Chemico-Biological Interactions 302 (2019) 190–195

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

# Phase II metabolism of betulin by rat and human UDP-glucuronosyltransferases and sulfotransferases

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ARTICLE INFO

*Keywords:*
Betulin
Glucuronidation
Sulfation
Isoenzymes

ABSTRACT

The natural product betulin is under investigation for several therapeutic indications, however little is known about its metabolism. In the present study, the glucuronidation and sulfation of betulin in human and rat liver microsomes and cytosol were tested. We further identified the main UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) involved in these two metabolism pathways. Results showed that one betulin glucuronide metabolite was observed after incubation with human and rat liver microsomes. The glucuronidation of betulin in human liver microsomes had a $K_m$ value of 21.1 ± 5.93 μM and a $V_{max}$ value of 6.39 ± 0.66 pmol/min/mg protein. The glucuronidation activity in rats was too low to get enzyme kinetic parameters. Among the 11 recombinant UGT enzymes investigated, UGT1A3 and UGT1A4 were identified as the major enzymes catalyzing the glucuronidation of betulin [$K_m$ values of 10.12 ± 8.09 and 8.04 ± 3.96 μM, $V_{max}$ values of 6.71 ± 1.51 and 5.98 ± 0.76 nmol/min/(mg protein)]. Two betulin sulfate metabolites were found in human and rat liver cytosols. Human and rat liver had similar affinity for the formation of these two metabolites, the apparent $V_{max}$ for betulin sulfate I was higher than that for betulin sulfate II in both species. Among the SULT isoforms studied, SULT2A1 was the major isoenzyme involved in the betulin sulfation metabolism in human liver cytosol. The results suggest that glucuronidation and sulfation are important metabolism pathways for betulin, and UGT1A3, UGT1A4 and SULT2A1 play the major roles in betulin glucuronidation and sulfation.

## 1. Introduction

Betulin, 3β-lup-20(29)-en-3, 28-diol (Fig. 1) is an abundant naturally occurring pentacyclic triterpene, which is also named betulinol, trochton, birkenkampher, and (coryli-) resinol [1]. The main source of betulin is birch bark (up to 30% of dry weight). The dominance of betulin in extractives is reported to give white birch bark its white color [2].

Betulin has been identified as possessing a wide range of pharmacological effects such as antivirus [3], cytoprotective activity [4,5], anticonvulsant and antilithic activity [6,7]. Betulin exhibits antitumor activity against many types of tumor cell lines: cervix carcinoma HeLa cells, hepatoma HepG2 cells, lung adenocarcinoma A549 cells, breast cancer MCF-7 cells, human gastric carcinoma, and human pancreatic carcinoma [8,9]. Betulin triggers apoptosis of human cancer cells through the intrinsic apoptotic pathway [9]. As a promising clinical medication, betulin has received increasing attention over the past few decades. Hence, to understand the metabolism of betulin is essential to enhance the utilization of betulin.

According to our previous study, $C_{max}$ value of betulin in rat plasma was 59.6 ± 23.0 ng/mL after 500 mg/kg oral administration [10]. The solubility of betulin was only 80 ng/mL in water [11]. Due to the very poor water-solubility and bioavailability, it is difficult to directly study betulin metabolism and its metabolites *in vivo*. *In vitro* metabolism studies can target specific metabolic reactions that are expected to occur based on the structure of the drug substrate, and in vitro metabolism is suitable for drugs with low metabolic conversion rate and lack of sensitive detection *in vivo* [12]. For example, ursolic acid and oleanolic acid, two pentacyclic triterpenoids, were mainly metabolized by Phase II enzymes [13,14]. From its structural similarity with ursolic

*Abbreviations:* UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; UDPGA, Uridine diphosphoglucuronic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TLC, Thin Layer Chromatography; HTC, 7-hydroxy-4- (trifluoromethyl) coumarin; DHEA, dehydroepiandrosterone

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https://doi.org/10.1016/j.cbi.2019.02.009
Received 7 November 2018; Received in revised form 31 January 2019; Accepted 14 February 2019
Available online 15 February 2019
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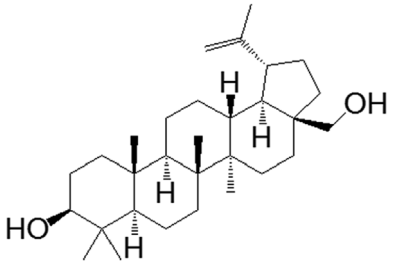


**Fig. 1.** Structure of betulin.

acid and oleanolic acid, we hypothesize that Phase II metabolism would be the main metabolic pathway for betulin.

Glucuronidation and sulfation are the two major phase II pathways for hydroxylated compounds. In the present study, human and rat liver microsomes and cytosol were used as the enzyme sources to examine the conjugation of betulin. A radiochemical method was used to discover and quantitatively analyze these new metabolites. A battery of recombinant human UGT and SULT enzymes were used to facilitate identification of the UGT and SULT enzymes involved in betulin glucuronidation and sulfation.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Betulin (purity > 95%) was purchased from Boyle Chemical Co., Ltd (Shanghai, China). Uridine diphosphoglucuronic acid (UDPGA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) was purchased from Dr. S.S. Singer, University of Dayton, OH, USA. $^{14}$C-UDPGA (313 mCi/mmol) and $^{35}$S-PAPS (2.01 Ci/mmol) were purchased from PerkinElmer (Boston, MA, USA). The $^{14}$C label was in the carboxyl group of glucuronic acid and the $^{35}$S was in the sulfate group of PAPS. The radiolabeled UDPGA was diluted with unlabeled material to a specific activity of 1.76 mCi/mmol, radiolabeled PAPS was diluted with unlabeled material to a specific activity of 140 mCi/mmol before use in assays. All other chemicals were of the purest grade and were purchased from commercial suppliers.

### 2.2. Liver subcellular fractions and enzymes

Female Sprague-Dawley rats of 52 weeks (adult) were purchased from Charles River Laboratories and housed under constant temperature and humidity conditions in conventional cages, kept on a 12-h light/dark cycle and given free access to food and water. Studies were performed after approval by the local Institutional Animal Care and Use Committee. Rat liver was harvested after rats were euthanized by carbon dioxide. Human liver samples from 4 male donors, ages 31–49 years, were obtained from tissue banks under an exempt protocol approved by the University of Florida Institutional Review Board. Rat and Human liver samples were processed into subcellular fractions as described previously [15], and stored at −80 °C under the protection of nitrogen until use.

hUGT Supersomes: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15 from cDNA-transfected insect cells expressing individual human UGTs were obtained from BD Gentest (Woburn, MA).

hSULTs: Expression in *Escherichia coli* of hSULT1A1, 1A3, 1B1, 1E1, and 2A1 have been described previously [16]. Cell lysates for each enzyme were used without purification.

### 2.3. Glucuronidation assays

Assay conditions were optimized to ensure that product formation was linearly relate to protein concentration and reaction time. The concentrations of betulin were selected with the requirement of no inhibition to the reaction. All studies were conducted with hepatic microsomes or isoenzymes from three different individuals, for each assay condition.

A radiochemical method was used with saturating concentrations of UDPGA, 1 mM, in the present study. Assay tubes contained, in a volume of 100 μL, betulin (5–75 μM), 100 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.6), 0.25 mg human liver microsomal protein or 0.1 mg hUGT isoenzyme protein. The microsomes were mixed with an optimal ratio of Brij 58, 0.5:1, and placed on ice for 20 min before use. In all experiments, betulin was added to tubes in methanol solution (10 μL) and methanol was removed under nitrogen before the addition of other components. The tubes were then incubated in a 37 °C shaking water bath for 1 min. To start the reaction, 1 mM $^{14}$C-UDPGA was added to each tube. Both incubated blanks with no substrate and zero time blanks with substrate were included. After 0 min (zero time blank) or 2 h (samples and incubated blanks with no substrate), the reaction was stopped by the addition of 150 μL of ice-cold methanol, followed by vortexing and centrifugation. The supernatant was transferred into a new tube and analyzed by Thin Layer Chromatography (TLC).

### 2.4. Sulfotransferase assays

The reaction conditions were optimized so that product formation was linearly related to protein concentration and reaction time. A saturating PAPS concentration of 20 μM was used. The concentrations of betulin were selected with the requirement of no inhibition to the reaction. All studies were conducted with hepatic cytosol from three different individuals, or expressed isoenzymes, for each assay condition.

Betulin sulfation was assessed with a radiochemical assay using $^{35}$S labeled PAPS. A concentration series of 0.5–20 μM betulin were incubated with human liver cytosol (0.25 mg protein), or rat liver cytosol (0.1 mg protein), or hSULT1A1, 1A3, 1B1, 1E1, and 2A1 (1–5 μg lysate protein), 100 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 20 μM $^{35}$S-PAPS (diluted with unlabeled PAPS to a specific activity of 140 mCi/mmol), and 0.4% BSA in a volume of 100 μL. The reaction mixture was pre-incubated at 37 °C for 1 min; then the reaction was initiated by adding PAPS. After incubation for 30 min at 37 °C with gentle shaking, the reaction was terminated by adding 150 μL of ice-cold methanol. Blanks were stopped immediately after the addition of PAPS. After centrifugation (3500 rpm for 10 min), the supernatant was transferred into new tubes and analyzed by TLC.

### 2.5. TLC detection of betulin metabolites

Betulin glucuronidation and sulfation assay samples (50 μL) were loaded to the pre-adsorbent phase of LK5DF Plates and developed in a solvent system containing 1-butanol: acetone: acetic acid: ammonium hydroxide: water, 50:25:9:1:15 (v/v/v/v/v). Dried plates were placed in a Packard Instant Imager to visualize and quantitate the radioactivity by electronic autoradiography. From the known total radioactivity and the specific radioactivity of betulin metabolites, the rate of metabolism was calculated as pmol product/min/mg protein.

### 2.6. Data analysis

Enzyme activity was calculated using Excel software. Enzyme kinetic parameters ($K_m$, $V_{max}$) were determined by fitting the data to the Michaelis-Menten equation using the GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). The efficiency of an enzyme was calculated by dividing $V_{max}$ by $K_m$. Results were presented as mean values

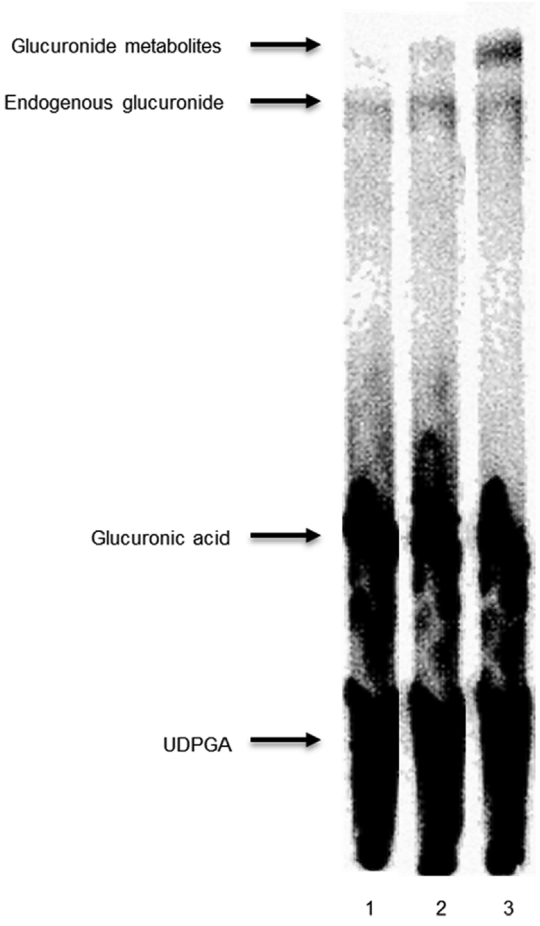


**Fig. 2.** TLC autoradiography of incubation of rat and human liver microsomes with betulin or no substrate. Lane 1 - Incubation of human liver microsomes with no substrate; Lane 2 - Incubation with 50 μM betulin in rat liver microsomes; Lane 3 Incubation with 50 μM betulin in human liver microsomes.

with standard deviation for results from three different individuals.

## 3. Results

### 3.1. Identification of betulin glucuronidation

The $^{14}$C-glucuronide conjugates were separated from unreacted $^{14}$C-UDPGA, as shown by TLC analysis and autoradiography (Fig. 2). Incubation of human liver microsomes with no betulin substrate showed no betulin glucuronide conjugate (Fig. 2, lane 1). There was one betulin glucuronide conjugate formed in rat liver microsomes, with $R_F$ of 0.9 (Fig. 2, lane 2). Fig. 2 (lane 3) shows the glucuronide of betulin using human liver microsomes. In the absence or presence of betulin, an endogenous substance in human liver microsomes formed a glucuronide with $R_F$ of 0.82 (Fig. 2, lane 1). This band was also found with rat liver microsomes (not shown).

### 3.2. Identification of the human UGTs capable of betulin glucuronidation

Glucuronidation of betulin was characterized using 11 human recombinant UGT enzymes. In order to verify that the recombinant hUGT enzymes were active, β-estradiol (150 μM) was used as a positive control substrate for hUGT1A1, and hUGT1A3, triclosan (1000 μM) was a test substrate for hUGT1A4, and 50 μM 7-hydroxy-4- (trifluoromethyl) coumarin (HTC) was for hUGT1A6, hUGT1A7, hUGT1A8, hUGT1A9, hUGT1A10, hUGT2B4, hUGT2B7, and hUGT2B15. The results showed that hUGT1A3, and 1A4 were able to catalyze the production of betulin glucuronide (Table 1), whereas other UGTs did not show any catalyzing activity in betulin glucuronidation. All studied UGTs were active with their positive control substrates (Supplemental Table 1).

**Table 1**
Glucuronidation of betulin by recombinant human UGTs.

| UGTs | Activity of catalyzing betulin glucuronidation (pmol/min/mg protein) |
|---|---|
| UGT1A1 | – |
| UGT1A3 | 7.75 |
| UGT1A4 | 7.03 |
| UGT1A6 | – |
| UGT1A7 | – |
| UGT1A8 | – |
| UGT1A9 | – |
| UGT1A10 | – |
| UGT2B4 | – |
| UGT2B7 | – |
| UGT2B15 | – |

- not detectable (Limit of detection for betulin was 5 μM).

### 3.3. Kinetics of glucuronidation of betulin

The kinetics of glucuronidation of betulin were examined in human liver microsomes, hUGT1A3, and 1A4 (Fig. 3, Table 2). The variation of rate with substrate concentration fits the Michaelis–Menten equation for all samples. A comparison of hUGT1A3 and hUGT1A4 data revealed that there is no obvious difference for $K_m$ ($p < 0.05$) and $V_{max}$ ($p < 0.05$) between these two isoenzymes.

### 3.4. Assay validation of betulin sulfation

The $^{35}$S-sulfate conjugates were separated from unreacted $^{35}$S-PAPS by TLC analysis and autoradiography (Fig. 4). Incubation with no substrate showed no sulfate conjugate (Fig. 4, lane 1). Fig. 4 (lane 2) shows the formation of two betulin sulfates using human liver cytosol. There were also two betulin sulfate conjugates formed in rat liver cytosol (Fig. 4, lane 3).

### 3.5. SULT isoforms involved in betulin sulfation

A panel of 5 recombinant human SULTs was used to evaluate their ability for catalyzing the formation of betulin sulfates Triclosan was used as the positive control substrate to verify activity for hSULT1A1, hSULT1A3, hSULT1B1 and hSULT1E1, and dehydroepiandrosterone (DHEA) was used for hSULT2A1. The expressed hSULTs were all demonstrated to show activity with the test substrates (Supplemental Table 1). Of all hSULTs tested, hSULT2A1 was the only isoform involved in betulin sulfation (Table 3).

### 3.6. Kinetic analysis of sulfation of betulin

The kinetics of betulin sulfation were examined in adult rat liver cytosol, human liver cytosol, and individual human SULTs (Fig. 5). The rates of conjugate formation at different concentrations of substrate fitted the Michaelis–Menten equation and the results are shown in Table 4.

In adult rat liver cytosol, apparent $V_{max}$ and efficiency of betulin sulfate I were 9-fold ($p < 0.05$) higher than that of betulin sulfate II. There was no significant difference in $K_m$ between the two betulin sulfate metabolites ($p > 0.05$).

The apparent $V_{max}$ for the formation of betulin sulfate I in adult human liver cytosol was 3.7-fold higher than that for betulin sulfate II. As for $K_m$ and efficiency, no remarkable difference was observed between betulin sulfate I and betulin sulfate II.

In the comparison of species differences, for betulin sulfate I, the

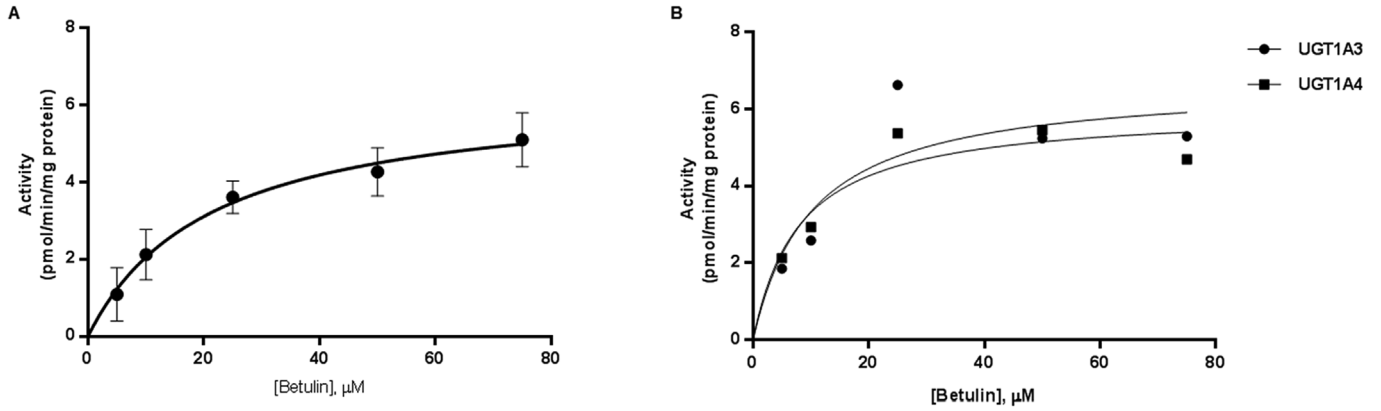


**Fig. 3.** Representative kinetic plots of the glucuronidation of betulin. A, Michaelis-Menten plot for betulin by human liver microsomes. B, Michaelis-Menten plots for betulin by hUGT1A3 and 1A4.

**Table 2**
Kinetics of betulin glucuronidation.

| | $K_m$ | $V_{max}$ | Efficiency |
|---|---|---|---|
| | (μM) | (pmol/min/mg protein) | (μl/min/mg protein) |
| Human liver | 21.10 ± 5.93 | 6.39 ± 0.66 | 0.32 ± 0.12 |
| UGT1A3 | 10.12 ± 8.09 | 6.71 ± 1.51 | 0.66 |
| UGT1A4 | 8.04 ± 3.96 | 5.98 ± 0.76 | 0.74 |

Values shown are mean ± S.D., n = 3.

apparent $V_{max}$ and efficiency values in rats were 3.3 and 3.4-fold ($p < 0.01$) higher than that in humans, whereas $K_m$ showed no significant difference in rats and humans. Similarly, for betulin sulfate II, kinetic parameters show no obvious difference in the two species.

hSULT2A1 converted betulin into both betulin sulfate I and II. In the comparison of kinetic parameters of two sulfate metabolites, $K_m$ and efficiency showed no obvious difference, and the apparent $V_{max}$ for betulin sulfate I was higher than that for betulin sulfate II.

## 4. Discussion

In the present study, betulin glucuronidation was investigated in vitro. The metabolite formed from the incubation of betulin with human liver microsomes and UDPGA was identified by radiochemical and autoradiography methods. One band that appeared to be a single glucuronide conjugate was formed from betulin. We hypothesize that the glucuronidation is at the C-3 hydroxyl, as a similar molecule, ursolic acid, formed a C3- hydroxyl glucuronide [12] but more structural strudies are needed to identify the site of conjugation. Enzyme kinetics of betulin in human liver microsomes showed a typical Michaelis–Menten kinetics model with $K_m$ of 21.10 ± 5.93 μM and $V_{max}$ value of 19.18 ± 1.97 pmol/min/(mg protein). Compared with human liver microsomes, the activity of glucuronidation in adult rat liver microsomes with 50 μM betulin was only 15%, which was too low to obtain enzyme kinetic parameters, implying that rats might be not a good animal model to study betulin glucuronidation *in vivo*.

hUGT enzymes responsible for the formation of betulin glucuronide were identified using 11 recombinant human UGT enzymes. hUGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 appear to be the most important human liver drug metabolizing UGT forms. hUGT1A7, hUGT1A8 and hUGT1A10 are expressed mainly in the gastrointestinal tract but not in human liver, and are employed to study extrahepatic glucuronidation [17,18]. Our results showed hUGT1A3 and hUGT1A4 mainly catalyzed the glucuronidation of betulin. This finding was similar with another pentacyclic triterpene, ursolic acid [12]. hUGT1A3 and 1A4 had similar affinity to betulin with the $K_m$ value of 10.12 ± 8.09 and 8.04 ± 3.96

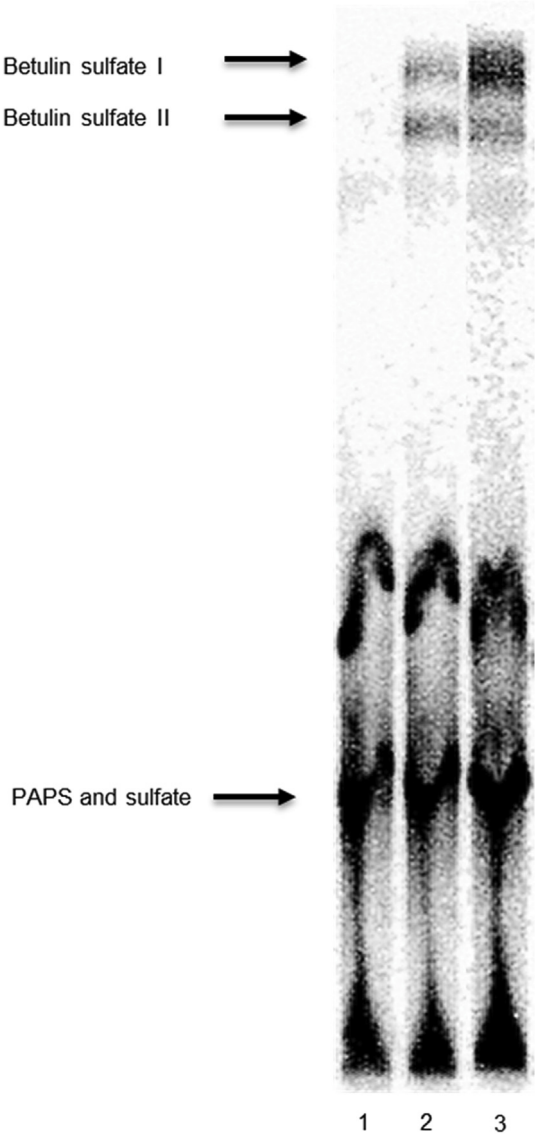


**Fig. 4.** TLC of incubation of rat and human liver cytosol with betulin or no substrate. Lane 1 - Incubation of human liver cytosol with no substrate; Lane 2 - Incubation with 200 μM betulin in human liver cytosol; Lane 3 - Incubation with 200 μM betulin in rat liver cytosol.

**Table 3**
Sulfation of betulin by recombinant human SULTs.

| SULTs | Activity of catalyzing betulin sulfation (pmol/min/mg protein) | |
|---|---|---|
| | Betulin sulfate I | Betulin sulfate II |
| SULT1A1 | – | – |
| SULT1A3 | – | – |
| SULT1B1 | – | – |
| SULT1E1 | – | – |
| SULT2A1 | 1625.12 | 1932.26 |

- not detectable (Limit of detection for betulin was 0.5 μM).

respectively. According to a previous report, hUGT1A4 was expressed 3 fold higher than 1A3 in human liver [18]. Hence, hUGT1A4 should play a more important role than hUGT1A3 does in betulin glucuronidation in humans. As UGT1A4 gene is a pseudogene in rats, UGT1A4 could not express in rats [19], which would explain why betulin glucuronidation in rat liver microsomes was much lower than that in human liver microsomes.

In studies of betulin sulfation, two betulin sulfate metabolites were found with both rat and human liver cytosol, which means betulin sulfates were formed at both C-3 and C-28 sites. Further studies are needed to ascertain the structures of each botulin sulfate metabolite.

Both rat and human liver showed similar patterns for the formation of these two metabolites, the apparent $V_{max}$ for betulin sulfate I were both higher than that for betulin sulfate II in these two species. The only difference was that the apparent $V_{max}$ values for betulin sulfate I in rats were 3.3-fold higher than in humans ($P < 0.01$).

The SULT isoforms including SULT1A1, 1A3, 1B1, 1E1, and 2A1 were tested. SULT1A, 1B, and 1E have phenols and catechols as their preferred substrates, whereas SULT2A1 preferentially catalyzes sulfation of aliphatic hydroxyl groups in steroids [20]. hSULT2A1 was the only isoenzyme that showed sulfation activity toward betulin in this study. SULT2A1 shows high expression level in liver, adrenal, duodenum and fetal adrenal [13]. hSULT2A1 can catalyze the formation of both betulin sulfate metabolites, and the catalyzing efficiency for betulin sulfate I was higher than betulin sulfate II. This finding was consistent with what we found in human liver cytosols, which further demonstrated that hSULT2A1 was the major isoenzyme involved in betulin sulfation in human liver cytosols. Unlike humans, which have only one solitary isoform (SULT2A1), rats have multiple isoforms, rSULT2A1, 2A2, 2A3, and 2A4 [21]. Other isoenzymes of the 2A family might be involved in sulfation metabolism of betulin as well, which might account for higher apparent $V_{max}$ values in rats than that in humans.

It was interesting that the efficiency of sulfation was considerably higher than that of glucuronidation in human liver. In rat liver we were unable to measure efficiency of glucuronidation as rates were very low, but the efficiency of sulfation was higher than in humans. These results suggest that in both humans and rats, betulin will be more readily sulfated than glucuronidated.

In conclusion, betulin can be metabolized to one betulin glucuronide in human and rat liver microsomes. The activity of glucuronidation in rat liver microsomes was much lower than that in humans. hUGT1A3 and hUGT1A4 were the main metabolic enzymes catalyzed the glucuronidation of betulin in human liver microsomes. Two betulin sulfate metabolites were found in human and rat liver cytosols. hSULT2A1 was the major isoenzyme involved in betulin sulfation in humans.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

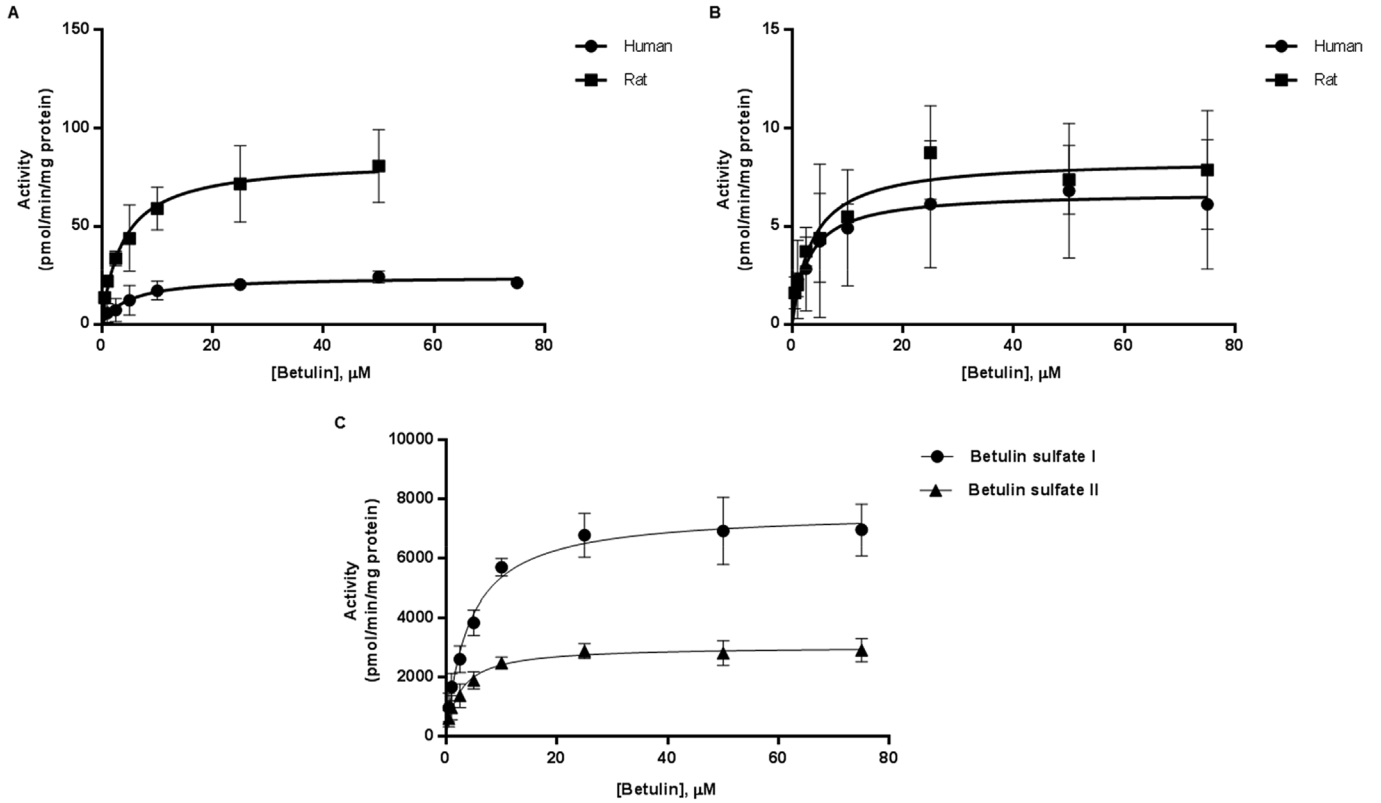


**Fig. 5.** Representative Michaelis-Menten kinetic plots of the sulfation of betulin. A, betulin sulfate I by human and rat liver cytosol. B, betulin sulfate II by human and rat liver cytosol. C, betulin sulfate I and II by hSULT2A1.

**Table 4**
Kinetics of betulin sulfation.

| | Metabolites | $K_m$ | $V_{max}$ | Efficiency |
|---|---|---|---|---|
| | | (μM) | (pmol/min/mg protein) | (μl/min/mg protein) |
| Rat liver | Betulin sulfate I | 3.7 ± 0.3 | 83.6 ± 12.5 | 22.5 ± 1.7 |
| | Betulin sulfate II | 3.6 ± 0.9 | 8.5 ± 1.5 | 2.5 ± 0.4 |
| Human liver | Betulin sulfate I | 6.8 ± 3.4 | 25.2 ± 1.6 | 6.6 ± 3.5 |
| | Betulin sulfate II | 4.6 ± 1.6 | 6.9 ± 1.9 | 2.4 ± 1.5 |
| SULT2A1 | Betulin sulfate I | 4.2 ± 0.7 | 7593 ± 632 | 1899 ± 276 |
| | Betulin sulfate II | 2.9 ± 1.2 | 3081 ± 252 | 1358 ± 455 |

Values shown are mean ± S.D., n = 3.

## Acknowledgements

This work was funded by the Fundamental Research Funds for the Central Universities (2572017BA05) in China.

We thank Marci Smeltz, Guo Zhong, and Laura Rowland Faux for assistance.

## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2019.02.009.

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