Supplementary Information

A highly selective probe for UDP-glucuronosyltransferase 2B7 (UGT2B7) in human microsomes: isoform specificity, enzyme kinetics, and applications†

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Materials and Methods

Chemicals and reagents. ECB (compound **5**), EDCB (compound **6**), ERB (compound **7**), EBF (compound **8**), and other bufadienolides (compounds **1-4** and **9-16**) used for screening tests were isolated and prepared from crude materials of Chansu, microbial transformation of natural bufadienolides or from the biological samples isolated from rat after administration of Chansu by the authors (X.C. Ma, X.G. Tian, C. Wang and J. Ning).[1-7] Their chemical structures were unambiguously identified using NMR and MS techniques; and their purities were above 98% as determined by HPLC-DAD analysis. Androsterone, bilirubin, Brij 58, β-D-glucuronoside, estradiol, magnesium chloride, morphine, codeine, AZT, mefenamic acid, fluconazole, phenylbutazone and UDPGA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Millipore water (Millipore, Bedford, MA, USA), HPLC grade acetonitrile and methanol (Tedia, USA) were used. In addition, all other reagents used were either of HPLC grade or of the highest commercially available grade.

Enzyme sources. Pooled HLMs (prepared from the livers of 25 human donors of Mongoloid race, Batch number: LEV), lung (n=5, FOQ), kidney (n=5, FBY) and intestine (n=3, ZJL) extracts were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). In addition, pool male intestine (n=5, Batch number: LQS), male kidney (n=5, LLL), female kidney (n=3, OJG), male lung (n=5, VIN), female lung (n=3, MSX), and male liver (n=50, IGO) microsomes, were also purchased from Rild Research Institute for Liver Diseases (Shanghai, China). Animal liver microsomes used in

this experiment, including Swiss–Hauschka (ICR) mice (n=20, male, 18 to 20 g), Sprague–Dawley (SD) rats (n=10, male, 180–220 g), Cynomolgus monkeys (n=5, male, 4 years old), beagle dogs weighing about 10 kg (n=5, male, 12 months old) and Colony-bred Chinese Bama minipigs weighing 10 to 12 kg (n=8, male, 6 months old), were obtained from Rild Research Institute for Liver Diseases (Shanghai, China). A panel of recombinant human UGT Supersomes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) expressed in baculovirus-infected insect cells were purchased from BD Gentest Corp (Woburn, MA). The glucuronidation activity of each of the expressed UGTs was substantiated using the following substrates: bilirubin and estradiol (UGT1A1), 7-hydroxy-4-trifluoro-methylcoumarin (UGTs 1A3, 1A8, 1A10, 2B4, and 2B15), trifluoperazine (UGT1A4), 1-naphthol (UGTs 1A6 and 1A10), propofol (UGT1A9), morphine (UGT2B7), octylgallate (UGT1A7) and eugenol (UGT2B17). The catalytic activities for 12 recombinant UGT supersomes ranged from 250 to 12,000 pmol/min/mg protein. All microsome samples and recombinant human UGT isoforms were stored at -80℃ before using. A membrane preparation from insect cells infected with wild-type baculovirus (BD Biosciences) was used as a negative control.

Analytical instruments and conditions. The quantification of glucuronides by UFLC for screening were carried out using an a Shimadzu (Kyoto, Japan) Prominence ultra-fast liquid chromatography (UFLC) system, which was equipped with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, and a CTO-20AC column oven, as well as a diode array detector (DAD) detector. A Shim-pack XR-ODS column (150 mm × 2.0 mm,

2.2μm, Shimadzu) was kept at 40 °C. The mobile phase consisted of CH_3CN (A) and 0.2 % (v/v) formic acid (B). B used the following gradient condition, 0-2 min, 95-65 %; 2-7 min, 65-40 %; 7-7.5 min, 40-10%; 9.5-9.8 min, 10-5%; 9.8-14 min, 95%. The flow rate was set at 0.4 mL/min. The injection volume was 20 μL and the detection wavelength was set at 300 nm. The limits of detection and quantification were determined at signal to noise ratios of 3 and 10 times, respectively. The accuracy and precision of the back-calculated values for each concentration were less than 15 %.

Agilent 1200 HPLC system consisted of a quaternary delivery system, a degasser, an auto-sampler and UV-detector. The chromatograph was equipped with an Elite Kromasil C_{18} (150 mm × 2.1 mm, 3.5 μm) analytical column. The mobile phase was consisted of acetonitrile-0.1% formic acid aqueous solution (37:63, v/v) at a flow rate of 0.5 mL/min. The run time was 5.0 min for each injection, and the column temperature was controlled at 30^{o}C.

An Applied Biosystems MDS Sciex API 3200 Triple Quadrapole mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source was used and the system was operated in negative-ion mode from *m/z* 100 to 800. Optimization of MS conditions was carried out using standard solutions containing 500 ng/mL of EDCB, EDCBG and IS, delivered via a Harvard syringe pump at a constant flow rate of 8 μL/min. The quantification assay was performed using multiple reactions monitoring (MRM). The transition (precursor to product) used for EDCBG and EDCB were *m/z* 575.3 to 539.0, and *m/z* 401.3 to 365.0, respectively. The optimized ionspray voltage and temperature were

set at 5000 V and 500 °C, respectively. Nitrogen was used as the curtain gas and collision gas, which were controlled at 13 and 6 psi, respectively. The declustering potential (DP), entrance potential (EP) and collision energy (CE) for EDCB were 55, 9 and 27 V, respectively.

Data measurement was performed using the Analyst software version 1.6.1.

EDCB metabolism assay with recombinant UGTs. EDCB glucuronidation was measured in reaction mixtures containing eleven recombinant human UGT isoforms, namely the UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 isoforms. The incubations were performed as described for the HLMs study. Three substrate concentrations (5, 50 and 500 μM) and protein concentrations of 0.1-0.5 mg of protein/mL were used. All of the isoforms with different concentrations of EDCB were incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 mL of acetonitrile, and the reaction mixtures were then centrifuged to precipitate the proteins before using HPLC-DAD-MS/MS to monitor the *in vitro* metabolites.

Chemical inhibition studies. The glucuronidation of EDCB (100 μM) catalyzed by HLMs (0.05 mg/mL protein concentration) was measured in the absence and presence of known UGT-selective inhibitors/probes performed for 30 min. Fluconazole (3 mM), AZT (1 mM), mefenamic acid (100 μM), diclofenac (400 μM), estradiol (200 μM), phenylbutazone (500 μM) and bilirubin (50 μM) were used to determine the contribution of UGTs 2B7, 2B7, 1A1/1A9/2B7, 1A9/2B7, 1A1/2B7, 1A subfamily and 1A1, respectively, to glucuronidation of EDCB. One group without inhibitors but the same volume of solvent was used as the control group. Using the same conditions, the inhibitory effect

of increased concentrations of mefenamic acid (0.1-10 μM) and diclofenac (1-100 μM) were then evaluated on EDCB glucuronidation in HLM and rhUGT2B7 isoforms, to determine the individual IC_{50} values.

EDCBG formation kinetic study. The formation of EDCB glucuronide was linear with incubation times up to at least 120 min and microsomal protein concentrations, up to at least 0.3 mg/mL using both HLM and rhUGT2B7. The maximal solubility of EDCB in the incubation system was 400 μM. To estimate the kinetic parameters, EDCB (0.5-400 μM) was incubated with different sources of pooled human liver microsomes with a 0.05 mg/mL protein concentration for 30 min. For recombinant UGT isoforms, EDCB was incubated with rhUGT2B7 (0.05 mg/mL) during 30 min for the kinetic analysis using the same experimental conditions. Substrate concentrations and reaction velocities were analyzed using Eadie-Hofstee plots to determine the kinetic type. The V_{max} and K_m values were obtained from a nonlinear regression between substrate concentrations and reaction velocities using the Michaelis-Menten equation. All incubations were performed in duplicate in three independent experiments.

Data analysis and statistics. The kinetic constants for EDCB glucuronidation by HLMs or rhUGT2B7 were obtained according to the Michaelis-Menten equation using the Origin software (OriginLab Corporation, Northampton, MA). The CL_{int} value was calculated based on the ratio of the V_{max} and K_m values, where V_{max} is the maximum reaction velocity and K_m is the Michaelis-Menten

constant representing the substrate concentration at which the velocity was half of that at V_{max}. The IC_{50} value, representing the inhibitor concentration that inhibits 50% of the control activity, was determined by nonlinear curve fitting using the Origin software. The kinetic constants and IC_{50} values are reported as the value ± S.E. of the parameter estimate.

Preparation of EDCB and its predominant metabolite (EDCBG) by in vitro biosynthesis. Briefly, the preparative scale biotransformation of cinobufagin (CB, a major bufadienolide) by *Penicillium aurantigriseum* AS 3.4512 was performed in 1000 mL Erlenmeyer flasks. After 36 h of pre-culture, 10 mg of substrates and 0.5 mL acetone were added into 350 mL of culture medium. In total, 100 mg of CB was used for preparative biotransformation. The incubation was continued for another 36 h. The culture was then filtered, and the filtrate was extracted with the same volume of EtOAc five times. The organic phase was collected and concentrated *in vacuo*. The residues were applied to an ODS column and eluted with MeOH-H_2O (75:25) to obtain EDCB (65 mg, yield 65%) with a high purity level above 98%, and its NMR data was listed in Table S1 (Fig.S6) .

EDCB (400 μM) was incubated in 1 mL reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 50 mM $MgCl_2$, 4 mM UDPGA, and DLM (5 mg/mL) purchased from the Rild Research Institute for Liver Diseases (Shanghai, China). The incubation lasted for 5 h at 37℃ in a Thermo-shaker. The reactions were terminated by the addition of methanol (500 μl), followed by centrifugation at 20,000 g for 20 min at 4℃. Then, the supernatant was concentrated to 200 μL using a rotary evaporator at 37 ℃ for further preparation of the EDCB metabolite. In total, 10 mg of EDCB were used to prepare

EDCBG. The extraction method mentioned above was performed using a HPLC-DAD equipped with an Inertsil ODS-3 chromatographic column (4.6 × 250 mm, 5μm), and eluted with acetonitrile and water containing 0.3% TFA (35:65) to obtain EDCBG (9.5 mg, yield 95%). The mobile phase was injected at a flow rate of 1.5 mL/min. Its purity was approximately 98% by HPLC-UV analysis at 300 nm of wavelength. (Fig. S6 E)

Western blotting and correlation analysis. Recombinant UGT2B7 and human liver microsomes from 14 individuals were analyzed by western blotting using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% acrylamide gels) and transferred onto PVDF membranes (Millipore, Bedford, MA) to determine the expression levels of UGT2B7 in these human liver microsomes. The blots were probed with an anti-UGT2B7 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 UGT2B7 isoform expression values were measured using a ScanImage software. In addition, the formation rates of EDCB (50μM, near the Km of EDCB in HLM) were determined using a series of HLMs from 14 individual donors. Then, the UGT2B7 isoform expression values in these 14 individuals, obtained from western blotting, were compared. Substantial correlation parameters between UGT2B7 protein expression and EDCB glucuronidation levels were also measured using a linear regression coefficient (r). A value of $P<0.05$ was considered statistically significant.

Computational modeling. Molecular modeling has been performed to simulate the interaction modes between the *epi*-isomers of 3-OH (EDCB and DCB) and UGT2B7 to investigate the differences between their metabolic behaviors. Because the integral 3D structure of UGT2B7 has already been crystallized and described in a previous publication, we first constructed a homology model for UGT2B7 using the FUGUE and ORCHESTRAR packages within the SYBYL 7.3 package. The developed model took into consideration the Glu donor and the substrate binding domain. A UDP glycol donor was extracted from the UDP-glucosyltransferase GtfB (PDB code: 1IIR) template to represent UDPGA (UDP-glucuronosyltransferase). With the homology model, the possibly interacting bioactive conformations of the ligand can be mimicked using the Surflex-Dock package, implanted within the SYBYL package. Being a knowledge-based docking method, the Surflex-Dock package employs an idealized active site, named a protomol, as a target to generate the putative conformations of molecules or molecular fragments. Using this method, a substrate binding site was recognized within the homology model of UGT2B7, and the *epi*-isomers were docked into the active site to illustrate their binding conformations. When performing the docking simulations, the parameters of the protomol threshold and bloat were set at 0.5 Å and the float was set at 3 Å. The chemscore function was employed to evaluate the binding affinities. Generally, the EDCB conformations exhibited lower chemscore function values, suggesting a stronger binding affinity than that of DCB. First, the distribution of chemscore values for DCB and EDCB were compared (Fig. S12, ESI†). Subsequently, we analyzed the binding coordination of the isomers to determine the possible metabolic behavior. For

EDCB, among 30 binding conformations, 5 were located with the 3-hydroxyl group near the end hydroxyl group of the uridine diphosphate, within a distance of 5.03 Å from where the catalysis will probably take place during the transfer of the glucuronide by a nucleophilic attack. However, only 2 DCB conformations positioned the 3-hydroxyl group near the UDP. Unlike for EDCB in which the 3-hydroxyl group is located at the end hydroxyl group, the DCB 3-hydroxyl group is located towards the middle carbonyl group of the uridine diphosphate, at a distance of 6.93 Å from the end hydroxyl group, and is thereby a less probable glucuronide transfer site.

References

1. Xin XL, Su DH, Sa D, Tian Y, Zhang BJ, Ma XC, An L, Huang SS, Yao JH, Cui X. (2011). Novel microbial transformation of resibufogenin by *Fusarium solani*. J Asia Nat Prod Res. 13: 831-7.

2.Ma XC, Cui J, Zheng J, Guo DA. (2007). Microbial transformation of three bufadienolides by *Penicillum aurntigriseum* and its application for metabolite identification in rats. J Mol Cat B: Enzymatic. 48: 42-50.

3.Ma XC, Zheng J, Guo DA. (2007). Highly selective isomerization and dehydrogenation of three major bufadienolides at 3-OH by *Fusarium solani*. Enz Microb Technol 40:1585-91.

4. Ma XC, Xin XL, Liu KX, Han J, Guo DA. (2009). Microbial transformation of cinobufagin by *Syncephalastrum racemosum*. J Nat Prod 71:1268-70.

5.Ning J, Wu TH, Tian Y, Wang CY, Tian G, Zhang BJ, Liu KX, Ma XC. (2010) Identification of cinobufagin metabolites in the bile of rats. Xenobiotica 40:48-54.

6.Xin XL, Liu JY, Ma XC, Wei Q, Lv L, Wang CY, Yao JH, Cui J. (2010) Preparative Separation of Four Major Bufadienolides from the Chinese Traditional Medicine, Chansu, Using High-Speed Counter-Current Chromatography. *Natural Product Communications 5*:1031-1034.

7. Li JK, Ma XC, Li FY, Wang JK., Chen HR, Wang G, Lv X, Sun CK, Jia JM., (2010) Preparative separation and purification of bufadienolides from Chinese traditional medicine of ChanSu using high-speed counter-current chromatography. *J. Sep. Sci. 33*, 1325-1330.

Supplementary Figures

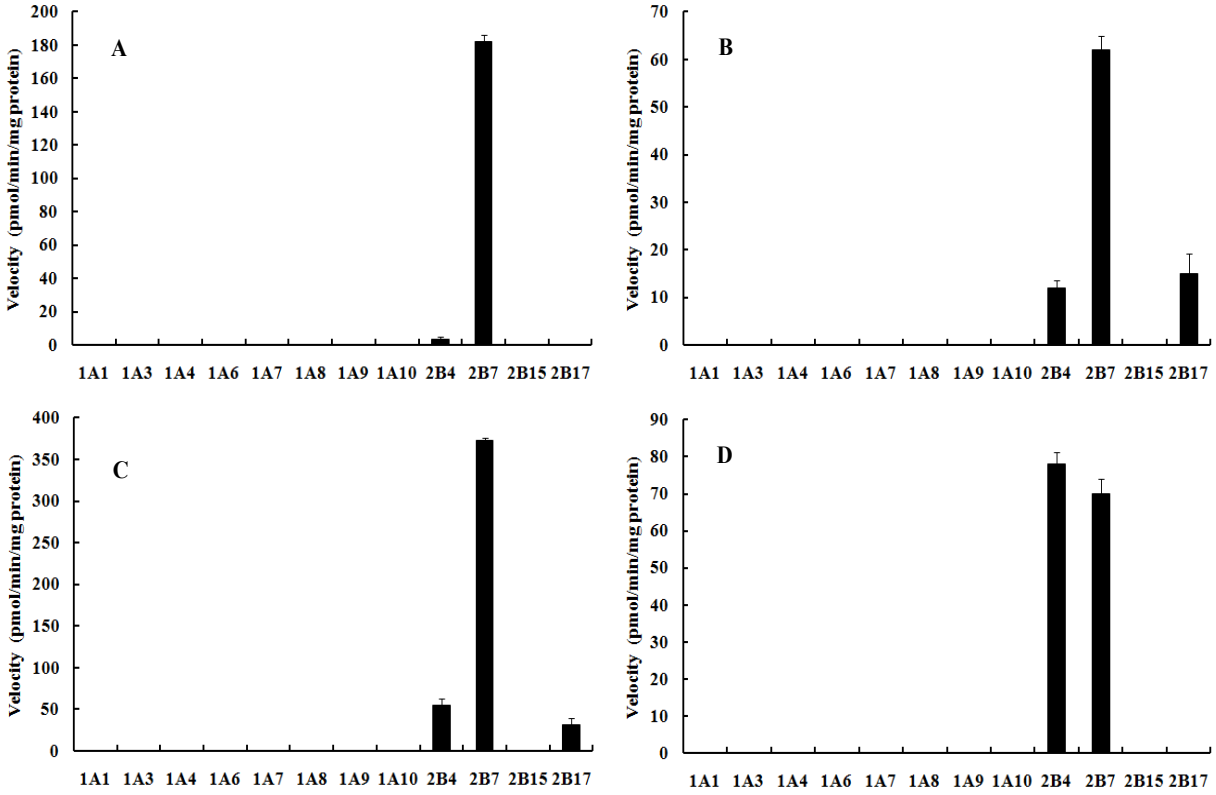

Fig. S1 The selectivity comparison of EDCB with UGT2B7 substrates (AZT, morphine and codeine) reported in previous investigation, by using individual probe reaction at the concentrations of 500 μM. (A) EDCB (for 3-glucuronidation), (B) AZT, (C) morphine (for 6-glucuronidation) and (D) Codeine. ECDB displayed a more significant isoform-selectivity than AZT, morphine and codeine.

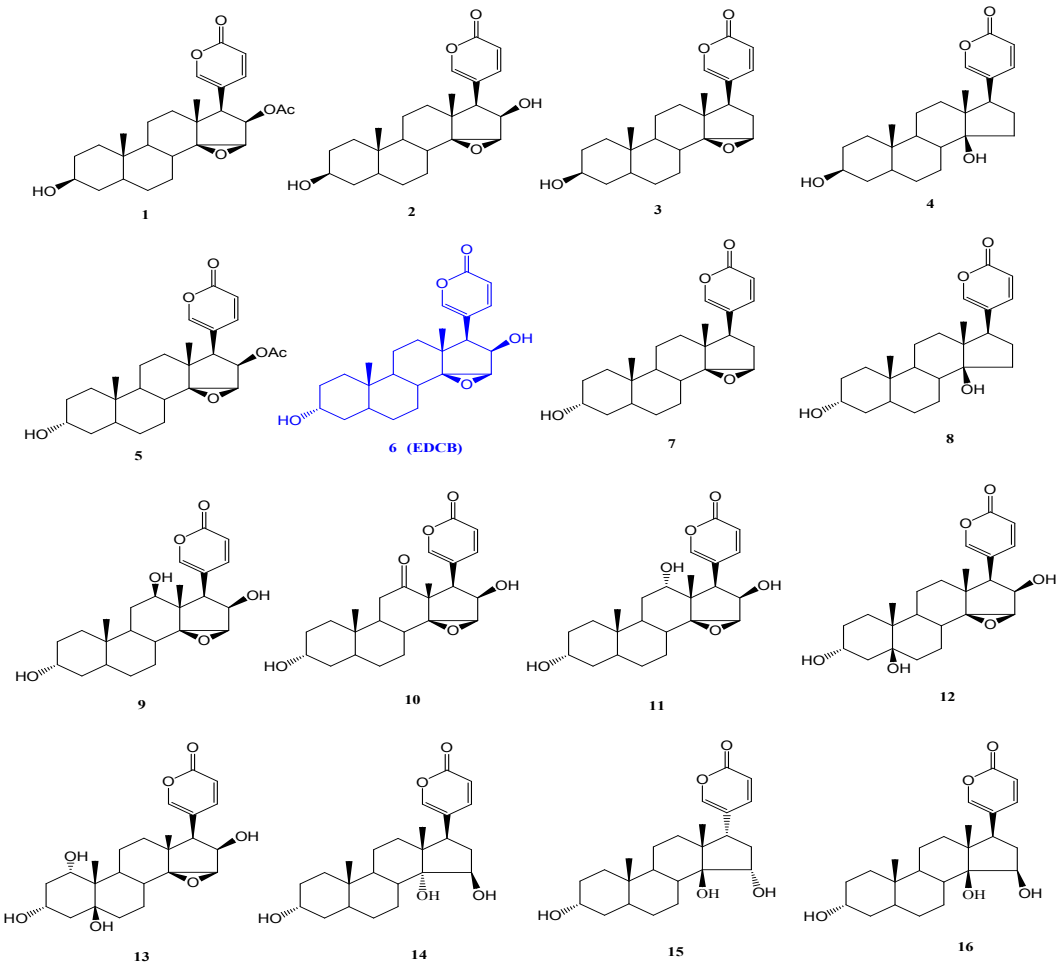

Fig. S2 Chemical structures of bufadienolides (**1-16**) used in present work

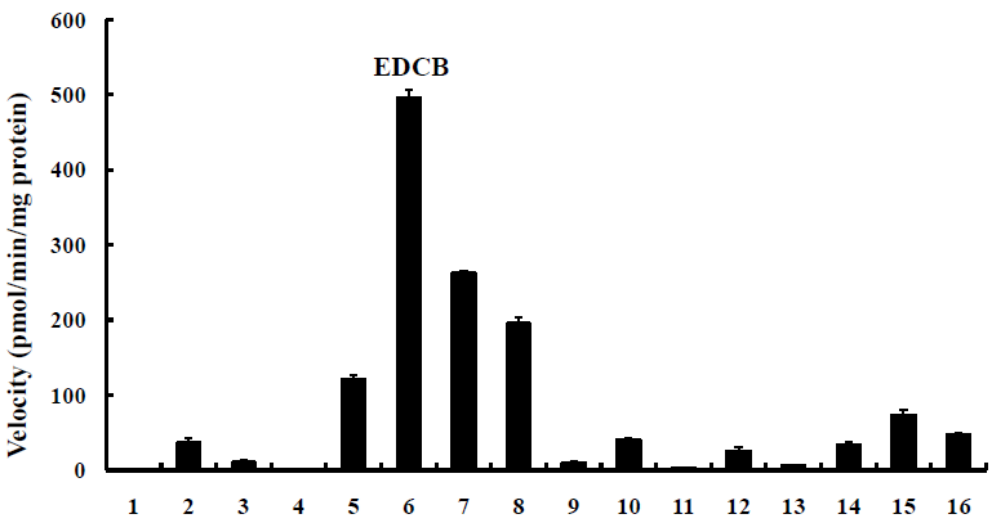

Fig.S3 The glucuronidation velocities of bufadienolides (**1-16**) by HLMs

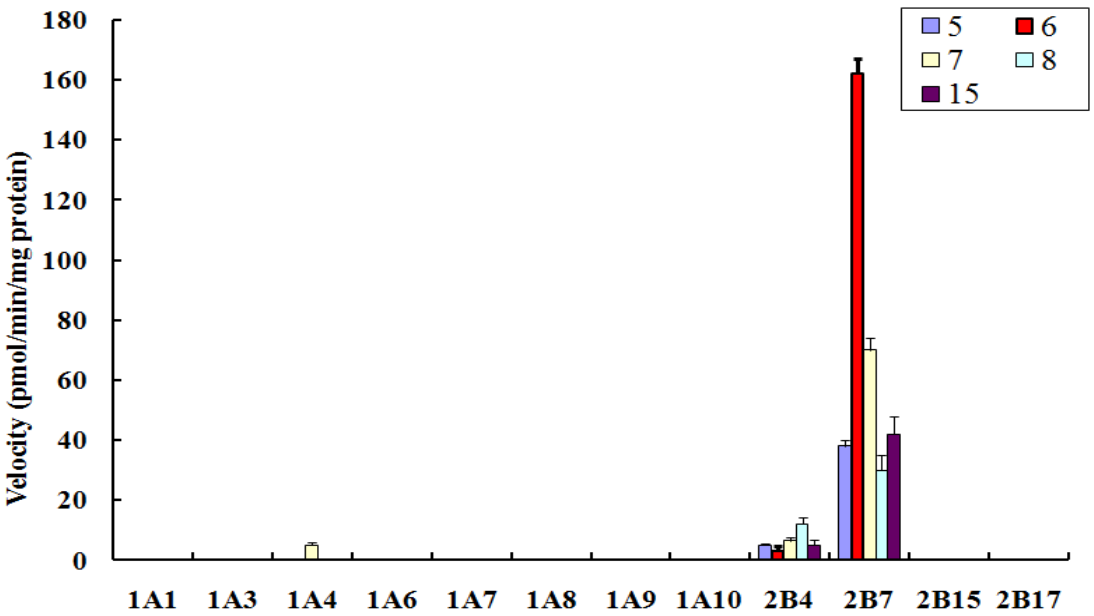

Fig. S4 The isoform-specificity of compounds **5-8** and **15** that had the high relative velocity of glucuronidation in HLMs at concentration of 400μM

5

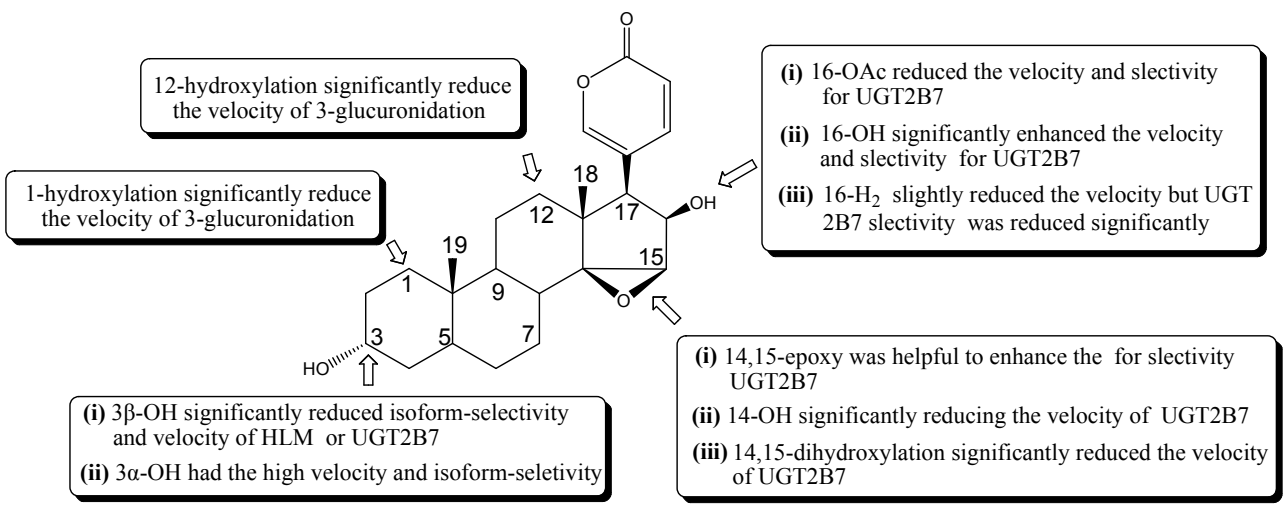

Fig. S5 The relationships of chemical structures of bufadienolides with isoform-selectivity and velocity for UGT2B7 enzyme

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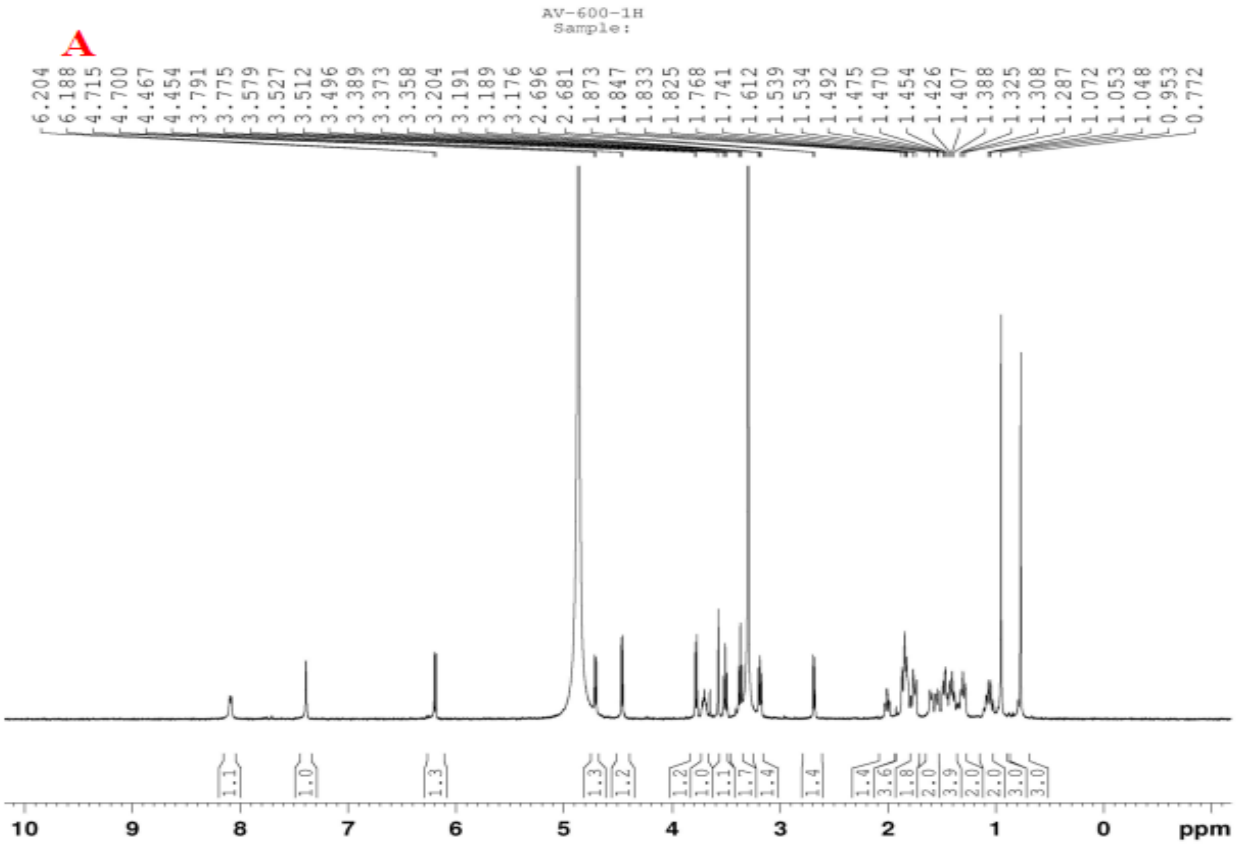
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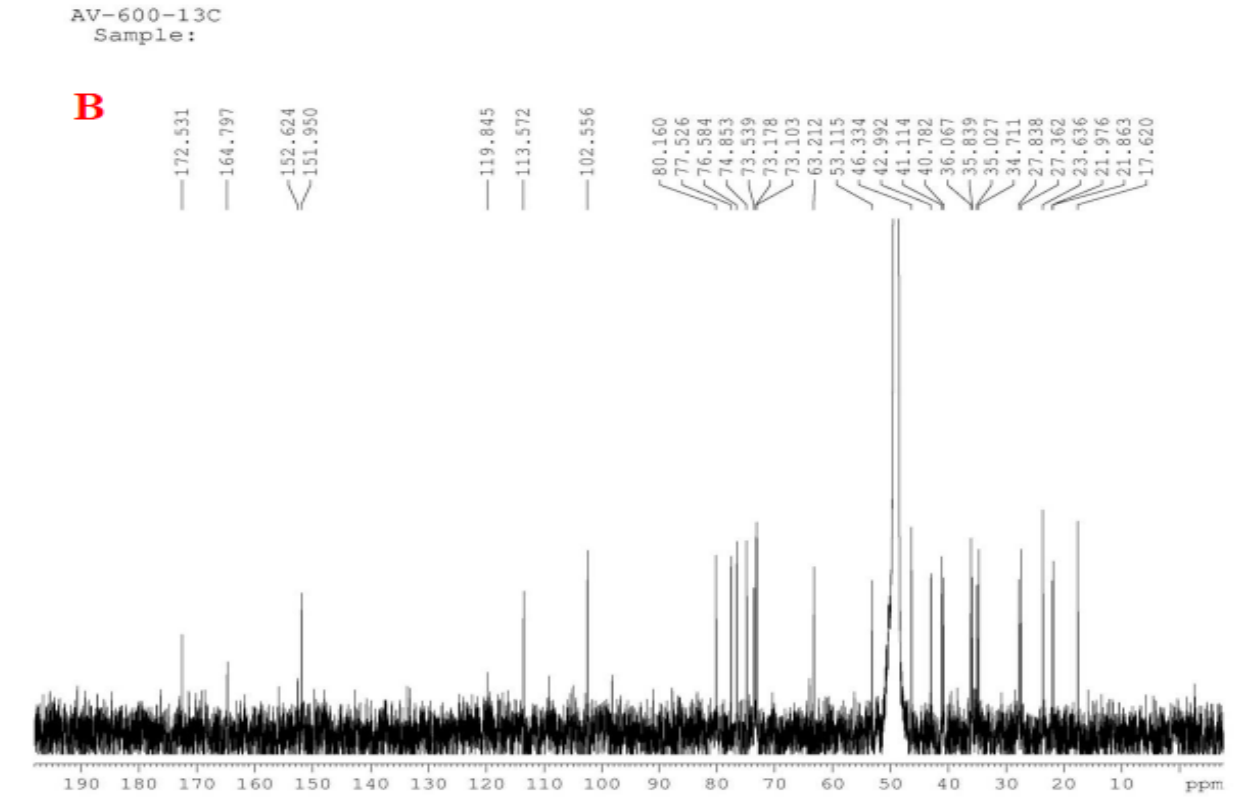
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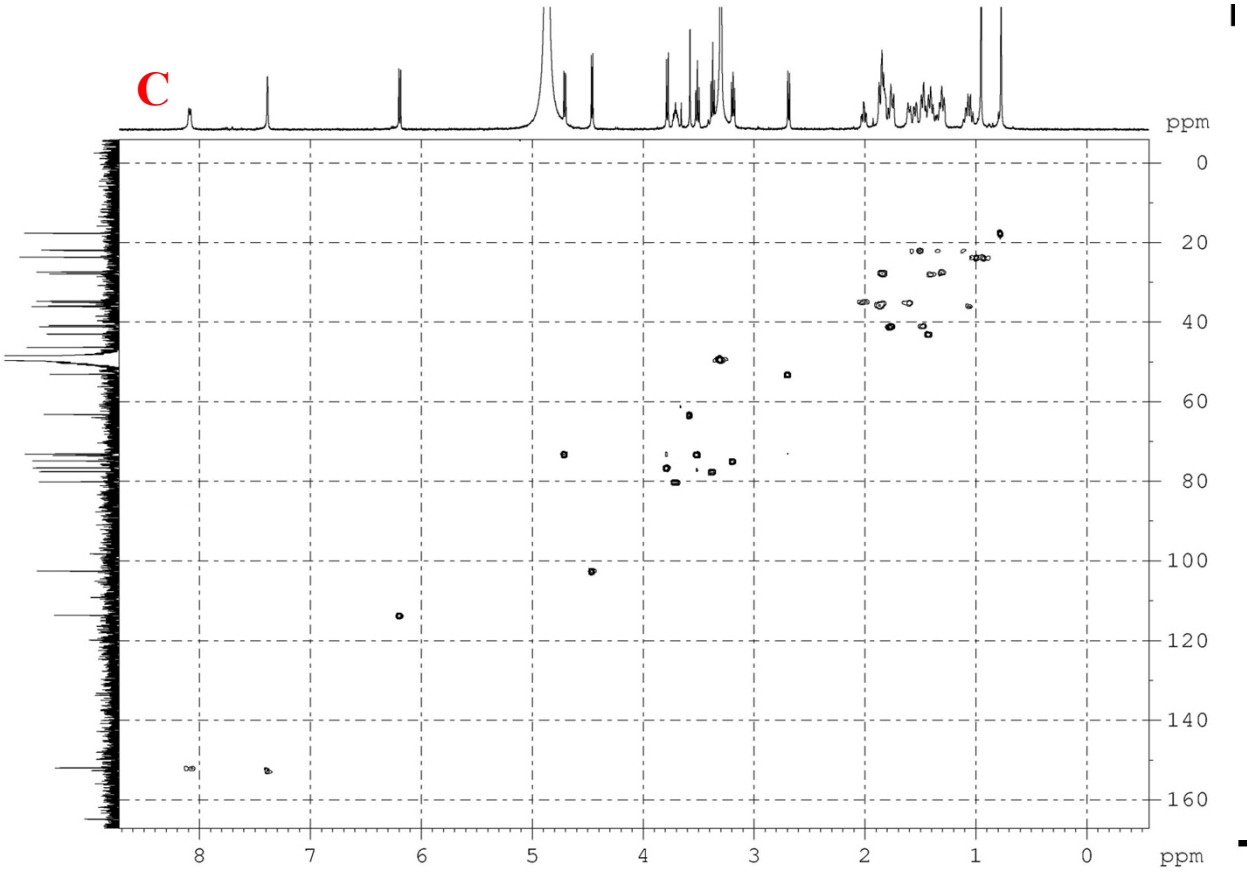
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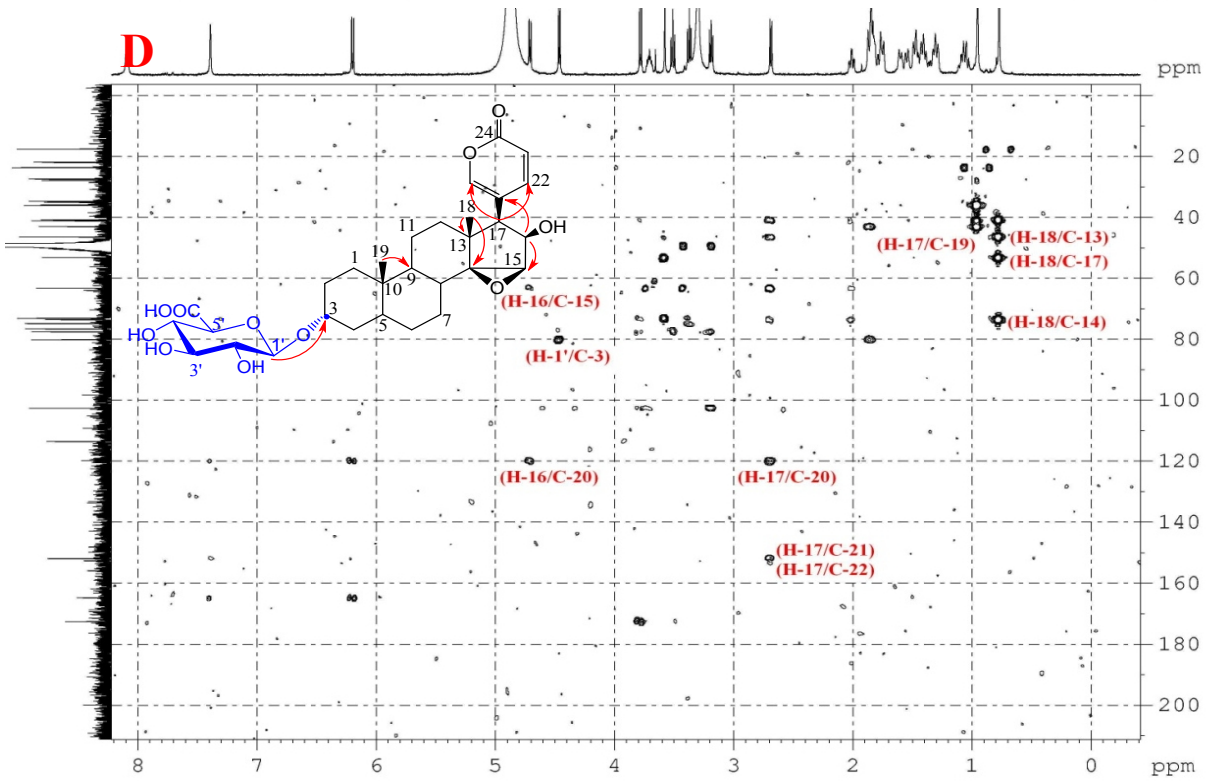
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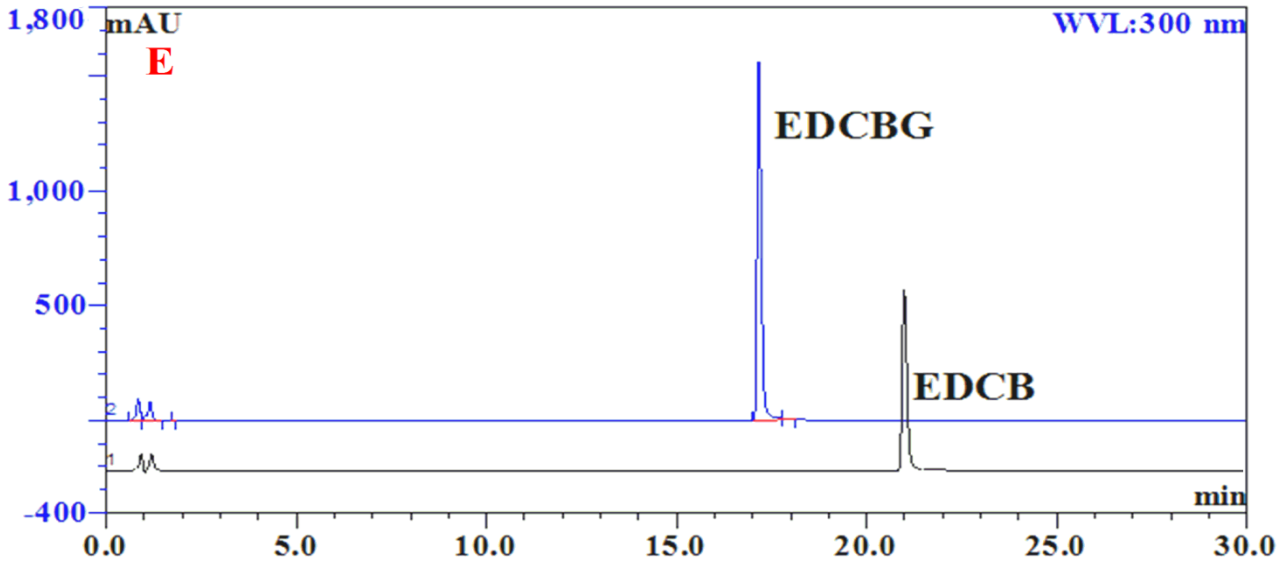

Fig. S6 NMR spectral data of 3-EDCBG in MeOD. (A) ^{1}H-NMR (600MHz); (B) ^{13}C-NMR (150MHz); (C) HMQC; (D) HMBC and HPLC chromatograms of EDCB and EDCBG at 300 nm (E).

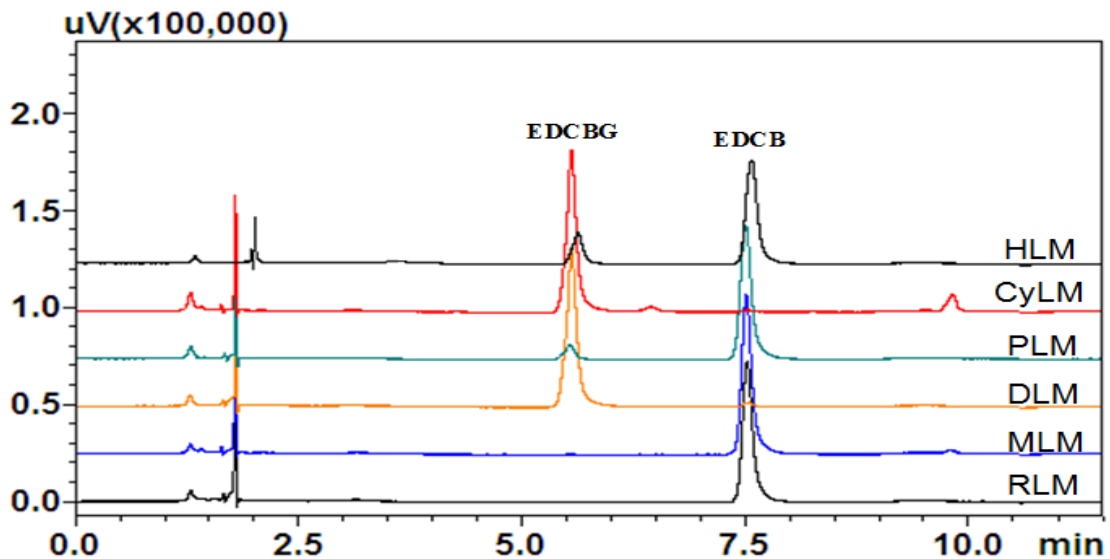

Fig. S7 Representative UFLC profiles of EDCB and its metabolites in liver microsomes from human (HLM), monkey (CyLM), minipig (PLM), dog (DLM), mouse (MLM) and rat (RLM). EDCB (200μM) was incubated with liver microsomes (0.3 mg/ml) from different species and an UDPGA-generating system at 37°C for 30 min. **The beagle dog liver microsomes (DLMs) could be used as an effective bioreactor for preparing the large amount of EDCBG as the standard compounds to determine UGT2B7 activity in the different biological samples.**

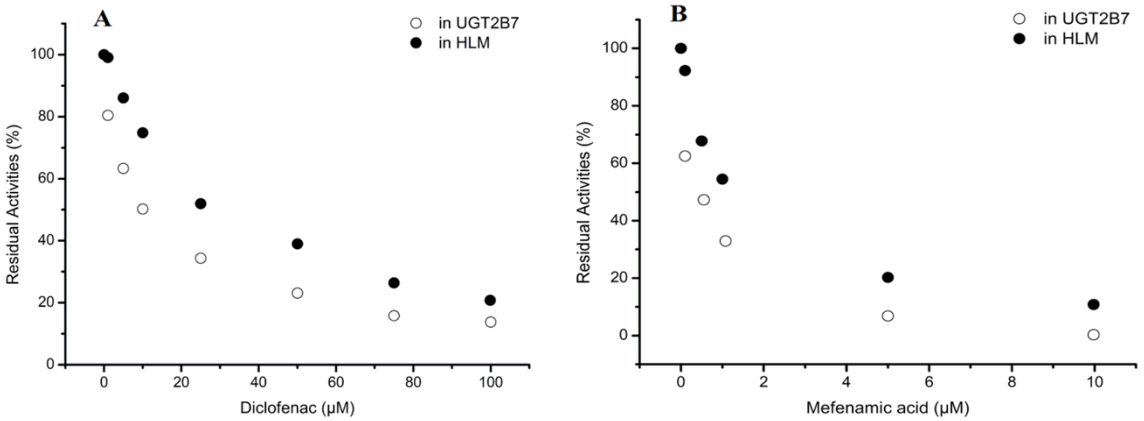

Fig. S8 The inhibition of Diclofenac (**A**) and Mefenamic (**B**) on UGT2B7 and HLMs. Each data point represents the mean of duplicate determinations.

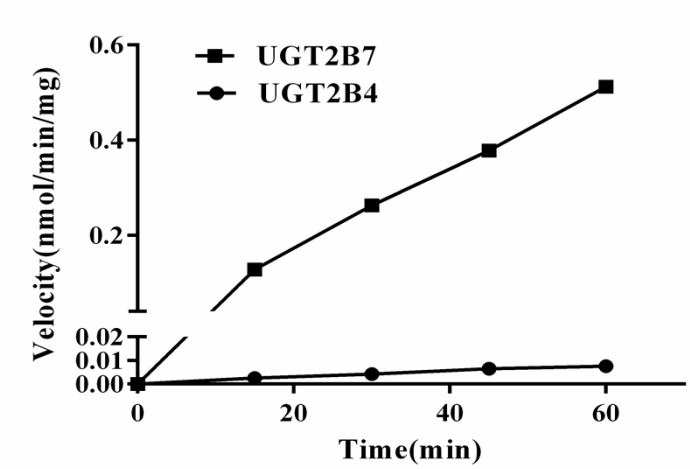

Fig. S9 Time courses of EDCB-glucuronidation by UGT2B7 (5 nM) or UGT2B4 (40 nM). The final substrate concentration was 50 μM. Each data point represents the mean of triplicate determinations.

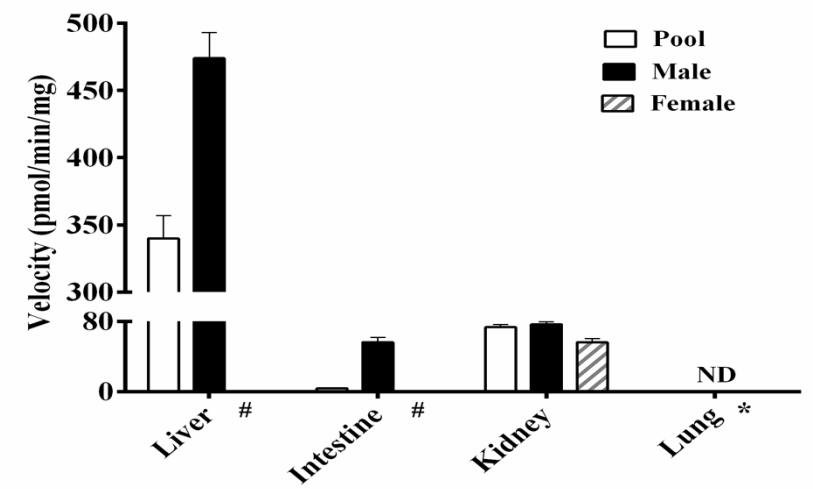

Fig. S10 EDCB 3-glucurondiation formation rate by human organ microsomes including liver, intestine, kidney and lung (male and female). Due to the unexpressed UGT2B7 in lung (Ref 3), EDCBG was not detected at all. (* the expression of UGT2B7 in human lung was very low; # liver and intestine microsomes of female were unavailable in present work)

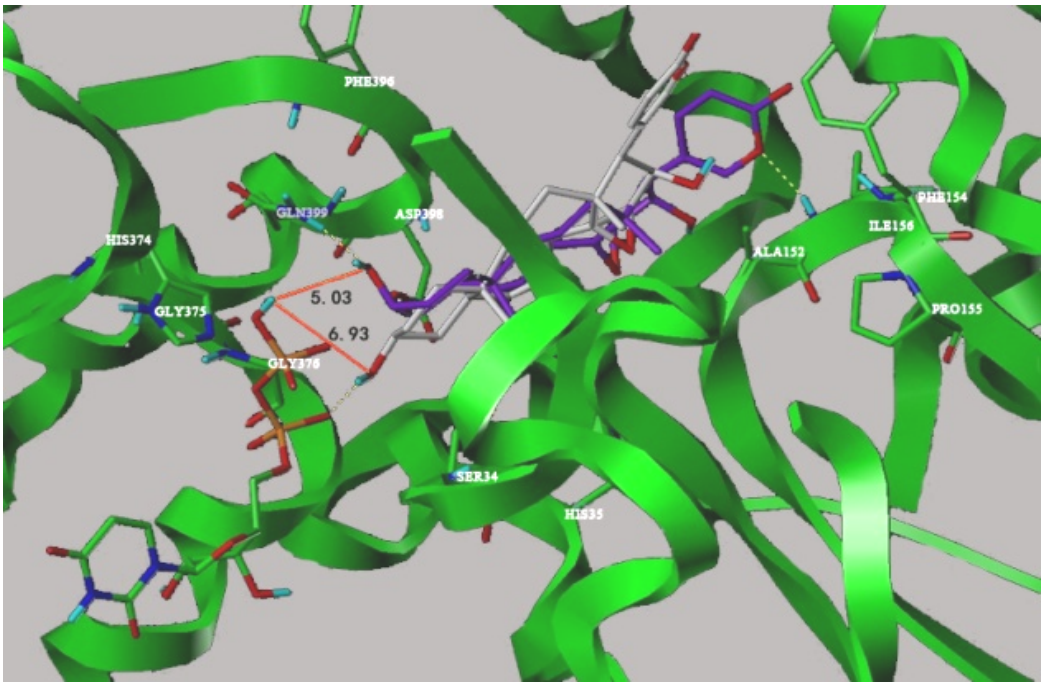

Fig. S11 Binding conformations of EDCB and DCB with UGT2B7. EDCB by violet (5.03 Å) and DCB is atom-type color (6.93 Å).

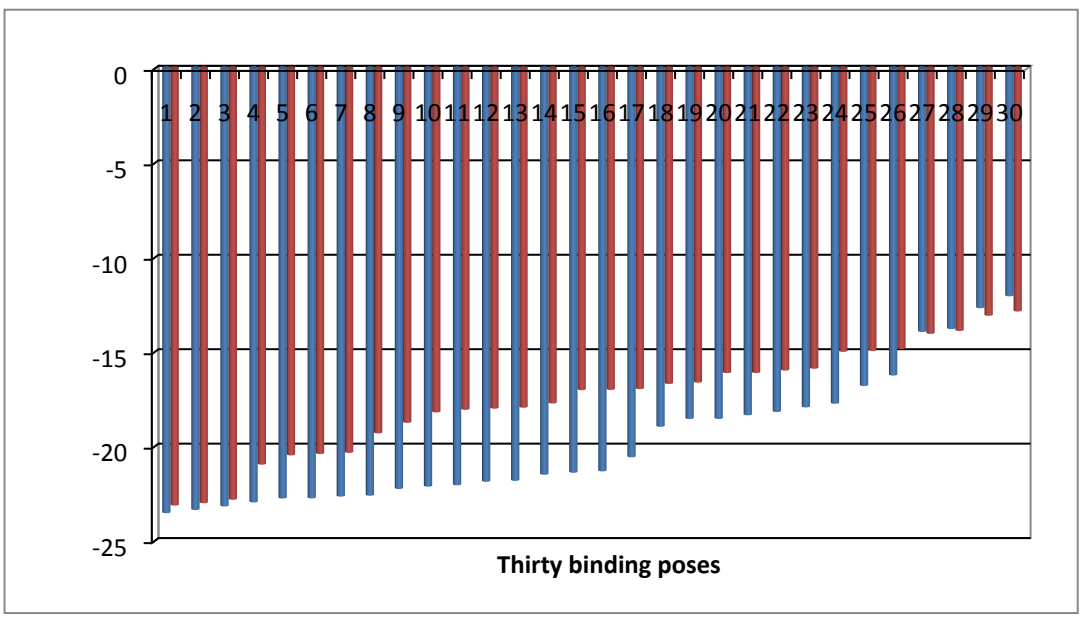

Fig. S12 The distribution of Chemscore values for the binding conformations of EDCB (blue) and DCB (red) within the human UGT2B7.

Table S1 ^{1}H NMR and ^{13}C NMR spectral data of **EDCB** and **EDCBG**

No.	EDCB (DMSO-d_6)		EDCBG (Methanol-d_4)	
	δ_H	δ_C	δ_H	δ_C
1	1.23 m 1.51 m	30.3	1.40 m 1.82 m	27.8
2	1.39 m 1.64 m	34.6	1.06 m 1.87 m	35.8
3	3.40 m	69.8	3.72 m	80.2
4	1.03 m 1.33 m	36.0	1.59 m 1.05 m	35.0
5	1.90 m	41.1	1.76 m	41.1
6	1.18 m 1.58 m	26.0	1.31 m 1.46 m	27.4
7	0.97 m 1.41 m	20.3	1.19 m 1.34 m	21.9
8	1.76 m	32.9	2.03 m	34.7
9	1.63 m	38.9	1.43 m	43.0
10	---	34.8	---	36.1
11	1.17 m 1.30 m	20.4	1.49 m 1.58 m	22.0
12	1.45 m	39.0	1.47 m	40.8
13	---	44.4	---	46.3
14	---	70.6	---	73.5
15	3.53 s	62.0	3.66 s	63.2
16	4.65 dd (9.0, 5.0)	71.7	4.71 d (9.0)	73.1
17	2.60 d (9.0)	50.8	2.69 d (9.0)	53.1
18	0.66 s	17.1	0.77 s	17.6
19	0.88 s	23.1	0.95 s	23.6
20	---	117.9	---	119.8
21	7.45 d (2.0)	151.1	7.39 s	152.6
22	7.92 brd (9.5)	150.0	8.09 d (9.0)	152.0
23	6.17 d (9.5)	112.0	6.20 d (9.0)	113.6
24	---	161.3	---	164.8
1'	---	---	4.46 d (7.8)	102.6
2	---	---	3.18 m	74.9
3'	---	---	3.36 m	77.5
4'	---	---	3.53 t (9.0)	73.2
5'	---	---	3.78 m	76.6
6'	---	---	---	172.6

Table S2 The3-glucuronidation related parameters derived from the molecular modeling of EDCB and DCB with UGT2B7 enzyme

Parameters	EDCB	DCB
Chemscore value	-22.11	-17.99
Site-UDP distance	5.03Å	6.93Å

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