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

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Bufalin 5β-hydroxylation was found to be an isoform-specific biotransformation probe substrate for cytochrome P450 3A4 (CYP3A4). The probe reaction was well-characterized and it can be used for measuring the real catalytic activities of CYP3A4 from different enzyme sources.

Cytochromes P450 (CYPs) are key enzymes responsible for the oxidative metabolism of drugs, environmental chemicals, and endobiotics.[1] The CYP3A subfamily is the most abundant group of CYP enzymes in the liver, and takes part in the metabolism of a large variety of drugs.[2] In humans, the CYP3A subfamily consists of at least 3 isoforms: CYP3A4, 3A5 and 3A7.[3] In the majority of adult human livers, CYP3A4 is the major contributor to CYP3A-mediated drug metabolism,[4] but other minor isoforms (especially CYP3A5) also contribute to a different extent.[5]

CYP3A4 and CYP3A5 share high amino acid sequence homology (>84%) and their substrates often overlap,[6] but the catalytic activities and the relative importance of these two isoenzymes in overall CYP3A-mediated metabolism differ among substrates.[7] In human liver, CYP3A4 was usually recognized as the major CYP3A isoform, but CYP3A5 is expressed in adults polymorphically. The relative amount of CYP3A5 to total hepatic CYP3A protein varies significantly among individuals, which is estimated to range from 0% to 50%, or even more than 50%.[8] The levels of CYP3As and their functions varied in different individuals, due to the high degree of genetic polymorphism of CYP3As and the different response to environmental, hormonal, or nutritional influences.[6] These factors have greatly limited the understanding of the precise role of CYP3A4 in the metabolism and detoxification of various substrates, and have impeded the accurate determination of the real catalytic activities of each CYP3A isoform in different enzyme sources.[8] Therefore, discovery of isoform-specific CYP3A probe substrates which can precisely define the role of a single member of CYP3As would be of tremendous benefit to the drug metabolism related fields.[9]

As a good probe substrate targeting a single metabolizing enzyme, the following rules should be obeyed at least: (1) high selectivity; (2) good sensitivity; and (3) measurable kinetic parameters.[10] Furthermore, for practical use, other features should also be considered, such as the probe reaction follows the Michaelis–Menten kinetics and has a relatively high k_{cat}, as well as the substrate and its metabolite(s) are chemically stable and commercially available. Unfortunately, most commonly used CYP3A4 probes (such as testosterone, nifedipine, and midazolam) are not highly selective, due to the participation of CYP3A5/3A7.[11]

Recently, we found that CYP3A4 played a predominant role in 1α- and 5β-hydroxylations of cinobufagin (one of the naturally occurring bufodienolides with a steroid skeleton and a unique six-membered lactone ring located at the C-17 site), and the isoform selectivity of CYP3A4 in cinobufagin hydroxylations was superior to typical steroids including testosterone and medroxyprogesterone acetate.[12] This finding motivated us to screen other bufodienolides to find out whether they can serve as ideal probe substrates for determining the real activities of CYP3A4 in biological samples. Herein, we report an isoform-specific probe substrate (Bufalin, Fig. 1) of human CYP3A4, following a screening campaign against a series of natural and derived bufodienolides (Fig. S1, ESI†). The major metabolic pathway of bufalin (BF) in humans is selectively catalyzed by CYP3A4, and this biotransformation follows Michaelis–Menten kinetics and has a relatively high k_{cat} (Scheme 1).

BF could be metabolized by human liver microsomes (HLM) to form two metabolites. The formation of these two metabolites was time-, NADPH-, and microsome-dependent. These two metabolites were biosynthesized and fully identified as 5β-hydroxy-bufalin (5-HBF) and 3-ketobufalin (3-KBF), respectively, based on the LC retention times, UV spectra and MS/MS spectra with the help of standards (Fig. S2 and Table S1, ESI†). The enzymes involved in the formation of 5-HBF and 3-KBF were investigated

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† Electronic supplementary information (ESI) available: Materials and methods, enzyme sources, analytical instruments and conditions, inhibition assays, correlation studies, and docking simulation. See DOI: 10.1039/c3cc45250f

‡ These authors contributed equally to this work.

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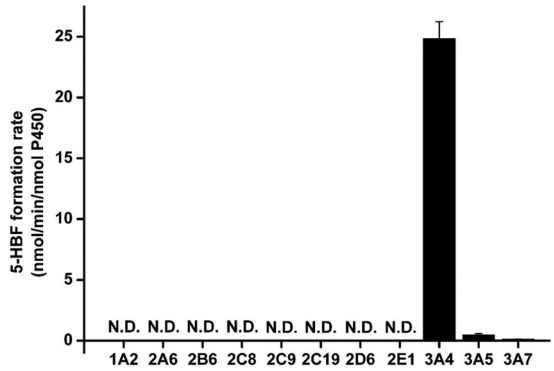
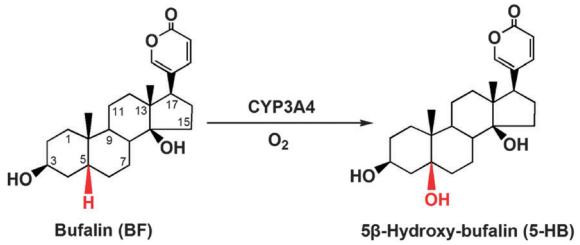


Fig. 1 Isozyme specificity of BF 5β-hydroxylation.



Scheme 1 BF 5β-hydroxylation by CYP3A4.

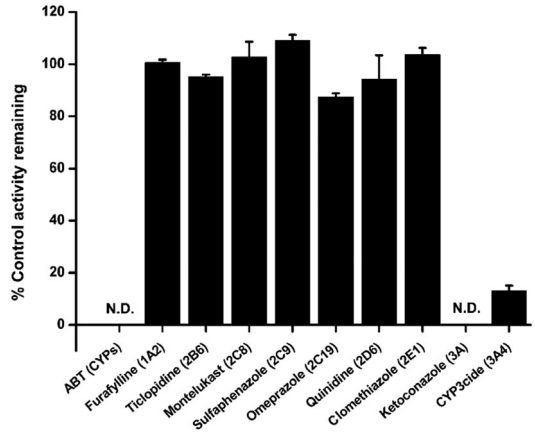


Fig. 2 Inhibition assays of BF 5β-hydroxylation by selective CYP inhibitors in HLM.

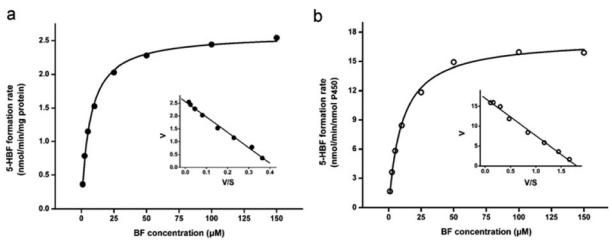


Fig. 3 Michaelis–Menten plots of BF 5β-hydroxylation in human liver microsomes (a) and in CYP3A4 (b). The corresponding Eadie–Hofstee plots are shown as insets. Each data point represents the mean of triplicate determinations.

using 11 cDNA-expressed human P450 isoforms. Surprisingly, as shown in Fig. 1, CYP3A4 demonstrated a good ability to catalyze the formation of 5-HBF, while CYP3A5 and CYP3A7 displayed very limited activities to catalyze BF 5β-hydroxylation. In addition, CYP2D6 and CYP3A4 participated in the formation of the minor metabolite 3-KBF (Fig. S3, ESI†).

The key role of CYP3A4 in BF 5β-hydroxylation was also confirmed by the chemical inhibition assays. The formation of 5-HBF could be potently inhibited by ABT (a broad specificity CYP inactivator), ketoconazole (a potent inhibitor of CYP3A) and CYP3cide (a potent and selective inhibitor of CYP3A4),[13] while inhibitors of other CYP isoforms had minor effects on the formation of 5-HBF (Fig. 2). These results demonstrated that BF 5β-hydroxylation was selectively catalyzed by CYP3A4 in humans.

To further characterize the isoform-specific biotransformation mediated by CYP3A4, a series of kinetic studies was performed using different enzyme sources including HLMs, HLM from a single genotyped donor possessing CYP3A5*1/*1, recombinant human CYP3A4 and CYP3A5. BF 5β-hydroxylation in CYP3A4, HLMs and HLM (CYP3A5*1/*1) followed the Michaelis–Menten kinetics, which are evidenced by the corresponding Eadie–Hofstee plots (Fig. 3). Furthermore, BF 5β-hydroxylation in HLMs from different origins displayed similar K_m values as in CYP3A4 (Table 1), implying that CYP3A4 was the major enzyme participating in BF 5β-hydroxylation. In contrast, the kinetics of CYP3A5-mediated BF metabolism could not be well characterized because the velocity of this reaction was not saturated at the maximum BF concentration (Fig. S4, ESI†). These results demonstrated that BF was a good substrate of CYP3A4 but a weak substrate of CYP3A5.

Compared to CYP3A5, CYP3A4 displayed high affinity and good reactivity in BF 5β-hydroxylation, it is estimated that there

Table 1 Kinetic parameters of BF 5β-hydroxylation determined in different enzyme resources

Enzyme resources	k_{cat}	K_m (μM)	k_{cat}/K_m
HLMs (Pooled)	2.62 ± 0.04	8.00 ± 0.52	327.50
HLM (CYP3A5*1/*1)	1.42 ± 0.03	9.07 ± 0.66	156.56
CYP3A4	17.33 ± 0.29	10.17 ± 0.67	1704.03
CYP3A5	0.25 ± 0.02[a]	>150	<1.67

k_{cat} values were in nmol min^{-1} mg^{-1} protein for liver microsomes, or in nmol min^{-1} nmol^{-1} CYP for CYP3A. The range of substrate concentrations was 1 to 150 μM. Each value was the mean ± S.D. of three determinations performed in duplicate. [a] The k_{cat} value was determined at the maximum substrate concentration (150 μM).

was more than a 1000-fold difference in clearance (k_{cat}/K_m) of BF 5β-hydroxylation between CYP3A4 and CYP3A5. To the best of our knowledge, it is the most selective probe reaction for CYP3A4 reported to date. The high specificity and good reactivity of BF for CYP3A4 can make BF serve as an ideal selective probe for evaluation of CYP3A4 activity in biological samples containing multiple enzymes.

Fig. 4 displays the activities of CYP3A4 in a panel of twelve HLMs from individuals, using BF 5β-hydroxylation as the probe reaction. In order to determine whether the formation rate of 5-HBF can reflect the levels of CYP3A4 in these biological samples, the correlation studies between the formation rates of 5-HBF and the concentrations of CYP3A4/5 in a panel of twelve HLMs from individuals were carried out using a

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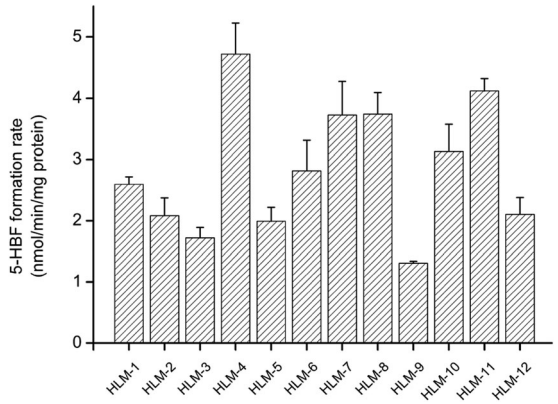

Fig. 4 BF 5β-hydroxylation in a panel of 12 individual HLMs. Data columns represent the mean of triplicate determinations.

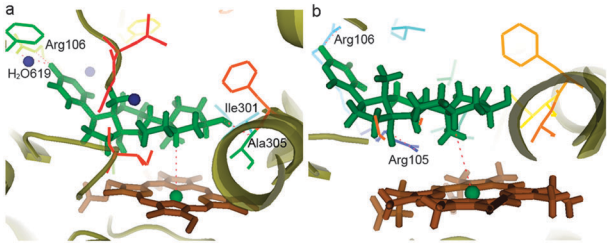

Fig. 5 Docking simulation of BF into CYP3A4 (a) and CYP3A5 (b). Water, heme and iron atoms are colored in blue, brown, and green, respectively.

proteomics-based approach. As expected, a strong correlation was observed between the formation rate of 5-HBF and the level of CYP3A4 in a panel of twelve individual HLMs, while a very poor correlation was obtained between the formation rate of 5-HBF and the level of CYP3A5 (Fig. S5, ESI†). These findings strongly suggested that CYP3A4 is selectively involved in BF 5β-hydroxylation.

Additionally, molecular modeling studies were carried out to explore the large difference in BF 5β-hydroxylation between CYP3A4 and CYP3A5, from the view of binding and recognition between the substrate and enzymes (Fig. 5). Compared to CYP3A5, more hydrogen bonds formed between CYP3A4 and BF (with Arg106, Ile301, Ala305, and a molecule of H_2O), explaining why BF had a higher binding affinity to CYP3A4. As a result, the bioactive pose of BF in 3A4 was given a higher hammerhead score value than in 3A5 (Table S2, ESI†). In addition, the distance between the H atom of the C-5 site and the heme of CYP3A4 (4.05 Å) was shorter than that in CYP3A5 (4.58 Å), implying that BF 5β-hydroxylation catalyzed by CYP 3A4 was easier than CYP3A5. These findings agreed well with the above-mentioned experimental results in which BF was a good substrate of CYP3A4 but a weak substrate of CYP3A5.

In summary, BF 5β-hydroxylation was found to be an isoform-specific probe reaction for CYP3A4, the most important drug-metabolizing enzyme in humans. The probe reaction was highly specific and followed Michaelis–Menten kinetics with relatively high k_{cat}, while BF and its metabolite (5-HBF) were commercially available and easily and sensitively detectable by using both LC-UV and LC-MS/MS.[14] Furthermore, the contribution of other human CYPs including CYP3A5 to BF 5β-hydroxylation is very limited. The reported isoform-specific probe reaction for CYP3A4 can be used to characterize the real functions of CYP3A4 in biological samples, as well as to evaluate the modulations of CYP3A4 activity by the influence of genetic and environmental factors.

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