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Supplementary Information

A highly selective probe for human cytochrome P450 3A4: isoform selectivity, kinetic characterization and its applications†

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Materials and Methods

Chemicals and reagents. Bufalin (BF), 5β-hydroxy-bufalin (5-HB), 3-ketobufalin (3-KB), telocinobufagin, bufotalin, and cinobufotalin were purchased from Shanghai Boyle Chemical Company (Shanghai, China). Other naturally bufadienolides were isolated from Chansu by the authors (J. Ning & H-R. Chen) and unambiguously identified by NMR and MS techniques. The commercially unavailable hydroxylated derivatives of bufodienolides were obtained by biotransformation using microorganisms (*Mucor polymorphosporus* AS 3.3443)，and fully characterized by NMR and MS techniques. The purity of each bufadienolide is above 98% determined by HPLC-UV. 1-Aminobenzotriazole (ABT), sulfaphenazole, quinidine, clomethiazole, furafylline, 8-methoxypsoralen, omeprazole, CYP3cide, glucose-6-phosphate dehydrogenase, $NADP^+$, D-glucose-6-phosphate and trypsin (TPCK-treated, from bovine pancreas) were purchased from Sigma (St. Louis, MO, USA). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). N-octyl glucoside, tris-(2-carboxyethyl)-phosphine and methyl methane thiosulfonates were from Applied Biosystems Sciex (Ontario, Canada). Montelukast was from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylenethiophosphoramide (TEPA) was purchased from Acros Organics (Geel, Belgium). All other reagents, fine chemicals and LC solvents with the highest grade commercially available were obtained from J&K Chemical Ltd. (China) and Tedia (USA).

Enzyme sources. cDNA-expressed recombinant human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7 derived from baculovirus-infected insect cells co-expressing NADPH-CYP reductase and

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cytochrome b5 were obtained from BD Biosciences (MA, USA). Human liver microsome (HLM) possessing *CYP3A5*1/*1* (HH47) and pooled human liver microsomes prepared from male Caucasians (25 donors) were also obtained from BD Biosciences (MA, USA). Pooled human liver microsomes prepared from male Mongulia (14 donors), and a panel of twelve HLMs from individuals (male Mongulia) were obtained from Research Institute for Liver Diseases (Shanghai) Co. LTD (China). All microsomal samples and recombinant human CYP isoforms were stored at -80ºC until use.

Analytical instruments and conditions. The UFLC system equipped with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, a CTO-20AC column oven and an SPD-M 20A diode array detector (DAD). A Shim-pack XR-ODS (75 mm × 2.0 mm, 2.2 μm, Shimadzu) analytical column with an ODS guard column (5 mm × 2.0 mm, 2.2 μm, Shimadzu) was used to separate CB and its metabolites. The mobile phase consisted of CH_3CN (A) and water containing 0.2% (v/v) formic acid (B), with the following gradient profile: 0–2 min, 90%–58% B; 2-8 min, 58%–38% B; 8–10 min, 5% B; 10-13 min, balanced to 90% B. The flow rate was 0.4 ml/min and the column temperature was kept at 40 ºC. BF and its metabolites (5-HBF & 3-KBF) were detected at 300 nm and quantified according to the calibration curves of authentic standards.

MS/MS detection was performed on an AB SCIEX 4000 QTRAP® equipped with Turbo V™ source. ESI technique was used for identification and quantification of BF and its metabolites. Mass detection was performed in positive-ion mode (ESI^+) from *m/z* 100 to 800. Selective and sensitive MRM mode was used for detection quantification of BF and 5-HBF.

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The transitions (precursor to product) used for BF and 5-HBF were *m/z* 387.8→255.3, and 403.3→349.3, respectively. The instrument was tuned and optimized for each analyte. The ionspray needle was maintained at 5.5 kV, and the turbo gas temperature was 500 °C. The declustering potential (DP), entrance potential (EP), collision energy (CE), and the collision cell exit potential (CXP) for BF were 100, 10, 33, and 7 V, respectively, and for 5-HBF were 105, 10, 32, and 9 V, respectively. The dwell time was 100 ms for each transition. Both quadruples were maintained at unit resolution. Data processing was performed using the software version Analyst 1.6.1.

Incubation conditions. The incubation mixture, with a total volume of 200 µl, consisted of 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM NADP+, 10 mM glucose-6-phosphate, 1 unit/ml of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$), and human liver microsomes or CYPs. In all experiments, BF (15 mM dissolved in acetonitrile previously) was serially diluted to the required concentrations and the final concentration of acetonitrile did not exceed 1% (v/v) in the mixture. After preincubation at 37ºC for 3 min, the reaction was initiated by adding NADPH-generating system and further incubated at 37ºC in a shaking water bath. The reaction was terminated by the addition of ice-cold acetonitrile (200 µl). The mixture was kept on ice until it was centrifuged at 20,000 × g for 10 min at 4ºC. Aliquots of supernatants were stored at -20ºC until analysis. Control incubations without NADPH-generating system or without substrate or without CYP enzyme sources were carried out to ensure that metabolites formation was CYP- and NADPH-dependent. All incubations throughout the study were carried out in three experiments performed in duplicate with S.D. values generally below 10%.

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Assignment of P450 isoform(s) involved in BF metabolism. Eleven cDNA-expressed human CYP isoforms co-expressing NADPH-P450 reductase (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were used to screen the involved isoform(s) for hydroxylation of CB in HLM. The incubations were carried out under the above mentioned incubation conditions with each of CYP isoform. To generate adequate metabolites for detection, a relative high substrate concentration (100 μM) was used and incubated with each of the recombinant CYPs (40-80 nM) at 37ºC for 30 min. The reaction mixtures were centrifuged to precipitate the protein as described previously. The supernatants were then analyzed by UFLC-DAD to quantify the metabolites of BF.

Chemical inhibition assays on BF 5β-hydroxylation. 5β-hydroxylation of BF in pooled HLM in the absence or presences of selective inhibitors for different CYP isoforms were measured to verify the involved enzyme(s) for this biotransformation. In brief, BF (10 μM, relevant to the K_m values) was incubated in HLM (0.125 mg protein/ml) with an NADPH-generating system in the absence (control) or presence of known CYP isoform-specific inhibitors/substrates. The selective inhibitors and their concentrations were as follows: montelukast (2 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, omeprazole (20 μM) for CYP2C19, quinidine (10 μM) for CYP2D6, clomethiazole (50 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A, CYP3cide (1 μM) for CYP3A4. Inhibition by furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, TEPA (50 μM) for CYP2B6 (Rae et al. 2002), ABT (500 μM) for broad CYPs and CYP3cide (1 μM) for CYP3A4 were assayed by pre-incubation with NADPH-generating system at 37ºC for 20-30 min.[1]

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Correlation studies. The formation rates of the metabolites described for bufalin (10 μM, near K_m value) were determined in a panel of HLMs prepared from 12 individual donors. The incubation conditions were described as above mentioned. These values were compared with the levels of CYP3A4 or CYP3A5 in 12 individual HLMs. The concentrations of CYP3A4 and CYP3A5 in HLMs were determined by liquid chromatography-tandem mass spectrometer (LC-MS/MS), using multiple reaction monitoring (MRM) mode and isotope labeled peptide as the internal standards. Specific peptides of EVTNFLR (for CYP3A4) and SLGPVGFMK (for CYP3A5) were selected for their quantification by using transition ion of 439.7/549.3, and 468.3/678.5, respectively. The correlation parameter was expressed by the linear regression coefficient (r). $P<0.05$ was considered statistically significant.

Enzyme kinetics of BF 5β-hydroxylation. To estimate the kinetic parameters of BF 5β-hydroxylation in different enzyme sources, the incubation conditions were optimized to ensure the formation rates of 5-HBF was in the linear range in relation to incubation time and protein concentration at 37 °C. BF (dissolved in ACN previously) was serially diluted to the required concentrations (1, 2.5, 5, 10, 25, 50, 100, 150 μM), and the final concentration of ACN was 1% (v/v). BF was incubated with pooled human liver microsomes (HLM, 0.05 mg protein/ml) for 30 min, or with recombinant CYP3A4 (5 nM) for 20 min, or with recombinant CYP3A5 (50 nM) for 60 min. All incubations were carried out in three independent experiments in duplicate. The apparent K_m and V_{max} values were calculated from nonlinear regression analysis of experimental data according to the following Michaelis-Menten equation, and the results were graphically represented by Eadie-Hofstee plots.

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$v = \frac{V_{max} \times [S]}{K_m + [S]}$, where v is the rate of the reaction, [S] is the substrate concentration, V_{max} is the maximum velocity estimate, and K_m is the Michaelis-Menten constant. Kinetic constants were estimated using Origin 7.5 and reported as the value ± standard error (S.E.) of the parameters estimate.

***In vitro* biosynthesis and isolation of predominant metabolite**. The predominant metabolite 5β-hydroxy-bufalin was biosynthesized by using the mixture of mouse liver microsomes (MLM, 90%) and HLM (10%). The incubation system was scaled up to 250 mL. BF (150 μM) was incubated with the liver microsomes (final protein concentration, 1.0 mg/mL) and the NADPH-generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/mL of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$) for 4 h at 37 °C. Under these conditions, about 30% of BF was converted to 5β-hydroxy-bufalin. Methanol (250 mL) was added to the reaction mixture to precipitate the protein. After centrifuged at 20, 000× g for 15 min at 4°C, the supernatant was separated and extracted with ethyl acetate (250 mL×3). The organic layer was combined and dried in vacuo, and the residue was re-dissolved in methanol (1.5 mL) and the solution was injected into the LC column. The HPLC system (SHIMADZU, Kyoto, Japan) consisted of a SCL–10A system controller, two LC–10AT pumps, a SIL–10A auto injector, a SPD–10AVP UV detector and a C18 column (4.6 mm×150 mm, 10 μ) was used to separate BF and its metabolite. The mobile phase was 65% methanol in water. The eluent was monitored at 300 nm with a flow rate of 2.0 mL/min, and the fractions containing 5β-hydroxy-bufalin were collected and dried *in vacuo*. The purity of 5β-hydroxy-bufalin was about 98% by HPLC-UV analysis.

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Docking simulation of bufalin into the reported structure of CYP3A4 and a homology model of CYP3A5. Docking simulation was performed by using the knowledge-based homology modeling package of Advanced Protein Modeling (APM) distributed within SYBYL (X-1.1). The homology model of CYP3A5 was constructed based on the template structure of CYP3A4 (PDB ID:3TJS). The sequence of both 3A4 (UnitProt ID: 08684) and 3A5 (UnitProt ID: 20815) were obtained from PubMed protein sequence database, exhibiting a similarity of 84%. After the backbone and variable modeling, a 1000-step minimization was carried out to obtain a low-energy conformation without any steric clashes between side chains. The model of CYP3A5 was analyzed and evaluated using the ProTable module which showed a high Ramachandran graphs score of 98.2%. With the established 3D-structure of 3A5 as well as the crystal structure of 3A4, the bioactive binding conformations of bufalin were generated using Surflex-Dock, which were evaluated by an empirical function ChemScore, one of the most suitable scoring functions for P450s super-family.[2]

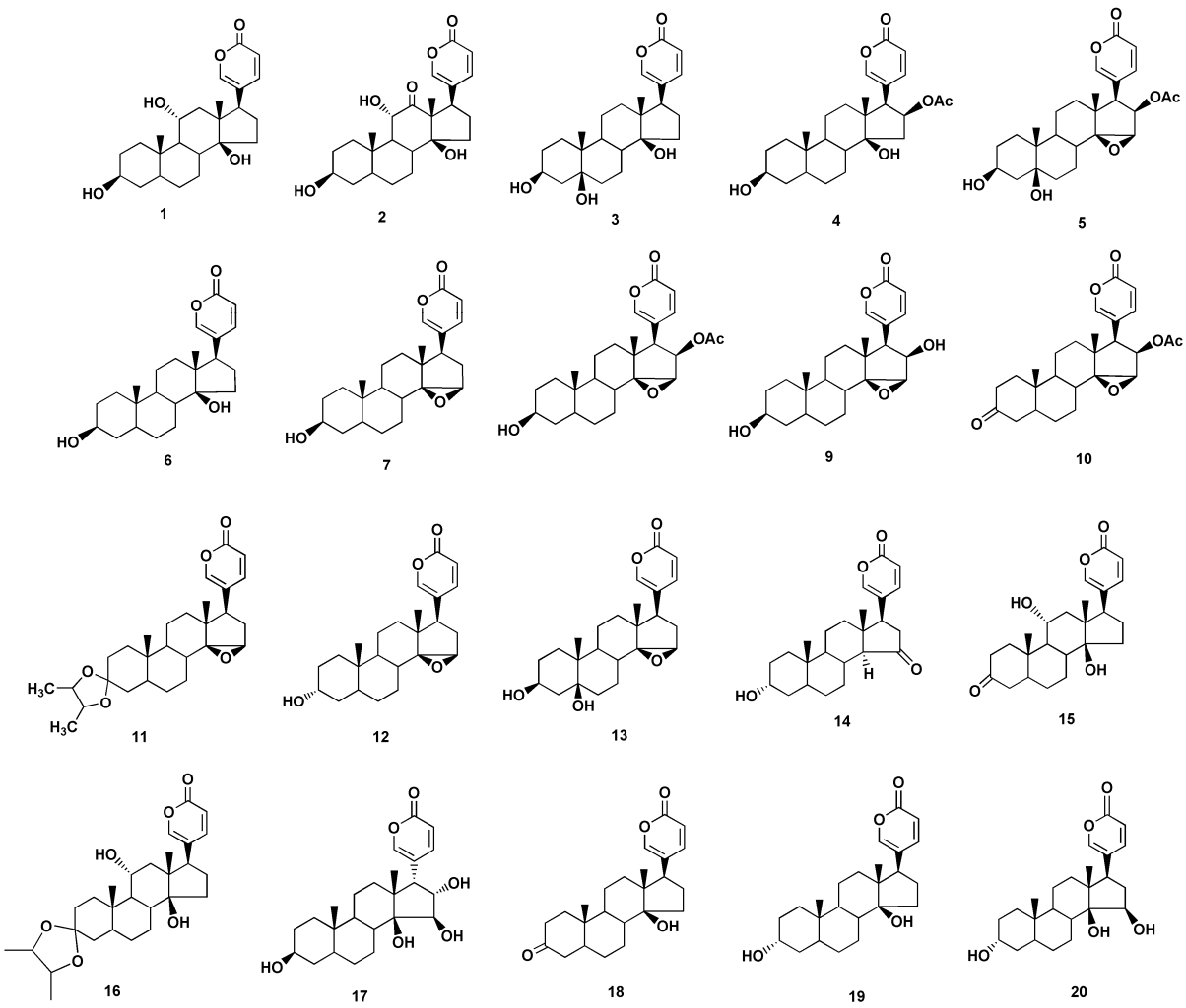

Fig. S1 Chemical structures of bufodienolides used in this study

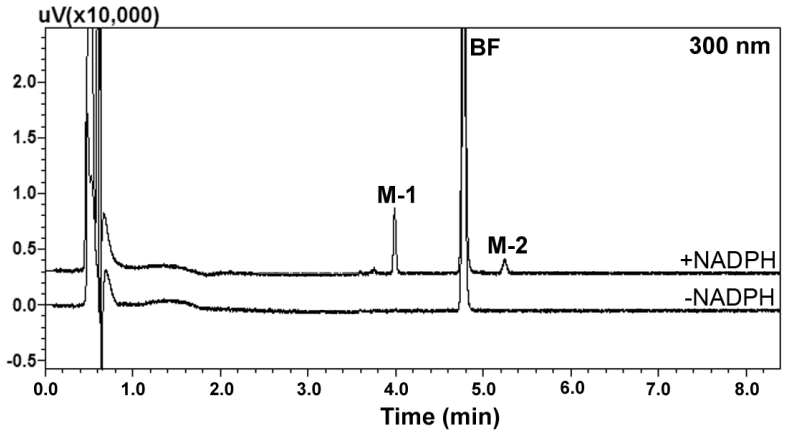

Fig. S2 The metabolic profile of BF in human liver microsomes (HLM). The formation of these two metabolites was time-, NADPH-, and microsome-dependent.

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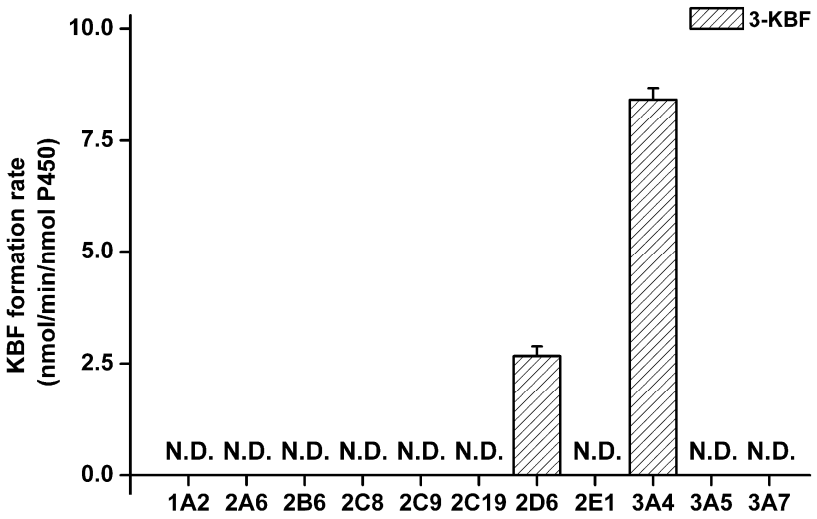

Fig. S3 Assignment of isozymes involved in the formation of 3-KBF

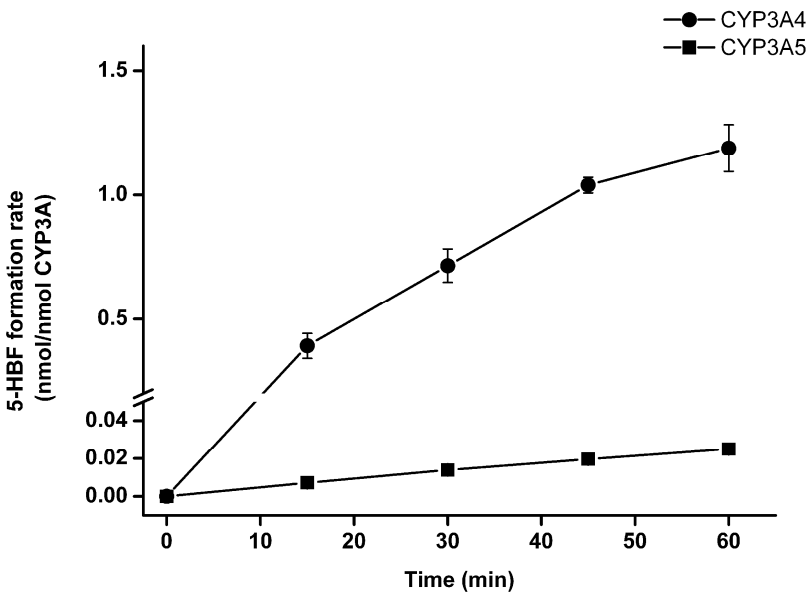

Fig. S4 Time courses of BF 5β-hydroxylation by CYP3A4 (5 nM) or CYP3A5 (50 nM). The final substrate concentration was 100 uM. Each data point represents the mean of triplicate determinations and is shown with S.D. bars.

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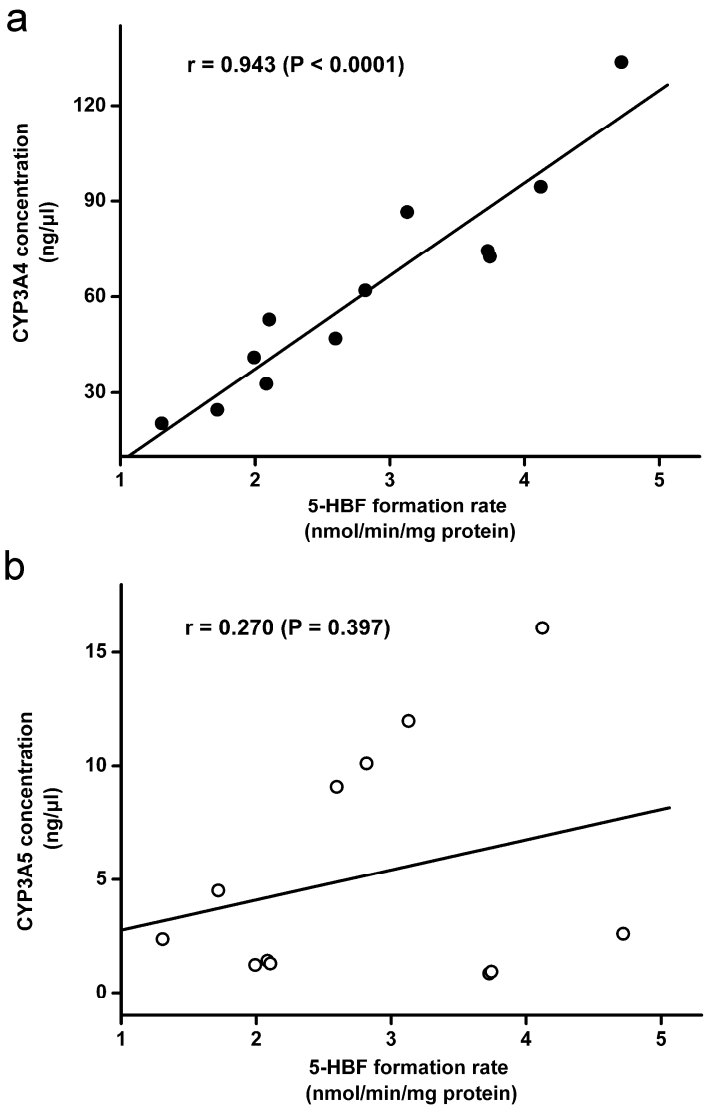

Fig. S5 Correlation studies between the formation rate of 5-HBF and the level of CYP3A4 (A), and the level of CYP3A5 (B), in a panel of twelve HLMs from individuals.

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Table S1 Retention times (t_R), UV λmax values, molecular weights (M.W.) and MS data for BF and its metabolites in HLM

Compound	t_R (min)	λ_{max} (nm)	M.W.	Identification	Product ions (ESI^+, *m/z*)
M-1	3.99	300	402	5β-hydroxy-bufalin	403.4, 385.3, 367.3, 349.3, 253.4, 161.2
Substrate	4.78	302	386	bufalin	387.3, 369.3, 351.3, 255.3, 161.2
M-2	5.25	310	384	3-keto-bufalin	385.3, 367.3, 349.2, 253.4, 161.2

Table S2 BF 5β-hydroxylation related parameters derived from the molecular modelling of BF with the crystal complex of CYP3A4, and a homology model of CYP3A5, respectively.

Parameters	CYP3A4	CYP3A5
Hammerhead score	**-35.46**	**-29.21**
Site-heme distance	**4.05 Å**	**4.58 Å**

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