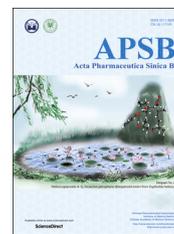




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ORIGINAL ARTICLE

Arenobufagin is a novel isoform-specific probe for sensing human sulfotransferase 2A1



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KEY WORDS

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Abstract Human cytosolic sulfotransferase 2A1 (SULT2A1) is an important phase II metabolic enzyme. The detection of SULT2A1 is helpful for the functional characterization of SULT2A1 and diagnosis of its related diseases. However, due to the overlapping substrate specificity among members of the sulfotransferase family, it is difficult to develop a probe substrate for selective detection of SULT2A1. In the present study, through characterization of the sulfation of series of bufadienolides, arenobufagin (AB) was proved as a potential probe substrate for SULT2A1 with high sensitivity and specificity. Subsequently, the sulfation of AB was characterized by experimental and molecular docking studies. The sulfate-conjugated metabolite was identified as AB-3-sulfate. The sulfation of AB displayed a high selectivity for SULT2A1 which was confirmed by *in vitro* reaction phenotyping assays. The sulfation of AB by human liver cytosols and recombinant SULT2A1 both obeyed Michaelis-Menten kinetics, with similar kinetic parameters. Molecular docking was performed to understand the interaction between AB and SULT2A1, in which the lack of interaction with Met-137 and Tyr-238 of SULT2A1 made it possible to eliminate substrate inhibition of AB sulfation. Finally, the probe was successfully used to determine the activity of SULT2A1 and its isoenzymes in tissue preparations of human and laboratory animals.

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1. Introduction

Human cytosolic sulfotransferases (SULT) are a superfamily of enzymes that catalyze the transfer of sulfonic groups from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxyl or amine groups in substrate molecules, therefore they play a vital role in the detoxification and elimination of exogenous and endogenous substances¹⁻⁴. SULT2A1 is one of the most important members of the SULT superfamily in human. It is extensively expressed in various human organs, especially abundant in liver^{2,5}. The important role of SULT2A1 in the sulfoconjugation of various drugs including raloxifene, lorcaserin, pentazocine and buprenorphine has been confirmed⁶⁻⁹. Most importantly, the SULTs-mediated sulfation can dramatically reduce the affinities of steroid hormone for their receptors, and thereby abolish the biological activity of these hormones¹⁰.

Considering the role of SULT2A1, it has been received increasing attention to investigate the regulation of SULT2A1 expression and its activity. The current results indicate that the expression of SULT2A1 could be regulated by various nuclear receptor including farnesoid X receptor, constitutive androstane receptor, and pregnane X receptor^{11,12}, resulting in activity variation of SULT2A1 among organs and species^{13,14}. Moreover, the inhibitory effects of small molecules derived from natural products, environmental contaminants and drugs on SULT2A1 activities are frequently reported¹⁵⁻¹⁷. Therefore, it is extremely urgent to measure the activities of SULT2A1 as well as to explore the interaction between SULT2A1 and its ligands. Most importantly, the functional level of SULT2A1 was a risk predictor for some diseases¹⁸. It has been observed that the activity of SULT2A1 is reduced in patients with Alzheimer's disease¹⁹. The levels of serum SULT2A1 exhibit superior characteristics for the health risk assessment of liver and kidney, especially in endotoxemia, ischemia/reperfusion, chemical and drug-induced liver and kidney injury²⁰. Thus, there is an urgent need for a highly sensitive, non-invasive pathogenically relevant molecular tool for selective detection of SULT2A1.

The detection methods of SULT2A1 including enzyme-linked immunosorbent assay, immunofluorometric activity-based probe analysis and mass spectrometry based proteomic assay have been subsequently developed²¹. Although these methods can be used to detect the levels of SULT2A1 in biological samples, only activity-based probe assay can be used to characterize the biological functions of SULT2A1. Therefore, the detection of SULT2A1 with the aid of specific activity-based probe has a potential for phenotypic study of SULT2A1 and diagnosis of SULT2A1-related diseases. However, there are some factors that affect the accuracy of the developed probe method for SULT2A1, the prominent one of which is the poor selectivity of the probe substrate for SULT2A1. For example, dehydroepiandrosterone (DHEA), a widely used probe for SULT2A1, can also be sulfated by SULT1E1. And the catalytic efficiency of SULT1E1 is approximately equal to that of SULT2A1. Another prominent problem in activity-based detection of SULT2A1 is that SULT2A1 frequently shows substrate inhibition during the sulfation of high concentrations of substrates. Herein, the activities of SULT2A1 may be underestimated or inaccurate due to the substrate inhibitory effect. The sulfation of DHEA and the another commonly used probe reaction, 17 β -estradiol sulfation, are both subject to substrate inhibition^{22,23}. Therefore, developing a probe with an excellent selectivity and Michaelis-Menton kinetics is necessary for accurate quantitative analysis of activity of SULT2A1 in complex biosamples.

Bufadienolides are the major bioactive constituents of toad venom, characterized by a unique steroid skeleton²⁴. With the aid of microbial transformation which is an effective method for the structural modification of natural products²⁵⁻²⁷, a series of bufadienolides have been isolated and identified as the bioactive molecules with potential impacts on a wide range of biological investigation^{24,28,29}. Notably, bufadienolides can be selectively sulfated by SULT2A1 at the C-3 position²⁹. Therefore, it is possible to seek a probe substrate for SULT2A1 among bufadienolides. The relative results prompted us to explore the possibility of bufadienolide derivatives as probes for the selective detection of human SULT2A1.

In this study, after charactering the sulfation of bufadienolides and their derivatives, excepting for arenobufagin (AB), the velocities of SULT2A1-mediated sulfation of various bufadienolides and DHEA were shown to be decreasing with increase of the concentrations of substrates. Then, AB was identified as a preferred probe substrate for SULT2A1 with the high selectivity and appropriate kinetic parameters, according to a panel of reaction phenotyping assays and molecular docking studies. Combined with a sensitive analysis method, AB was used for measuring the activities of SULT2A1 in various biological samples.

2. Materials and methods

2.1. Materials

3'-Phosphoadenosine-5'-phosphosulfate (PAPS), DHEA, dehydroepiandrosterone sulfate (DHEA-S), quercetin, estrone, dithiothreitol (DTT), nimesulide, ibuprofen, mefenamic acid (MFC), and 2,6-dichloro-*p*-nitrophenol (DCNP) were purchased from Sigma-Aldrich (MO, USA). Bufalin (BF), resibufogenin (RB), cinobufagin (CB), bufotalin (BFT), desacetylcinobufagin (DCB), telocinobufagin (TCB), gamabufagin (GB), 3-epi-DCB (EDCB), 3-epi-RB (ERB), 3-epi-BF (EBF), 9-oxo-cinobufagin (OCB) and AB were isolated from *Venenum Bufonis* and identified by NMR and ESI- mass spectrometry as described previously²⁹. Their purity was greater than 98% which were determined by high-performance liquid chromatography with diode-array detection (HPLC/DAD). Rabbit monoclonal anti-SULT2A1 antibody and mouse monoclonal anti- β -actin antibodies were purchased from Abcam (MA, USA). Pooled mixed-gender cytosols obtained from different human organs, pooled mixed-gender experimental animal liver cytosols and individual human liver cytosols were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). Recombinant human SULTs (rhSULTs) were obtained from BD Gentest (MA, USA).

2.2. Incubation system and analysis method

The standard incubation system for the sulfation reaction included potassium phosphate buffer (50 mmol/L, pH 7.4), tissue cytosols or recombinant human SULT, DTT (8 mmol/L), PAPS (4 mmol/L), MgCl₂ (5 mmol/L), and substrates. After preincubation at 37 °C for 3 min, the reaction was initiated by adding PAPS. The reaction was terminated by the addition of ice-cold acetonitrile (100 μ L). The mixture was kept on ice until it was centrifuged at 20,000 \times g for 10 min at 4 °C. Control incubations without PAPS or without substrate or without tissue preparations were carried out to ensure that metabolite formation was enzyme- and PAPS-dependent.

The Agilent 1200 high-performance liquid chromatography (HPLC) system consisted of a quaternary delivery system, a degasser, an auto-sampler and a UV-detector. An Elite SinoChrom ODS-BP (150 mm × 2.1 mm, 5 μm) analytical column was used for quantification. The mobile phase consisted of acetonitrile–0.1% formic acid aqueous solution at a flow rate of 450 μL/min. An Applied Biosystems MDS Sciex Qtrap 4500 Triple Quadrupole Mass Spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source was used to analyze target metabolites, and the system was operated in negative mode for AB-S (*m/z* 494.6–495.6). The negative ion spray voltage and temperature were set at –4500 V and 600 °C, respectively. The curtain gas (CUR) and collision-activated dissociation gas (CAD) parameters were set at 12 psi and 10 psi, respectively; gas1 and gas2 (nitrogen) were set at 20 and 60 L/min, respectively. The dwell times were 150 ms. And the quantification assay was performed using multiple reaction monitoring.

2.3. Sulfation of bufadienolides by SULT2A1

A series of bufadienolides were incubated with SULT2A1 at different substrate concentrations (1, 10 and 100 μmol/L), respectively. The incubation system was used as previously described at a final protein concentration of 0.1 mg/mL for 60 min at 37 °C.

2.4. Preparation of AB and AB-3-sulfate

The isolation and purification of AB from *Venenum Bufonis* was based on preparative high-speed counter-current chromatography method with two-phase solvent system composed of *n*-hexane/ethyl acetate/methanol/water. The purity was determined to be >98.5% by HPLC analysis. The sulfated metabolite of AB was produced *in vitro*. Briefly, AB was added to the pyridine reaction mixture (2 mL), which contained sulfuric acid (11.9 mg, 0.12 mmol/L) and acetic anhydride (0.12 mmol/L). After each mixture was stirred for 60 min at 60 °C, the reaction was quenched with 2 mL aqueous NH₃ (25%). The sulfated metabolite of AB was isolated and purified by reversed-phase-column chromatography (methanol:H₂O = 1:2) from incubation systems. The purity of this product (AB-S) was determined to be above 98% by HPLC-diode array detector (DAD) analysis.

The chemical structure of AB and the metabolite was determined by spectroscopic methods including ¹H and ¹³C NMR. All spectra were recorded on a Bruker AV-600 (Newark, Germany). Chemical shifts of ¹H and ¹³C between AB-S and parent AB were used to identify the conjugation site.

2.5. Characterization of AB sulfation by recombinant SULT

Sulfation of AB was measured in individual reaction system containing seven recombinant human SULT isoforms including rhSULT1A1*1, rhSULT1A1*2, rhSULT1A2, rhSULT1A3, rhSULT1B1, rhSULT1E1 and rhSULT2A1, respectively. The sulfation activities of DHEA in seven recombinant human enzymes were evaluated under identical condition.

2.6. Kinetic characterization

To estimate the kinetic parameters of AB sulfation, AB was incubated with pooled human liver cytosols (HLC) and rhSULT2A1, respectively. The incubation conditions were

optimized to ensure that the formation rates of the metabolites were in the linear range over the incubation time. The kinetic models used to analyze the experimental results were Michaelis-Menten (Eq. (1)) and substrate inhibition kinetic model (Eq. (2)), respectively.

$$V = V_{\max} \times [S]/(K_m + [S]) \quad (1)$$

$$V = V_{\max} \times [S]/(K_m + [S] + [S]^2/K_{si}) \quad (2)$$

where [S] is concentration of substrate; V_{\max} represented the maximum reaction rate and K_m was the substrate concentration at the half-maximal rate. The kinetic type was determined by Eadie-Hofstee (EH) plots. The V_{\max} and K_m values were obtained from a nonlinear regression between substrate concentrations and reaction velocities using the fit equation. All incubations were performed in duplicate in three independent experiments.

2.7. Chemical inhibition studies

AB was incubated with HLC in the presence or absence of different specific inhibitors including imesulide, mefenamic acid (MFC), ibuprofen, estrone, DHEA and glycyrrhetic acid (GA), 2,6-dichloro-4-nitrophenol (DCNP) and quercetin^{30,31}. The inhibitory effect of DHEA and GA towards sulfation of AB in HLC and rhSULT2A1 were also performed.

2.8. Immunoprecipitation assay

To determine the vital role of SULT2A1 in the sulfation of AB, immunoprecipitation was performed as previously described. Two μg of specific rabbit monoclonal antibody to SULT2A1 or nonimmune rabbit IgG were added to the protein A/G magnetic beads. The mixture was rotated for 1 h at room temperature. After washing the protein A/G magnetic beads-Ab complex, the samples (HLC, 400 μg) were added and incubated with rotation for 30 min. Finally, the supernatants were collected to detect the metabolic activity, and used for Western blot. The activity of SULT2A1 was evaluated using our selective probe AB, and other SULTs were evaluated using 6-hydroxymelatonin (6-OM), which was a broad spectrum substrate for several SULTs.

2.9. Correlation study

The liver preparations were analyzed by Western blotting using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% acrylamide gels) and transferred onto PVDF membranes (Millipore, Bedford, MA). The blots were probed with the anti-SULT2A1 antibody, followed by incubation with a horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody. The membranes were analyzed on a FluorChem FC Imaging System and the grey levels were measured using ScanImage software. The correlation between the protein contents of SULT2A1 and reaction velocities of AB sulfation was measured using a linear regression coefficient (*r*). All the experiments were performed in duplicate in three trials and the *r* value was reported as the mean ± SD of the parameter measured.

2.10. Docking studies

The molecular docking studies were performed using Surflex-Dock procedure, from the SYBYL suite. Surflex-Dock used an empirical scoring function and a patented search engine to dock ligands into a protein's binding site. The crystal structure of SULT2A1 with ligand DHEA (PDB: 1J99) was used as receptor. The active pocket for substrate binding was generated around the crystallographic ligand in an automatic mode with the float radius set to zero. AB was docked into the active site of SULT2A1. Then, the molecular dynamics (MD) simulation was performed to refine the docking result using the GROMACS 4.5.3 package. The system was solvated in a cubic box of TIP3P water molecules and neutralized with counterions. Equilibration of the solvated complex was performed by carrying out a short minimization procedure (500 steps of steepest descent and then a 50 ps position restrained molecular dynamics). Finally, 20 ns of production run were performed. Long-range electrostatics interactions were treated using the Particle Mesh Ewald (PME) method. The van der Waals and short-range electrostatic interactions employed a cutoff of 1.0 nm. The topology file for the compound was generated using ACPYPE. The trajectory was analyzed using GROMACS package, VMD 1.9.1 and PyMOL 1.7.1.

2.11. SULT2A1 activities analyses

The SULT2A1 activities of liver cytosols obtained from several animal species, included monkey, pig, dog, rabbit, guinea pig, rat and mouse were measured. The kinetic analyses were also performed. To apply AB for measuring the activity of SULT2A1 in various tissue cytosols, we established a LC-MS method. Then, AB was used as the probe substrate to assay the activity of SULT2A1 in various human cytosols obtained from intestinal, kidney and brain.

2.12. Data analysis and statistics

All data represent the means \pm SD. The significant differences were identified using the statistical program SPSS 17.0. To test for statistically significant differences among multiple treatments for a given parameter, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test was used for comparison among various groups. Differences with P value <0.05 were considered to be statistically significant.

3. Results

3.1. Sulfation of bufadienolides by SULT2A1

Inspired by our previous study on the *in vitro* metabolism of natural bufadienolides²⁹, a series of natural bufadienolides or their derivatives (Supplementary Information Fig. S1A) were used to develop the probe substrate for SULT2A1. After incubated with SULT2A1, the formation rates of the sulfonated product of bufadienolide derivatives and DHEA were determined, respectively. It was found that most of the tested compounds were metabolized by SULT2A1 (Supplementary Information Fig. S1B). The sulfation rates of CB, DCB, AB, BF and RB were higher than those of other bufadienolides, implying a superior sensitivity of these compounds to detect SULT2A1. Notably, a decreasing trend

of the sulfation rates was observed with increasing concentration of some bufadienolides (including CB, BF and RB), suggesting the non-classical kinetics of SULT2A1 mediated sulfation of these bufadienolides. However, the velocity of AB sulfation kept enhancing as the substrate concentration was increased to 100 μ mol/L. Herein, AB was selected as the SULT2A1 probe substrate for further investigation.

3.2. Identified the metabolite of AB sulfation

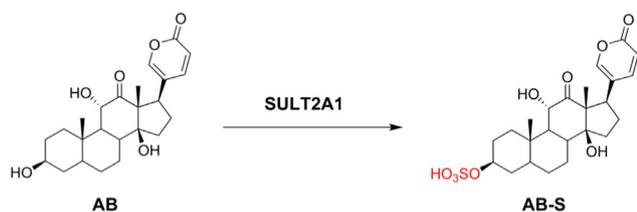
As shown in Supplementary Information Fig. S2, LC-MS spectrum of the metabolite of AB showed an ion peak of $[M-H]^-$ at m/z 495.2. Furthermore, a neutral loss (m/z 80) of a sulfonate group was observed from m/z 495.2 to m/z 415.4, indicating that the formed metabolite was AB monosulphate. Subsequently, NMR analysis was employed to identify the sulfate conjugation site of AB (Fig. S2B-C). The carbon signals were assigned and listed in Table 1. For the bufadienolides, the C-3 shift with β -OH was usually at δ 62–63, while the chemical shift of C-3 (α -OH) was approximately at δ 68–69, due to the γ -gauche effect^{32,33}. In comparison with AB, a significant downfield shift from δ 64.3 to δ 71.3 ($\Delta\delta+7.0$) was observed at C-3 of AB monosulphate. Due to chemical shift of C-3 at δ 71.3, the additional sulfate group should be still in β -configuration of AB monosulphate. All these spectroscopic data demonstrated that a sulfate group has been introduced at the C-3 position of AB. Therefore, the conjugated metabolite of AB was identified as AB-3 β -sulfate (AB-S, Scheme 1).

Table 1 ¹³C NMR and ¹H NMR (DMSO) spectral data for sulfation metabolite and parent compound AB.

No. ^a	AB	AB-S	
	¹³ C NMR	¹³ C-NMR	¹ H NMR ^b
1	31.5	31.3	1.83 m; 1.23 m
2	28.6	27.7	1.62 m; 1.55 m
3	64.3	71.3	4.34 brs
4	33.7	32.3	1.98 m; 1.46 m
5	37.4	36.3	1.70 m
6	26.6	26.2	1.86 m; 1.26 m
7	21.3	21.3	1.87 m; 1.56 m
8	38.7	38.1	2.34 brd ($J = 13.0$ Hz)
9	39.5	39.5	1.67 m
10	36.6	36.3	–
11	73.3	73.3	4.26 brd ($J = 11.0$ Hz)
12	213.5	213.5	–
13	62.2	62.2	–
14	84.5	84.2	–
15	31.8	31.9	1.85 m; 1.68 m
16	27.6	26.4	1.68 m; 1.23 m
17	40.3	38.8	3.96 dd ($J = 8.5, 7.0$ Hz)
18	17.5	17.3	1.07 s
19	23.5	23.4	0.80 s
20	120.9	120.9	–
21	150.5	150.2	7.58 brs
22	147.3	147.2	7.82 dd ($J = 10.0, 2.0$ Hz)
23	114.6	114.6	6.32 d ($J = 10.0$ Hz)
24	161.1	161.1	–

^aThe number of carbon position in the structure of AB and AB-S

^b¹H NMR data was belonging to AB-S.



Scheme 1 AB 3-O-sulfation by SULT2A1.

3.3. Recombinant SULTs assay

To further characterize the reaction specificity for SULT2A1, the sulfation of AB by series of rhSULTs were investigated. Meanwhile, the selectivity of DHEA sulfation (a widely used probe reaction for SULT2A1) for SULT2A1 was reevaluated. At three substrate concentrations, the AB sulfate was exclusively catalyzed

by rhSULT2A1, while no metabolite was observed in samples incubated with other rhSULTs (Fig. 1A). The sulfation rate of AB at the substrate concentration of 1, 10 and 100 $\mu\text{mol/L}$ was 0.021, 0.263 and 1.088 nmol per min per mg rhSULT2A1, respectively. However, SULT1E1 and SULT2A1 were both involved in DHEA sulfation, displaying similar activity at three substrate concentrations (Fig. 1B). Because of the inevitable interference of high SULT1E1 activity, it is hard to require the real SULT2A1 activity in human tissue preparations by using DHEA. However, the results showed the high selectivity of AB for sensing SULT2A1.

3.4. Chemical inhibition study

The inhibitory effects of selective inhibitors/substrates on AB sulfation by HLC were evaluated. As shown in Fig. 2, the sulfation of AB was significantly inhibited by DHEA and GA (the selective inhibitor for SULT2A1) with a residual activity of less than 10%.

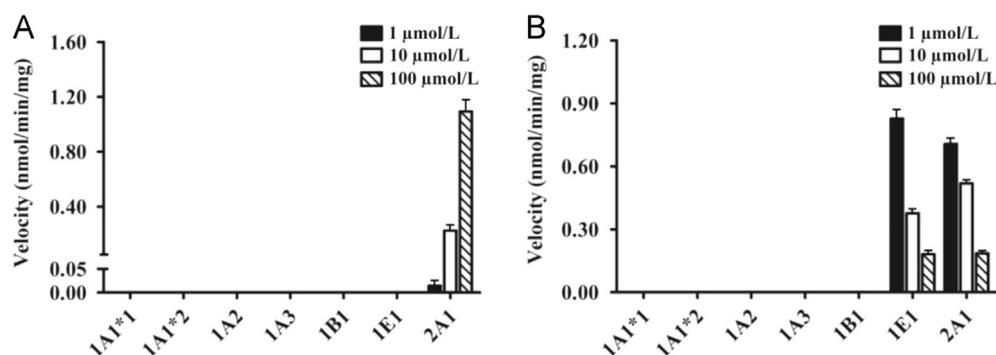


Figure 1 Sulfation of AB (A) and DHEA (B) by various recombinant SULTs at three different concentrations.

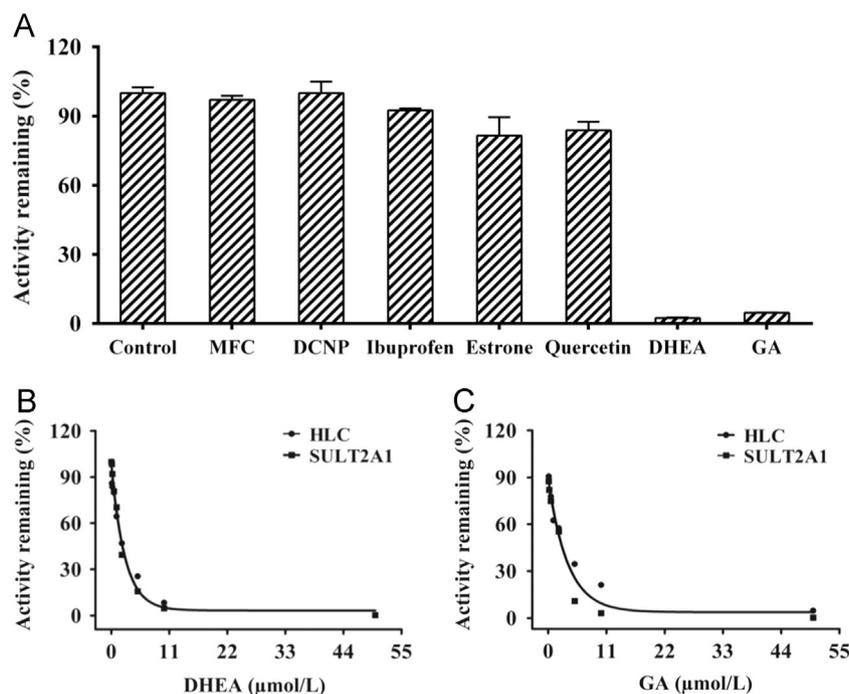


Figure 2 Assignment of isozyme(s) involved in AB sulfation. (A) Inhibition of AB sulfation by selective SULT inhibitors in HLC. (B) and (C) The concentrations dependent inhibitory effects of DHEA (B) and GA (C) toward AB sulfation in HLC and rhSULT2A1.

However, the selective inhibitors/substrates of other SULT isoforms, including MFC and nimesulide (the selective inhibitors for SULT1A1), ibuprofen and estrone (the selective inhibitors for SULT1E1), and DCNP (a general inhibitor of SULT1A1 and SULT1E1), showed negligible inhibitory effects against AB sulfation (less than 20% inhibition, $P < 0.05$).

Additionally, the concentration dependent inhibitory effects of DHEA and GA against the sulfation of AB were determined, respectively. The IC_{50} values of DHEA and GA for HLC-mediated AB sulfation were 1.88 and 2.21 $\mu\text{mol/L}$, which were close to the values determined with rhSULT2A1 (1.64 and 2.18 $\mu\text{mol/L}$), respectively (Fig. 2B–C).

3.5. Kinetic characterization

AB sulfation by HLC and rhSULT2A1 both obeyed the Michaelis–Menten model, as evidenced by an Eadie–Hofstee plot (Fig. 3). Meantime, the K_m value for the AB sulfation catalyzed by HLC ($69.02 \pm 11.01 \mu\text{mol/L}$) was close to that catalyzed by SULT2A1 ($39.55 \pm 9.18 \mu\text{mol/L}$). In contrast, as shown in Fig. S3, rhSULT2A1, rhSULT1E1 and HLC mediated the sulfation of DHEA, all of which were obeyed substrate inhibition kinetic model. As shown in Table 2, the K_m for DHEA sulfation catalyzed by SULT1E1 ($0.34 \pm 0.13 \mu\text{mol/L}$) was much lower than that catalyzed by rhSULT2A1 ($2.48 \pm 1.15 \mu\text{mol/L}$), and CL_{int} for DHEA sulfation catalyzed by SULT1E1 ($4264.7 \mu\text{L/min/mg}$) was

higher than that catalyzed by SULT2A1 ($2943.6 \mu\text{L/min/mg}$). The above results indicated a poor specificity of DHEA for SULT2A1 in comparison with AB.

3.6. Correlation study

The protein contents of SULT2A1 in a panel of human liver cytosols prepared from individual donors were determined by western blotting. These values were compared with the sulfation rates of AB by individual HLC. As shown in Fig. 4, the linear regression coefficient (r) for the formation rate of AB-3-sulfate and SULT2A1 protein concentration was high with $r=0.86$ ($P < 0.05$).

3.7. Immunoprecipitation assay

The highly selective monoclonal antibody of SULT2A1 was used for specific immunoprecipitation for SULT2A1 in our experiment. As shown in Supplementary Information Fig. S4A and B, after immunoprecipitation, the amount of SULT2A1 protein in HLC was decreased nearly 50% of the control group. Consistently, the activity of SULT2A1 also decreased to 54% of control group as shown in Fig. S4C. All these evidence strongly demonstrated that AB could serve as a highly specific probe for SULT2A1.

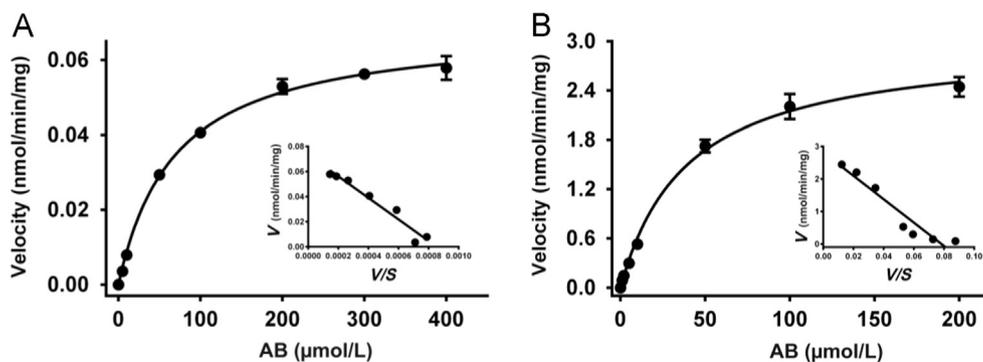


Figure 3 The kinetic profiles of 3-*O*-sulfation of AB in HLC (A) and rhSULT2A1 (B). An Eadie–Hofstee plot is shown as an inset to illustrate kinetic behavior. Data points represent the mean of triplicate determinations.

Table 2 Kinetic parameters and CL_{int} values of AB in different human cytosols (liver and small intestine), recombinant SULT2A1, mixed recombinant SULTs and kinetic parameters and CL_{int} values of DHEA in HLC, SULT2A1 and SULT1E1.

Compound	Enzymes	V_m (nmol/min/mg)	K_m ($\mu\text{mol/L}$)	K_{si} ($\mu\text{mol/L}$)	CL_{int} ($\mu\text{L/min/mg}$)
AB	HLC	0.069 ± 0.001	69.0 ± 4.5	–	1.00
	SULT2A1	3.00 ± 0.09	39.6 ± 3.8	–	75.9
	Mixed isoform	0.63 ± 0.025	40.2 ± 5.1	–	15.7
DHEA	HLC	0.052 ± 0.004	1.08 ± 0.17	17.2 ± 2.9	48.2
	SULT2A1	7.30 ± 2.46	2.48 ± 1.15	2.2 ± 0.9	2943.6
	SULT1E1	1.45 ± 0.29	0.34 ± 0.13	2.9 ± 1.2	4264.7

Data are represented as the mean \pm SD, $n = 3$.

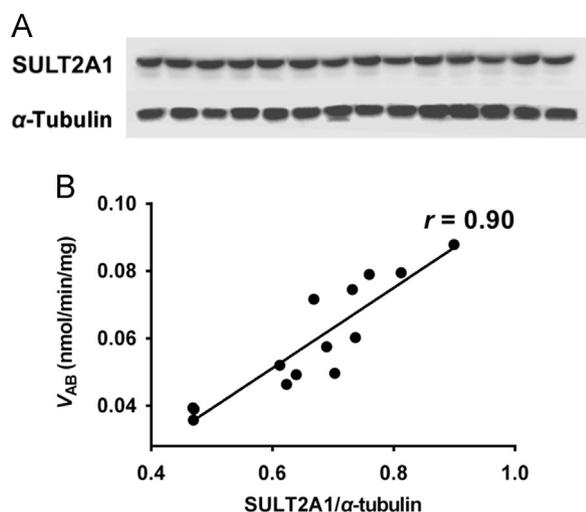


Figure 4 (A) Western blots of SUL2A1 using a panel of individual human liver cytosol. (B) The correlation between mean gray level ratio of SUL2A1 protein to α -tubulin and AB sulfation rates. The correlation parameter was expressed by the linear regression coefficient (r).

3.8. Molecular modeling study

To better elucidate the binding mode of AB with SUL2A1, a docking study was performed using Surflex-dock which was refined by a molecular dynamics simulation. SUL2A1-AB complex was equilibrated after a 20 ns MD simulation, and the plot of RMSD (in \AA) of the complex was shown in Fig. 5. The analysis suggested that the catalytic orientation of AB was different to that of DHEA. The steroid scaffold of AB fitted into the hydrophobic substrate binding site of SUL2A1. The α -pyranone of AB interacted favorably with

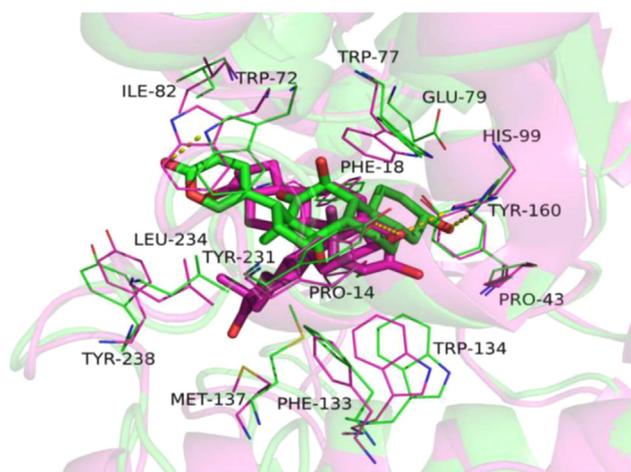


Figure 5 Superposition of the SUL2A1/DHEA (magenta) and the SUL2A1/AB (green) complex structures. The DHEA molecule was in two orientations and colored in red; AB molecule was colored in green.

the Trp72 side chain, giving rise to a positive π - π stacking interaction and a hydrogen bond between α -pyranone and Trp72. As shown in Fig. 5, after superimposing the two molecules, we found that AB was closed to the location of DHEA, while only DHEA interacted with Met-137 of SUL2A1. It should be pointed that the C ϵ atom of the Met-137 residue imposed steric hindrance against DHEA binding, thus increased the incidence of substrate inhibition kinetic. Moreover, the interaction of the two molecules with Tyr-238 exacerbated the binding difference of AB and DHEA (Fig. 5). Taken together, our molecular simulation allowed us to rationalize the concentration-dependent increase in the activity of AB sulfation, due to the lack of interaction of AB with Met-137 and Tyr-238 of SUL2A1.

3.9. Real sample analysis

We evaluate the SUL2A1 activity of cytosols from different human organs (including liver, kidney, small intestine and brain) using the novel probe AB. Consistent with the previous study³⁴, the variation of SUL2A1 activities in a variety of individual human liver cytosols was not exceeding 2-fold (Fig. 6A). As shown in Supplementary Information Fig. S5, the strong inhibitory effect of DHEA and GA toward AB sulfation indicated the high selectivity of AB for SUL2A1 in different organs. The rates of AB sulfation by cytosols of different human organs were as follows: liver (58.69 pmol/min/mg protein) > intestine (16.45 pmol/min/mg protein) > kidney (1.88 pmol/min/mg protein) > brain (0.023 pmol/min/mg protein). It was found that the SUL2A1 activities of human kidney cytosols were 31.1- and 8.7-fold lower than that of human liver and intestine cytosols. The activity tendency of SUL2A1 in cytosols of liver, intestine, kidney and brain was consistent with the previous reported SUL2A1 protein expression level in tissue preparations^{34,35}. Interestingly, we found that the AB sulfation rate of pooled male kidney cytosols was significant lower than that of pooled female kidney cytosols, implying the gender difference on functional activity of SUL2A1 in kidney.

The activities of SUL2A1 in liver cytosols of different animal species were also evaluated. As shown in Fig. 6B, there were no detectable activity of AB sulfation in liver cytosols of dog and mouse. Additionally, the liver cytosols of other animal species including monkey (CyLC), pig (PLC), rabbit (RaLC), guinea pig (GLC) and rat (RLC) displayed significant difference on activities of AB sulfation. AB sulfation in animal liver cytosols obeyed Michaelis-Menten kinetic model (Fig. 7). The apparent kinetic parameters including K_m , V_{max} for AB-3-sulfate were determined and listed in Table 3. In liver cytosols of five animal species, the K_m values for AB sulfation ranged from 19.3 to 233.2 $\mu\text{mol/L}$, while the V_{max} values ranged from 0.013 to 0.100 nmol/min/mg protein. According to CL_{int} values, the SUL2A1 metabolic efficiency for liver cytosols of different animal species was following the order: RaLC > GLC > CyLC > PLC > RLC. The CL_{int} of AB sulfation for CyLC was 1.27 $\mu\text{l/min/mg}$, which was approximately 9-fold higher than that for RLC. Our results implied the significant functional difference on the isoenzymes of human SUL2A1 in various animal species.

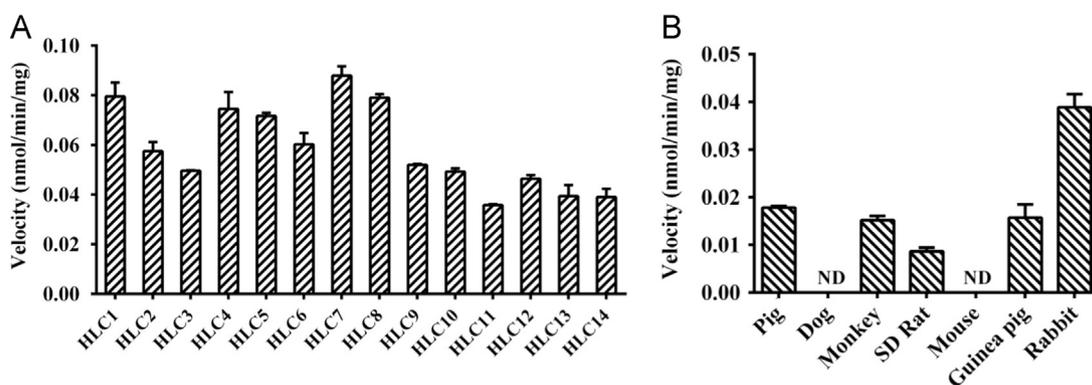


Figure 6 (A) 3-*O*-sulfation of AB in 14 individual HLCs. (B) 3-*O*-sulfation of AB in liver cytosols of different species.

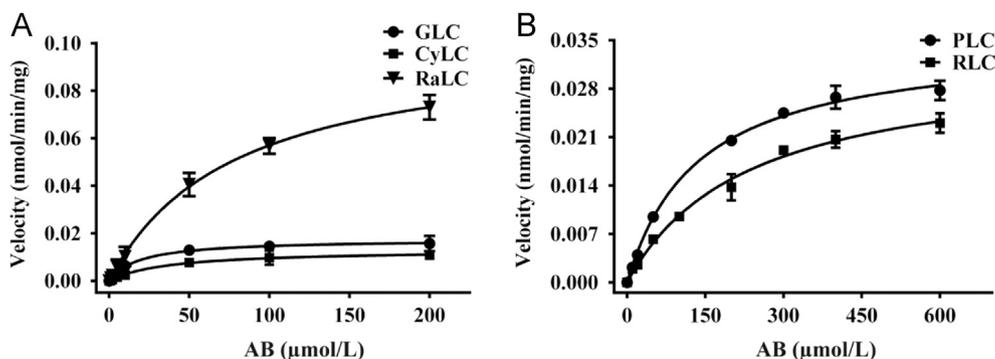


Figure 7 The kinetic profiles of AB sulfation in liver cytosols of different species, including guinea (GLC), monkey (CyLC), rabbit (RaLC), pig (PLC) and rat (RLC). Each data point represents the average of three replicates.

Table 3 Kinetic parameters of AB in different experimental animal cytosols.

Enzyme resource	V_m (nmol/min/mg)	K_m ($\mu\text{mol/L}$)	CL_{int} ($\mu\text{L/min/mg}$)
PLC	0.035 ± 0.001	136.7 ± 10.9	0.26
CyLC	0.013 ± 0.001	37.2 ± 3.5	0.35
RLC	0.032 ± 0.002	233.2 ± 28.4	0.14
GLC	0.017 ± 0.001	19.3 ± 1.6	0.88
RaLC	0.100 ± 0.002	78.5 ± 3.6	1.27

Data are represented as the mean \pm SD, $n = 3$.

4. Discussion

In addition to responsible for sulfation of hydroxysteroids, SULT2A1 also plays a vital role in the metabolism of xenobiotics^{36–39}. That is, the activity levels of SULT2A1 regulate the system and local exposure of endogenous and exogenous substances. Furthermore, SULT2A1 is involved in the individual genetic disposition, species differences, and organotropisms for toxicological effects of chemicals⁴⁰. The activity modulation of SULT2A1 with the aid of selective inhibitor may prove to be preventive or therapeutic method for metabolic activation mediated toxicological effects^{41–43}. Therefore, a specific activity-based probe for SULT2A1 is a valuable tool for measuring the function level of SULT2A1 and searching for the novel therapeutic agent that targeted to SULT2A1.

After a series of experiments, our results suggested that AB was a better probe for SULT2A1 than the widely used DHEA. Firstly, the sulfation rate of DHEA could not represent the activity of

SULT2A1 due to the involvement of SULT1E1 in sulfation of DHEA at a wide range of substrates concentration (Fig. 1B). It was found that SULT1E1 displayed a high activity toward DHEA sulfation, which was almost half of the activity of SULT2A1. Kinetic studies showed that the K_m value of SULT1E1-mediated DHEA sulfation was 7.2-fold lower than that for SULT2A1, implying the binding preference for SULT1E1 of DHEA. Meanwhile, the CL_{int} value of SULT1E1-mediated DHEA sulfation was 1.5-fold higher than that for SULT2A1. These results suggested that the contribution of SULT1E1 to DHEA sulfation could not be ignored, especially in some organs where SULT1E1 were highly expressed, such as small intestine, lung and liver^{34,44}. It was easily conceivable that the detection accuracy of DHEA for SULT2A1 activities was debatable. Considering the practicability of probe for SULT2A1, the difference on kinetic behavior of the two probes also demonstrated the superior of AB than DHEA. The sulfation of DHEA obeys to substrate inhibition kinetic (Fig. 3) which could influence the measurement accuracy of SULT2A1 in complex

biological samples. Thus, DHEA was not the good probe for assaying SULT2A1 activity.

Subsequently, several different experiments were designed to clarify the specificity of AB sulfation for the detection of SULT2A1. In the rhSULT isoforms screening assay, only rhSULT2A1 exhibited the capability of sulfation of AB among six rhSULT isoforms which were widely expressed in human tissues³⁴. Due to that some SULT isoforms cannot be obtained, chemical inhibition assay using isoform-specific chemical inhibitor or antibody, which were regarded as classical methods to determine the contribution of the specific metabolic enzymes for reaction, were performed⁴⁵. It was observed that the selective inhibitor or substrate of SULT2A1 all exerted strong inhibitory effects toward AB sulfation in HLC. Furthermore, the IC_{50} values of DHEA (the substrate for SULT2A1) and GA (the selective inhibitor for SULT2A1) on AB sulfation in HLC (1.88 $\mu\text{mol/L}$ for DHEA, 2.19 $\mu\text{mol/L}$ for GA) was closed to the values determined in rhSULT2A1 (1.64 $\mu\text{mol/L}$ for DHEA, 1.41 $\mu\text{mol/L}$ for GA). Additionally, correlation study and immunoprecipitation using the highly selective monoclonal antibody of SULT2A1 further verified the specificity of AB sulfation for SULT2A1. All of these evidences suggested that AB sulfation could be used as a probe to detect functional level of SULT2A1.

According to the molecular modeling simulation, the C3 oxygen atom of AB formed a hydrogen bond interaction with His99 (Fig. 5), which was the key site of SULT2A1 that could interact with the classical substrate DHEA²². Notably, the different kinetic profiles between the two steroid molecules DHEA and AB were elaborated, which was due to the lack of intermolecular interaction of AB with Met-137 and Tyr-238 of SULT2A1. In previous studies, Met-137 was seemed as a critical residue that could modulate the substrate binding orientation of ligands⁴⁶. Additionally, it was proposed that Tyr-238 acted as a gate residue to regulate the release of substrate from the substrate-binding cavity in a ternary dead-end complex^{46,47}. In the present study, after superimposing the two structures, it showed that AB was closed to the location of the DHEA; however, there was a different interaction with the key amino-acid residue of SULT2A1. It was lack of interaction of AB with Met-137 and Tyr-238. However, the Ce atom of the Met-137 residue imposed steric hindrance against DHEA binding. And the oxygen atom at C17 of DHEA donated one weak hydrogen bond to the carbonyl oxygen of residue Tyr-238 (3.4 Å), which may obstruct the release of molecule. Taken together, the molecular simulation allowed us to rationalize the difference on kinetic behavior of AB and DHEA, which provided valuable information for elucidation the interaction between AB and SULT2A1.

Animal models were widely used in preclinical studies to predict pharmacokinetics and toxicity in humans. However, due to the significant interspecies differences in their susceptibility to xenobiotic toxicity and the expression of metabolic enzymes, it is difficult to select a suitable animal model⁴⁸. Therefore, the assessment of metabolic difference of common experimental animal species was imperative^{49–51}. In the present study, the SULT2A1-like activities in different species were measured by using AB. It was found that the K_m and CL_{int} for AB sulfation of rabbit liver cytosols were closed to those of human liver cytosols, respectively. However, a significant difference on kinetic parameters for AB sulfation between liver cytosols of human and rat, with the CL_{int} for AB sulfation of RLC was approximately seventh of that of HLC. Additionally, SULT2A1-like activity of DLC could not be detected, which was consistent with the lack of

expression of SULT2A1 in dog^{10,52}. The undetectable SULT2A1-like activity of MLC could attribute to the significant interspecies differences on amino acid sequence of SULT2A1¹⁰. The multiple advantages made AB suitable as a probe for SULT2A1 even in tissue preparations of common experimental animal species. It could provide an important guidance for the further investigation of SULT2A1 mediated metabolism difference among species and the selection of rational model animals in preclinical studies for new drugs whose metabolism involves SULT2A1.

5. Conclusions

In conclusion, AB was identified as a highly selective probe for SULT2A1 throughout a series of reaction phenotyping studies. The interaction of AB with Met-137 and Tyr-238 of SULT2A1 were speculated as a cause for the elimination of substrate inhibition of SULT2A1. The sulfation of AB as a probe reaction of SULT2A1 exhibited high isoform selectivity and Michaelis–Menten kinetics which endowed the reaction with preferable accuracy and specificity in the quantification of SULT2A1 activity in various biological samples, in comparison with the frequently-used probe reaction DHEA sulfation. Based on the established method, the activities of SULT2A1 in human or laboratory animal tissue preparations were measured, which were found to be significantly varied.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.apsb.2018.07.007>.

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