), which differ in size and hydrogen bonding capabilities.

Biotransformation 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 UPLC using an Agilent Technologies 2000 system and a Zorbax Eclipse XDB-C18 column (1.8 µm, 50 mm × 4.6 mm; Agilent Technologies, 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 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. The products and substrate were detected at 254 nm.

HRS analysis of biotransformation products 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[11]. 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 hybrid mass spectrometer for HR-MS equipped with an electrospray ionization (ESI) source. In short, the biotransformation product mixtures were separated on an 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 C (1 % methanol, 99 % water, and 0.01 % formic acid); eluent D (99 % methanol, 1 % water, and 0.01 % formic acid). The gradient was programmed as follows: from 0 to 2 minutes isocratic at 20% D; from 2 to 18 minutes, linear increase of eluent D from 20% to 90%; from 18 to 22 minutes, isocratic 90% D; from 22 to 23 minutes linear decrease from 90% to 20% of eluent D; from 23 to 30 minutes re-equilibration isocratic at 20% eluent D. 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-HR-MS 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. MS^2 and MS^3 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, MS^2 and MS^3 spectra were generated with manual precursor selection and data-dependent analysis, respectively. The IC_{50} 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[11].

Large-scale production and isolation of biotransformation products by preparative HPLC

The biotransformation products 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 5 h 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/H_2O and applied by manual injection on a preparative chromatography column Luna 5 µm C18 (250 × 100 mm i.d.) (Phenomenex, Torrance, CA, USA), which was previously equilibrated with 40% eluent B. A 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 methanol-d_4 and ^{1}H-NMR spectra were recorded at room temperature. ^{1}H-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.

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