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Deciphering the origins of molecular toxicity of combretastatin A4 and its glycoconjugates: interactions with major drug transporters and their safety profiles *in vitro* and *in vivo*†

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Cellular uptake and transport mechanisms directly correlate with the drug-like profiles of lead compounds. To decipher the molecular origin of the toxicity of combretastatin A4 (CA4), an important microtubule targeting agent, we investigated the interactions between CA4 and six key drug transporters, namely hOAT1, hOAT3, hOCT1, hOCT2, hOATP1B3, and hOATP2B1. Three combretastatin-based glycoconjugates, namely **Glu-CA4**, **Man-CA4**, and **Gal-CA4** with glucose, mannose, and galactose respectively, were synthesized and their *in vitro* and *in vivo* biological characteristics were evaluated. CA4 exhibited significant inhibition against hOAT3 and hOATP2B1, moderate inhibition of hOAT1 and hOCT2, and weak inhibitory effects on hOCT1 and hOATP1B3. Compared to CA4, the inhibitory activities of **Glu-CA4** on the six transporters were minimal. The glycoconjugates were found to have a superior safety profile with their maximum tolerated dose (MTD) values exhibiting a 16–34-fold increase compared to CA4. Given the drawbacks of CA4, the enhanced solubility and safety profiles of CA4 glycoconjugates augur well for further investigation into these intriguing candidates' *in vivo* efficacy.

1. Introduction

Combretastatin A4 (CA4) is a natural vascular-disrupting agent (VDA) that has shown potent inhibition of tubulin polymerization by binding to tubulin at the colchicine binding site 5.[1] Though CA4 exhibits strong cytotoxicity against various human cancer cell lines, studies revealed that it was not promising as a therapeutic agent in clinical trials due in large part to its poor water solubility and lack of tumor-specificity.[2,3] Additionally, CA4 is only able to induce tumor regression at doses that are close to its maximum tolerated dose (MTD), which has been hypothesized to be associated with its low tumor selectivity and narrow therapeutic margin.[4,5] For this reason, a large number of CA4 analogs have been synthesized and evaluated for improved water solubility and *in vivo* efficacy.[6] Among these compounds, a few analogs with improved water-solubility including CA4-disodium phosphate (CA4P) and serine-conjugated CA4 (AVE8062) are currently under clinical investigation.[2,7] Nonetheless, some early-phase clinical trials have revealed promising antineoplastic activity, however, from the monotherapy studies, all tested CA4 analogs have been found to cause systemic toxicity and severe side effects to the patients.[8] The data observed from pre- and clinical studies unequivocally confirm the shortcomings of this natural compound as a monotherapy for cancer treatment, and this has led us to investigate the cellular uptake and drug transport mechanisms that may potentially be involved in the toxic effect of CA4.

Drug uptake primarily relies on solute carrier (SLC) transporters by facilitated diffusion or ion-coupled secondary active transport. These transporters are localized in organs such as the small intestine, liver, and kidney as well as the central nervous system (CNS), and they play key roles in drug absorption, distribution, metabolism, excretion, as well as drug toxicity profiles (ADMET).[9] Based on their mode of action, transporters could be grouped into uptake transporters that mediate the transport of drug molecules into cells and efflux transporters that transport substances out of cells. Inhibition or induction of transporters may also cause severe drug–drug

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interactions (DDIs) in patients.[10] Most DDIs *via* uptake transporters involve the two gene superfamilies, namely the SLCO superfamily, made up of the organic anion transporting polypeptides (OATPs), as well as the SLC22A superfamily, containing the organic anion transporters (OATs) and organic cation transporters (OCTs).[11] As key indicators of a potential drug candidate, a variety of transporter assays for OATPs, OATs, and OCTs are required by both the FDA and EMA prior to testing of a New Chemical/Molecular Entity (NCE/NME). Concerning R&D activities with CA4, although the overwhelming majority of efforts have been expended in its pharmacological and clinical evaluations, so far, to the best of our knowledge, no study has investigated its uptake and transport profiles, as well as its potential interactions with active transporters.

In the current study, together with the evaluation of the transport mechanism of CA4, we also investigated the effects of glycoconjugation on CA4 *in vitro* and *in vivo* biological characteristics. As shown in Fig. 1, glucose, mannose and galactose derived CA4-glycosides were synthesized and the differences in the transport properties between CA4 and its glycoconjugates were compared, including their potential interactions with uptake transporters, namely OAT1, OAT3, OCT1, OCT2, OATP1B3 and OATP2B1. The data generated from the current study provide fundamental information that is potentially relevant to the origin of the intrinsic toxicity of CA4, and our study also highlights a promising new possibility for the development of a safer CA4-based VDA.

2. Experimental

2.1. Materials

All biological and chemical reagents were purchased from commercial companies and directly used unless stated otherwise.

2.2. Synthesis of the glycoconjugates of CA4

As shown in Fig. 1, the preparation of glucose and galactose derived glycoconjugates (**Glu-CA4** and **Gal-CA4**) were accomplished by a two-step sequence from CA4. The mannose derived glycoconjugate **Man-CA4** was synthesized by a one-step reaction from CA4. The final products were completely characterized by ^{1}H and ^{13}C NMR spectroscopy. The stereochemistry for both **Glu-CA4** and **Gal-CA4** was assigned to a pure β-anomer,[12,13] whereas that of **Man-CA4** was assigned to a pure α-anomer (see the ESI† for the detailed procedure and spectra data).

2.3. Solubility of CA4 and its sugar conjugates

Solubility testing of CA4, **Glu-CA4**, **Man-CA4** and **Gal-CA4** was conducted by placing an excess of the compounds in deionized water in a series of 10 mL stoppered volumetric test tubes. The tubes were shaken in an incubating shaker at 25 °C for 1 h. The test tubes containing equilibrated solutions were then removed and the solutions were filtered immediately by passing through 0.2 μm filters. The filtered samples (0.5 mL) were diluted appropriately with deionized water and concentration estimation was made by using HPLC-UV. All experiments were performed in the dark. A reversed phase column (Zorbax SB-C18, 5 μm, 4.6 × 150 mm, Agilent) was used at room temperature for all analyses. The mobile phase consisted of methanol and water (70 : 30, v/v), and the flow rate was 1.0 mL min^{-1}. The injection volume for CA4 was 20 μL and 2 μL for the conjugates. The average value of three trials was taken. The standard curve obeyed Beer–Lambert's law in the respective concentration range with $>R^2 = 0.999$.

2.4. Cell culture

Human colon cancer (HT29), human prostate cancer (DU145), human breast cancer (MB231) and human ovarian cancer (SKOV3) cell lines were obtained from the Central Institute of Pharmaceutical Research, CSPC Pharmaceutical

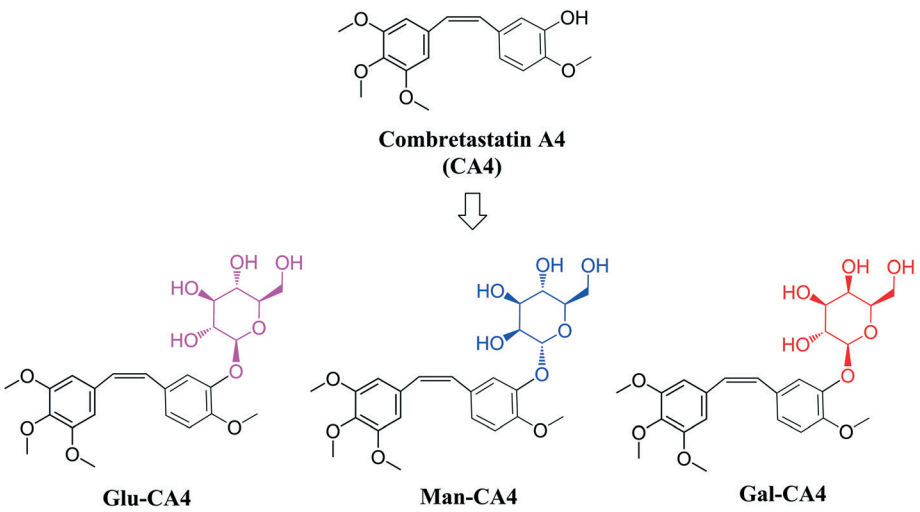


Fig. 1 Combretastatin A4 and its three sugar-conjugates studied in this project.

Group, China. SKOV3 was cultured in advanced Dulbecco's modified Eagle medium (DMEM), and the other cell lines were cultured as an adherent monolayer in RPMI-1640 supplemented with 1% (w/v) glutamine and 10% (v/v) fetal bovine serum (Gibco) at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air. Human embryonic kidney (HEK) 293 cells were purchased from ATCC, China and cultured in DMEM supplemented with 10% FBS at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air.

2.5. Transporter overexpression and cell transfection

Human embryonic kidney (HEK) 293 cells stably expressing human OAT1, OAT3, OCT1, OCT2 and OATP1B3 were established in the present study. Briefly, the open reading frame of each transporter was subcloned into a pcDNA3.1/Hygro (+) (Invitrogen) vector, which was then transfected into HEK293 cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Stable transporter-expressing cells were obtained by hygromycin B selection. The control (mock) HEK293 cells were obtained by transfecting an empty vector into HEK293 cells followed by hygromycin B selection. These transporter-expressing cells were validated by both mRNA expression of transporters and their uptake ability to their corresponding fluorescent substrates (Table 1 and Fig. 2). The OATP2B1-expressing and mock cells were kindly provided by Dr. Chunshan Gui from Soochow University (Suzhou, China). All stable transporter-expressing cell lines were maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 75 μg mL^{-1} hygromycin B at 37 °C with 5% CO_2. The transporter function was evaluated according to previous assays by using the corresponding fluorescent substrates (OAT1/3, 6-carboxyfluorescein (6-CF); OCT1/2, 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP^+); OATP1B3, fluorescein-methotrexate (FMTX); OATP2B1, 4′,5′-dibromofluorescein (DBF)).[14–18]

2.6. Cytotoxicity assay

Cells were seeded in a 96-well flat-bottomed microplate at 2000–7500 cells per well in 100 μL of growth medium solution on day 0. On day 1, the cells were treated either with a vehicle or the drugs that were dissolved in DMSO and diluted under cell culture conditions for an indicated time. The amount of DMSO used for dissolving the samples was kept below 0.1% (v/v) as the final concentration for each test. MTT (Sigma-Aldrich) was added to each well at a final concentration of 0.83 mg mL^{-1} and incubated overnight. Cells were lysed using MTT lysis buffer (15% SDS, 0.015 M HCl) and the lysate was measured at 570 nm using a multi-well-reading UV-vis spectrometer. For each drug, the cell survival rates were expressed as the relative percentage of absorbance compared to controls without drugs. The compounds were tested for 72 h in HT29 and 24 h in DU145, MB231 and SKOV3 cells. Each experiment was performed in five replicates (5 wells of the 96-well plate per experimental condition).

2.7. Transporter-mediated cellular uptake

Cells were seeded at a density of 7×10^4 cells per well in poly-D-lysine-coated 96-well culture plates. Transport assays were performed 16 h post seeding in preheated uptake buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM $MgSO_4$, 28 mM glucose, and 13 mM HEPES, pH 7.2). 6-Carboxyfluorescein (6-CF) was used as the fluorescent probe for both hOAT1 and hOAT3 according to a previous study.[15] Fluorescein-methotrexate (FMTX) and dibromofluorescein (DBF) were used as the fluorescent probe for OATP1B3 and OATP2B1, respectively. The fluorescent probe alone, with different inhibitors (probenecid for OATs, quinine for OCTs, and rifampicin for OATPs) or with different compounds was incubated for 5 min in HEK293-OAT1/3 cells, 10 min in HEK293-OCT1/2 cells, and 5 min in HEK293-OATP1B1/2B1 cells as well as in their corresponding mock cells. Uptake was terminated by adding ice-cold phosphate-buffered saline (PBS), and the mixture was quickly washed three times with ice-cold PBS. The cells in each well were lysed with 100 μL of 20 mM Tris–HCl containing 0.2% TritonX-100. An aliquot of 50 μL lysate was used to determine fluorescence by using a Tecan Infinite M200 (Austria, Swiss). The protein concentration of the cell lysate was determined using a BCA Protein Assay Kit (Cwbio, Beijing, China). All the uptake values were standardized against protein content, and were measured in triplicate. The uptake experiment for each sample was in triplicate in the same assay, and repeated 2–3 times on other plates.

2.8. Quantification of CA4 and Glu-CA4 by HPLC-UV

Analyses of CA4 and **Glu-CA4** concentrations were carried out using a Waters HPLC system with a UV detector at 294 nm. A reversed phase column (Zorbax SB-C18, 5 μm, 4.6 × 150 mm, Agilent) was used at room temperature for all analyses. The mobile phase consisted of methanol and water (70 : 30, v/v),

Table 1 The RT-PCR primer sequences. Primers were designed with Primer3 software (version 4)

Gene name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)
OAT1	GGAGCCAAATTGAGTATGGAGG	GTATGCAAAGCTAGTGGCAAAC	159
OAT3	CCCACAGTCATCAGGCAAACA	AGGGCGGTGATCCCGTAGA	137
OCT1	TGTCACCGAAAAGCTGAGCC	TCCGTGAACCACAGGTACATC	96
OCT2	GGCTCTATGAGTATCGGCTACA	TCCACGTATAGGTTGGGGAAAT	121
OATP1B3	TGGAGCAACAGTACGGTCAG	TGCTTTCGCAGATTAGAGGGAA	210

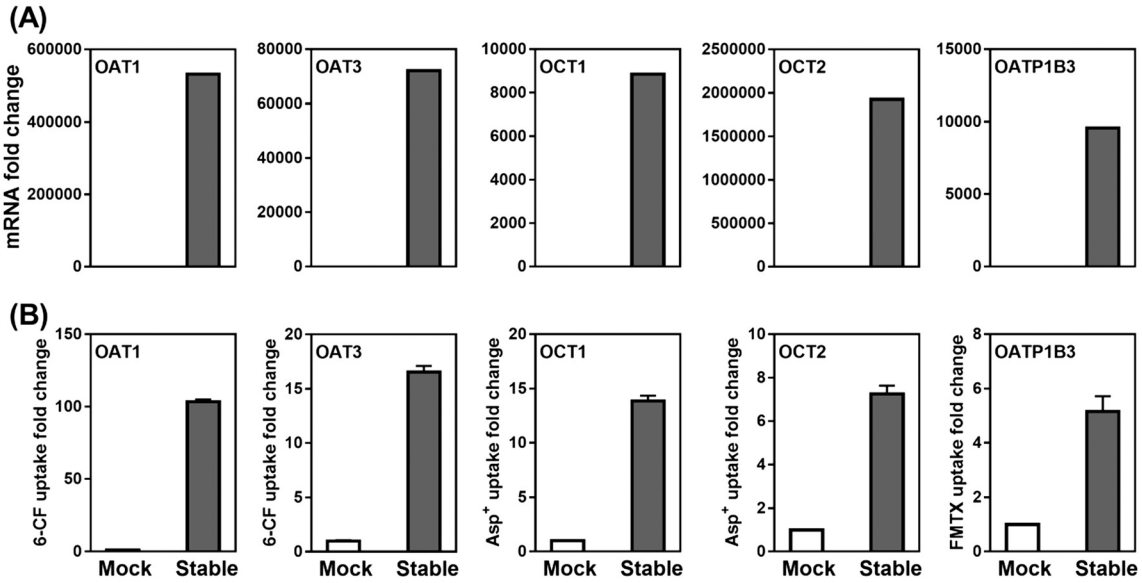


Fig. 2 Characterization of transporter protein transfected cells established in the present study. (A) The mRNA expression of transporters in stable over-expressing cells compared to control (mock cells). Total RNA was isolated with an RNAiso Plus reagent (Takara Biotechnology, Dalian, China) according to the manufacturer's protocol. RNA was then reverse transcribed to cDNA using a SuperScript II RT kit (Invitrogen). Quantitative reverse transcriptase PCR analysis was carried out using a SYBR green PCR mastermix (ABI Inc.). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH), and expressed as the relative fold change to control (mock) group. (B) The fluorescence uptakes in mock and transporter-expressing cells. Values were expressed as the relative fold change to the control (mock) group.

and the flow rate was 1.0 mL min^{-1}. The injection volume was 20 μL. **Glu-CA4** was used as the internal standard for CA4 quantification, and CA4 was used as the internal standard for **Glu-CA4** quantification.

2.9. Determination of maximum-tolerated dose (MTD)

Four-to-five-week old male DBA/2 mice from Beijing Vital River Laboratory Animal Technology Co. Ltd. were administered with CA4 and the sugar-conjugates by i.p. injection at daily intervals for 3 days. CA4 (10–100 mg kg^{-1}) and the sugar conjugates (200–600 mg kg^{-1} for **Glu-CA4**, **Man-CA4** and **Gal-CA4**) were administered *via* i.p. injection (10 mL kg^{-1}) to the randomly grouped mice. A total of five animals were used per treatment group, and eight animals for the control group. The control group was given an equivalent volume of sterile saline. The MTD was defined as the allowance of a median body weight loss of 15% of the weight before pharmacologic treatments causing neither death due to toxic effects nor remarkable changes in the general signs within 3 days after administration. The study was approved by the Committee for the Protection of Animal Care at Tianjin University. All experimental protocols were in accordance with the Guidelines for Experimental Animal Administration published by the State Committee of Science and Technology of the People's Republic of China.

2.10. Statistical analysis

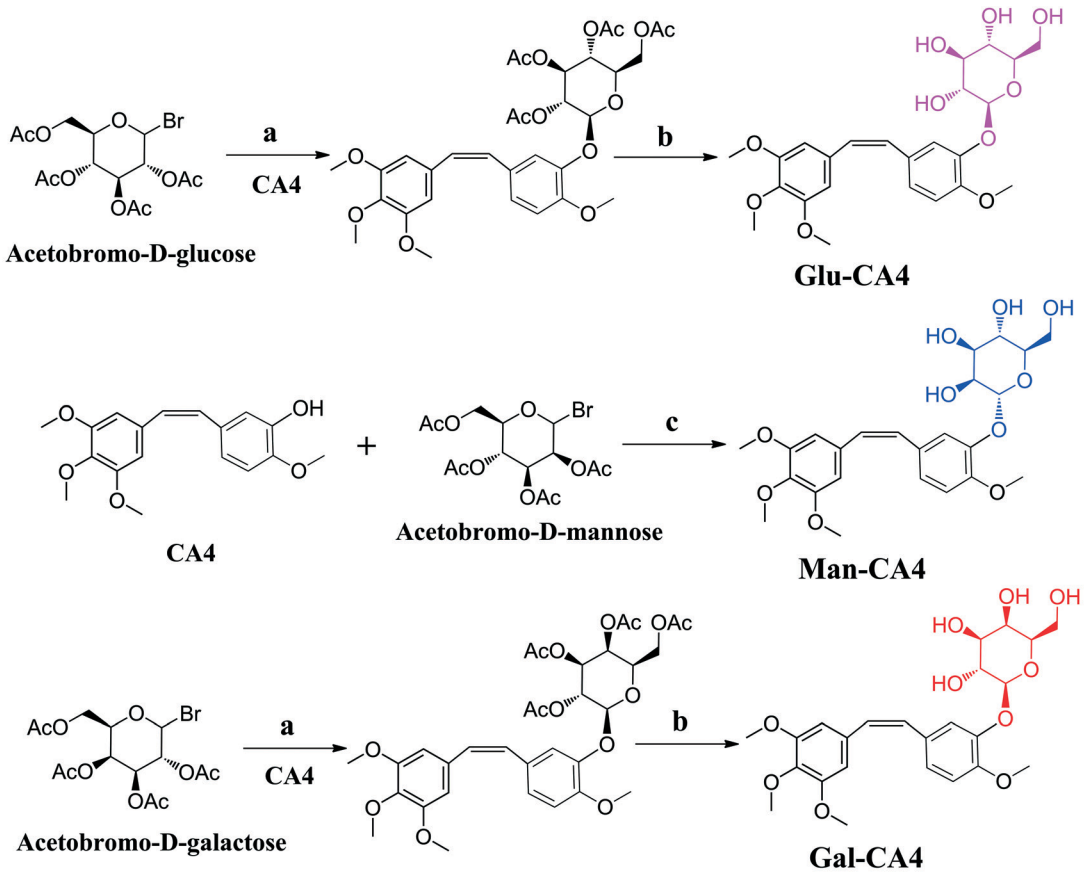
Data were analyzed by two-tailed unpaired Student's *t*-test. The *p* value for statistical significance was set to <0.05.

3. Results and discussion

3.1. Synthesis of the three sugar conjugates of CA4

As shown in Scheme 1, the coupling reactions of CA4 with tetra-*O*-acetyl-D-glucopyranosyl and D-galactopyranosyl bromide were carried out in a bilayer system using $CHCl_3$ and H_2O (3 : 1, v/v) in the presence of sodium hydroxide. After deprotection of the acetyl groups using NaOH in methanol, the desired products were afforded in 79% and 76% yields for **Glu-CA4** and **Gal-CA4**, respectively. To directly afford mannose conjugated **Man-CA4**, the coupling reaction of CA4 with tetra-*O*-acetyl-D-mannopyranosyl bromide was carried out using lithium hydroxide in methanol as a homogeneous reaction system. The stereochemistry of the conjugates was determined by ^1H NMR analysis with the proton on the 1-position of the sugar moiety. **Glu-CA4** and **Gal-CA4** were assigned to a pure β-anomer,[12,13] and the mannose derived glycoside **Man-CA4** was assigned to a pure α-anomer.[19]

The stereochemical outcomes for glycosylation of CA4 with different sugar-bromides can be explained by the role of "Neighbouring Group Participation". Thus, the protecting group, typically one with a carboxyl group at the 2-position of the glycosyl donor, will predominantly result in the formation of glycosides as an "anti-form" of the protecting group at position 2. In the current study, when sugar-bromide is derived from acetyl protected glucose and galactose (Scheme 1), both the acetyl protecting groups at position 2 will allow for the formation of an "acetoxonium ion" intermediate that blocks the attack of CA4 from the same side of the protecting group (α-side), therefore predominantly allowing for the formation of the β-glycosides. Similarly, when the glycosyl donor of the



Regents and Conditions: (a) TBAB, NaOH, $CHCl_3/H_2O$, 12h. (b) NaOH, MeOH, 1h. (c) LiOH, MeOH, 2h

Scheme 1 The synthetic scheme of CA4 glycoconjugates.

sugar-bromide is derived from mannose, the reaction will predominantly result in the formation of the α-glycoside. For a detailed mechanistic explanation, see Fig. S1.† Due to the steric hindrance imposed by the two neighbouring β-acetoxyl groups in acetobromo-D-mannose, the *O*-glycosylation of mannose with CA4 was found to be very slow in the bilayer system (Scheme 1, condition a) but occurs smoothly under the homogeneous conditions with lithium hydroxide in methanol.

3.2. Water solubility of CA4 and its sugar conjugates

One major purpose of the present study was to improve the solubility and the toxic profiles of CA4 by leveraging our expertise in drug-glycoconjugation and related technologies.[20–23] As summarized in Table 2, all three sugar conjugates had markedly increased water solubilities compared with CA4. Conjugation with glucose, mannose, and galactose resulted in a 610-, 1963-, and 853-fold increase in the water solubility of CA4, respectively.

3.3. *In vitro* cytotoxicity of CA4 and the sugar conjugates

CA4 has been found to have potent cytotoxic activity in colorectal cancer cell lines, such as HT-29 cells.[24] In this study, the cytotoxicity of CA4 and its sugar conjugates was first evaluated in HT-29 cells. As illustrated in Fig. 3A, the sugar conjugates of CA4 showed less cytotoxicity in HT-29 cells than CA4. The IC_{50} values of **Glu-CA4** (IC_{50} = 6.91 μM), **Man-CA4** (IC_{50} = 11.44 μM), and **Gal-CA4** (IC_{50} = 39.32 μM) were 9.59-,

Table 2 Solubility and safety improvement of glucose, mannose and galactose conjugated CA4 analogs

Compound	M.W. (g mol^{-1})	Solubility (mg mL^{-1})	MTD weight loss (mg kg^{-1})	Ratio (solubility)	Ratio (MTD)	HT29 cell IC_{50} (μM)
CA4	316.4	0.003	25	1	1	0.72 ± 0.10
Glu-CA4	478.5	1.83	400	610	16	6.91 ± 0.51
Man-CA4	478.5	5.89	400	1963	16	11.44 ± 1.70
Gal-CA4	478.5	2.56	550	853	34	39.32 ± 3.13

in vitro cytotoxicities tested with CA4 and the glycoconjugates

A: 72h	Compounds	B: 24h		
HT29		DU145	MB-231	SKOV3
√	CA4	√	√	√
√	Glu-CA4	√	√	√
√	Man-CA4			
√	Gal-CA4			

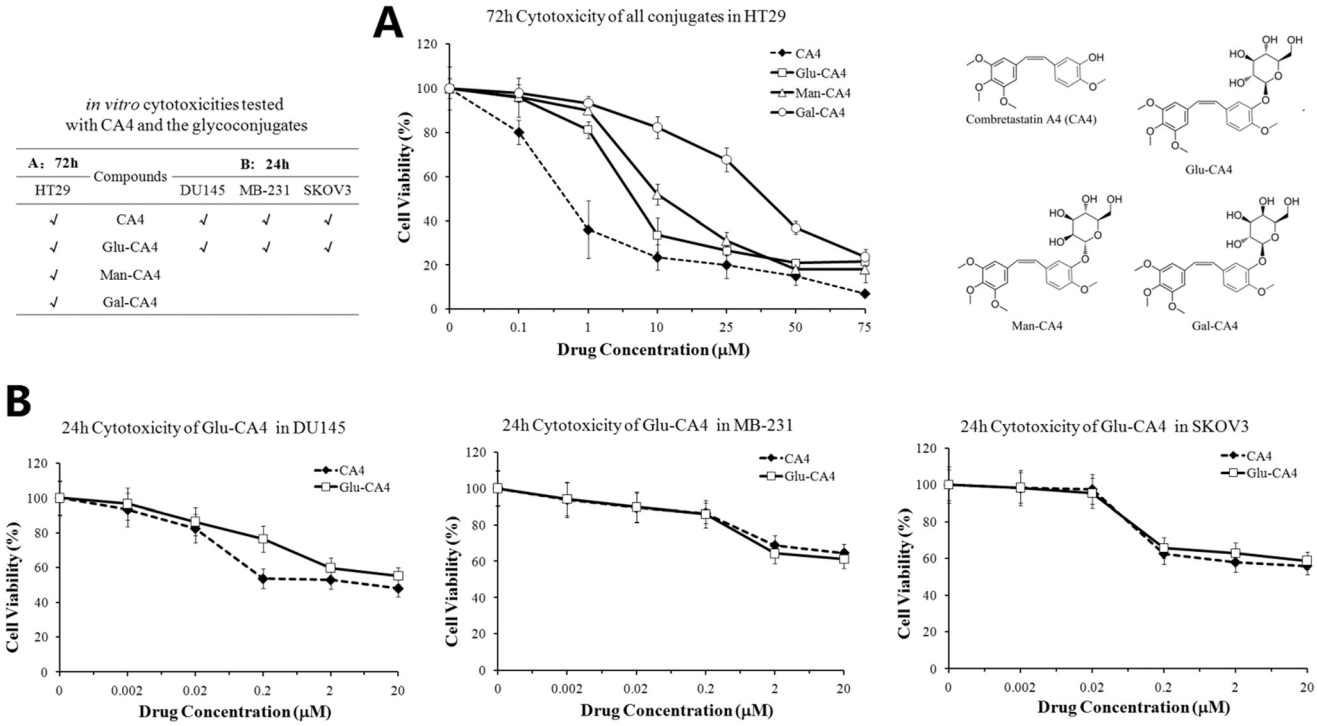


Fig. 3 Comparison of the cytotoxicity of CA4 with the glycoconjugates in different cell lines.

15.89-, and 54.61-fold higher than that of CA4 (IC_{50} = 0.72 μM) (Table 2). Since **Glu-CA4** has a slightly lower water solubility compared to **Man-CA4** and **Gal-CA4** (Table 2), this could possibly be one of the reasons for **Glu-CA4** exhibiting a relatively stronger cytotoxicity in HT-29 cells. To explain the decreased cytotoxicities of the sugar conjugates compared to

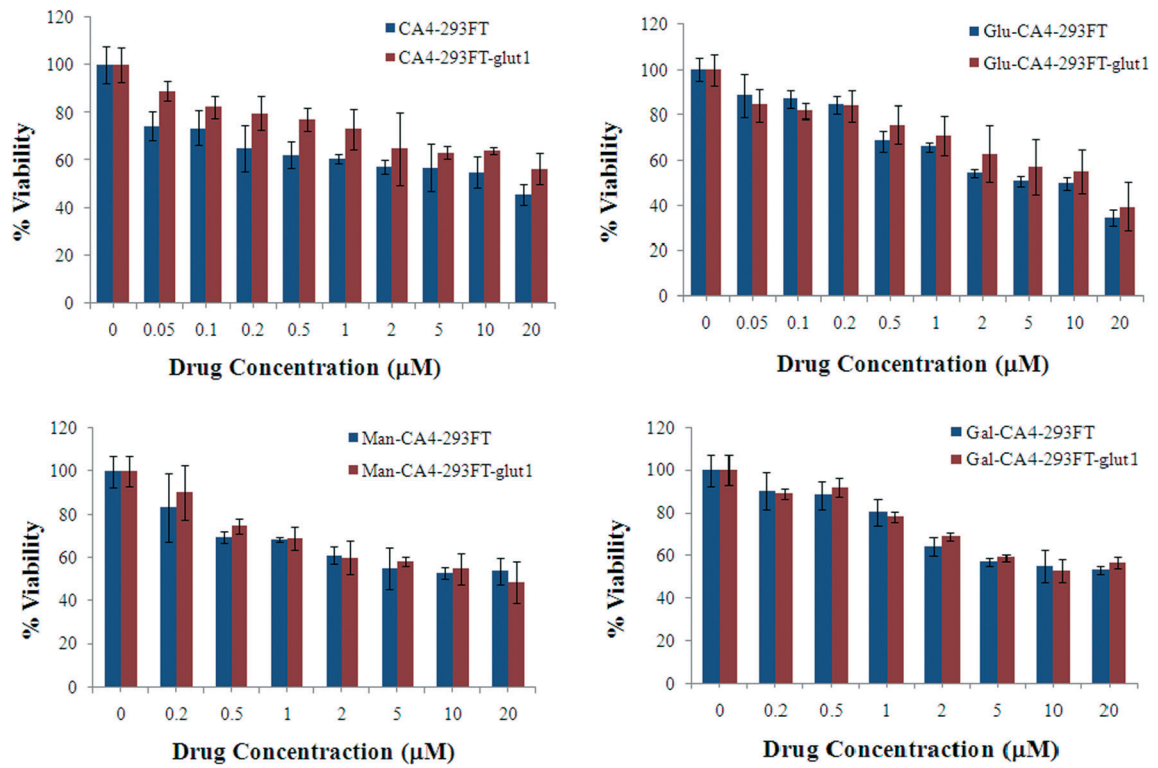
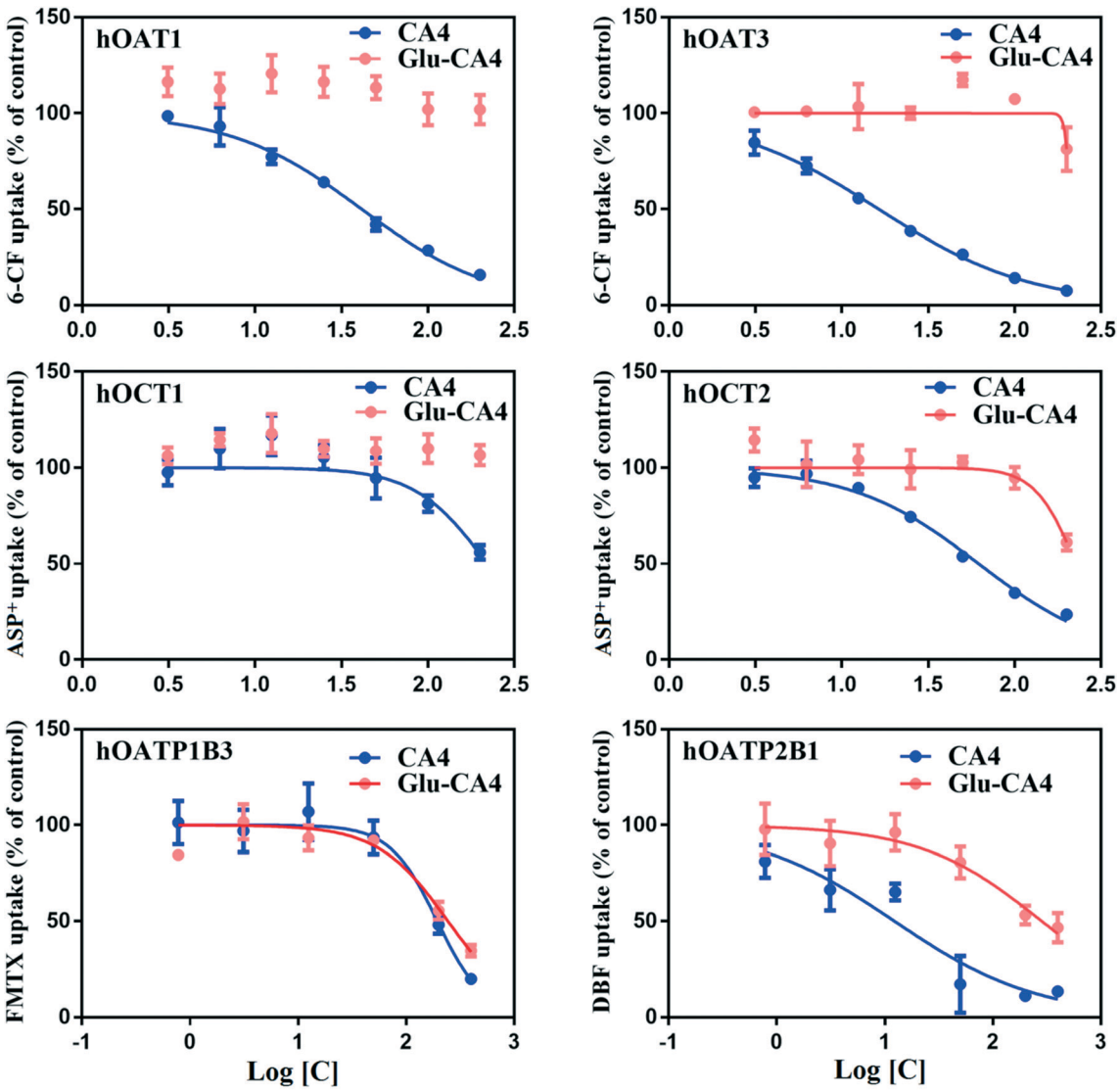


Fig. 4 Cytotoxicity comparison study of CA4 and glycoconjugates in the HEK293FT and GLUT1 overexpressing cell line: HEK293FT-GLUT1.

CA4, in section 3.5, we surveyed the cellular uptake properties of CA4 and **Glu-CA4** in different cell systems and demonstrated that **Glu-CA4** exhibited a more than ten-fold decrease in cellular accumulation. These results can be indicative of the lower IC_{50} values of the sugar conjugates, and the reduced cytotoxic effect of the conjugates can be also indicative of the potential for alleviating drug toxicity *via* glycoconjugation.

Among the three CA4 sugar conjugates, **Glu-CA4** was the most cytotoxic in HT-29 cells. Thus, **Glu-CA4** was chosen for further evaluation. The 24 h cytotoxicity studies of CA4 and **Glu-CA4** were conducted in DU145 (human prostate cancer), MB231 (human breast cancer), and SKOV3 (human ovarian cancer) cell lines (Fig. 3B). As discussed above, theoretically, more hydrophilic molecules have a lower ability to cross the cell membrane without the aid of transport proteins. However, in the 24 h cytotoxicity study, **Glu-CA4** did not show a marked difference in cytotoxicity with these cancer cell lines as compared to CA4. Some monosaccharides, such as D-glucose, D-mannose, and D-galactose, have been identified as substrates for glucose transporters (GLUTs). Human GLUT1-overexpressing cells were used to evaluate the potential role of GLUTs in the cytotoxicity of CA4 and its sugar conjugates. As a result (Fig. 4), the sugar conjugates of CA4 showed comparable cytotoxicity in mock and GLUT1-overexpressing cells, suggesting that GLUT1 had little effect on the cytotoxicity of the CA4 sugar conjugates.



Compounds	Overexpression Cell Lines (IC_{50} μM)					
	OAT1	OAT3	OCT1	OCT2	OATP1B3	OATP2B1
CA-4	41.36±1.05	16.35±1.03	>200	61.94±1.05	194.54±1.10	12.27±1.27
Glu-CA-4	>200	>200	>200	>200	>200	>200

Fig. 5 Dose-dependent inhibition of CA4 and **Glu-CA4** on six key drug transporters.

3.4. Concentration-dependent inhibition of CA4 and Glu-CA4 on cell uptake transporters

As shown in Fig. 5, using transfected cell lines, we evaluated the inhibitory effect of CA4 and **Glu-CA4** against six key drug transporters (hOAT1, hOAT3, hOCT1, hOCT2, hOATP1B3 and hOATP2B1). Since the toxicity of the drugs could cause potential false-positive results in the cellular uptake assays, HEK293-mock cells were incubated with CA4 or **Glu-CA4** at two high concentrations (100 and 200 μM) for 10 min, which was the maximal time for later cellular uptake assays, and the cytotoxicity was evaluated by both the MTT and total protein assays. As shown in Fig. 6, both high concentrations of CA4 and **Glu-CA4** had little effect on the cell viability and total protein amounts in HEK293-mock cells. The concentration-dependent inhibition of CA4 and **Glu-CA4** on the six key drug transporters was then examined to compare the inhibitory potencies of the two compounds. Dramatic differences between CA4 and **Glu-CA4** were observed for their inhibitory effects on OAT1/3-mediated 6-CF uptake, OCT2-mediated Asp^+ uptake, as well as OATP2B1-mediated DBF uptake (Fig. 5). Among the six transporters, CA4 showed very strong inhibition of OAT3 and OATP2B1 with IC_{50} values being 16.35 and 12.27 μM, respectively. Additionally, CA4 also showed moderate inhibition of OAT1 and OCT2, whereas its inhibitory effects on OCT1 and OATP1B3 were very weak. Compared to CA4, the inhibitory activities of **Glu-CA4** with the six uptake transporters were minimal, with IC_{50} values being larger than 200 μM. It is well known that both OATs and OCTs are ubiquitously expressed in tissues and organs of murines or humans, including kidney, liver, choroid plexus, olfactory mucosa, brain, retina, and placenta. Furthermore, biological studies using knockout mice demonstrated that both OAT and OCT systems are important for the transport of an extraordinarily broad range of molecules, including a number of endogenous hormones, nutrients and toxicants, as well as many clinically important drugs.[25,26] Besides the fact that DDIs might originate from the inhibition of both OAT and OCT transporters with CA4 as such a potent inhibitor of the most important organic anion and cation transporters for OAT1, OAT3, OATP2B1 and OCT2, there is a definite possibility that CA4 might trigger severe adverse effects and may cause systemic toxicities.

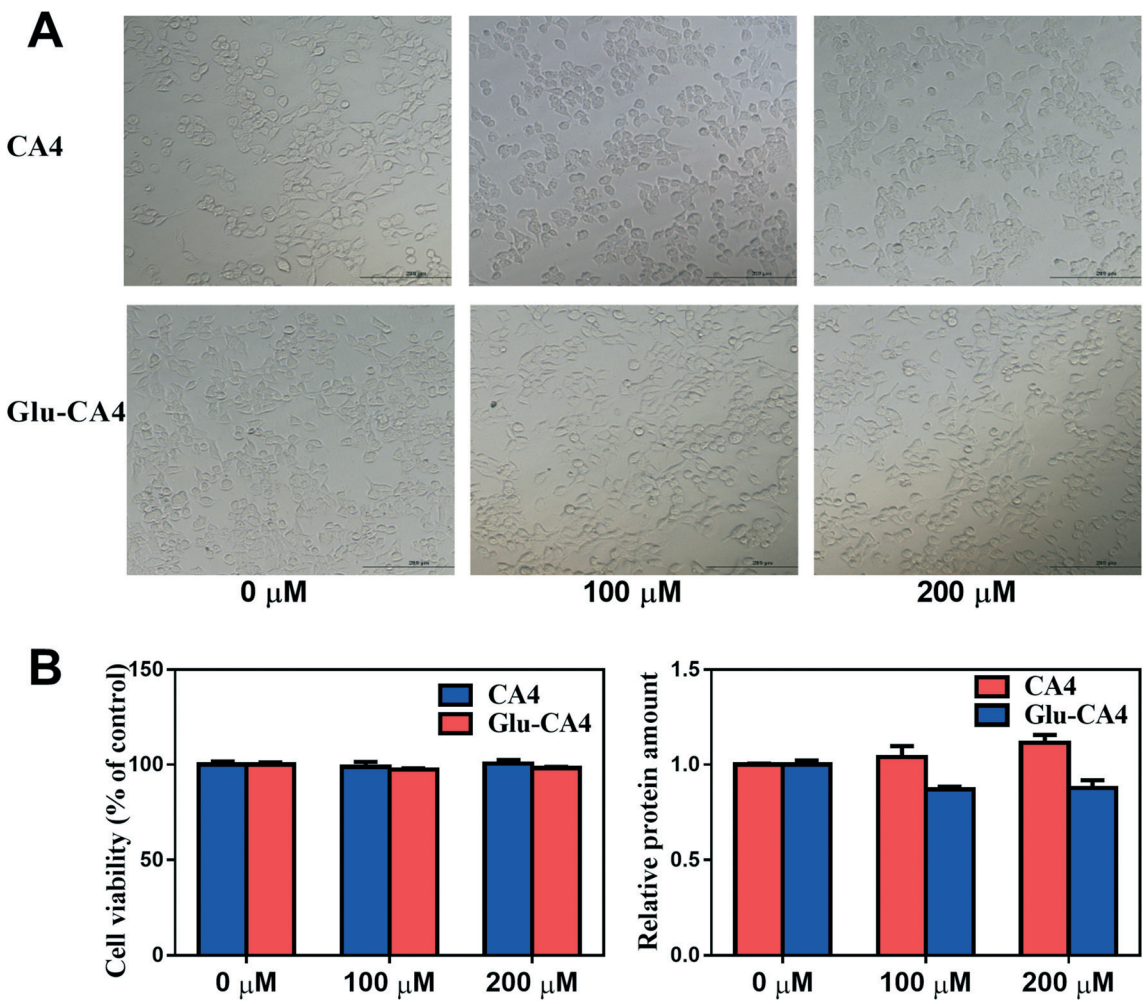


Fig. 6 Validation of the toxic effect that might occur under inhibitory assay conditions: high concentration (up to 200 μM) of CA4 and **Glu-CA4** do not induce toxicity in HEK293 cells for 10 min. A: Microscopic images of HEK293 cells treated with different concentrations of CA4 and **Glu-CA4**. B: Influence of different drug concentrations on cell viability.

3.5. Accumulation of CA4 and Glu-CA4 in transporter-overexpressing cells

To determine whether CA4 and **Glu-CA4** could be substrates of hOAT1/3, hOCT1/2, hOATP1B3, or hOATP2B1, the cellular uptake of CA4 and **Glu-CA4** in transporter-overexpressing cells and in their mock cells was investigated in the presence and absence of the well-known corresponding inhibitor for each transporter. As shown in Fig. 7A, uptake of CA4 in these transporters expressing cells and their corresponding mock cells showed no significant differences. Overexpression of OAT1/3, OCT1/2, and OATP1B3 did not increase the cellular uptake of **Glu-CA4** (Fig. 7B). Interestingly, the uptake of **Glu-CA4** increased by about 60% in hOATP2B1-expressing cells compared to that in the mock cells. However, co-incubation of rifampicin, a known inhibitor of OATP2B1, increased the uptake of **Glu-CA4** in both mock and hOATP2B1-expressing cells. Thus, **Glu-CA4** was not likely a substrate of hOATP2B1. Upon careful analysis of the cellular uptake data, we found that the cell concentrations of CA4 and **Glu-CA4** were significantly different. Except for the hOATP1B3 overexpressed cells, CA4 in all tested cell lines showed more than a 10-fold higher cellular accumulation than **Glu-CA4**, which parallels with the cytotoxicity of this compound in HT29 cells and reflects the effect of increased hydrophilicity through glycoconjugation.

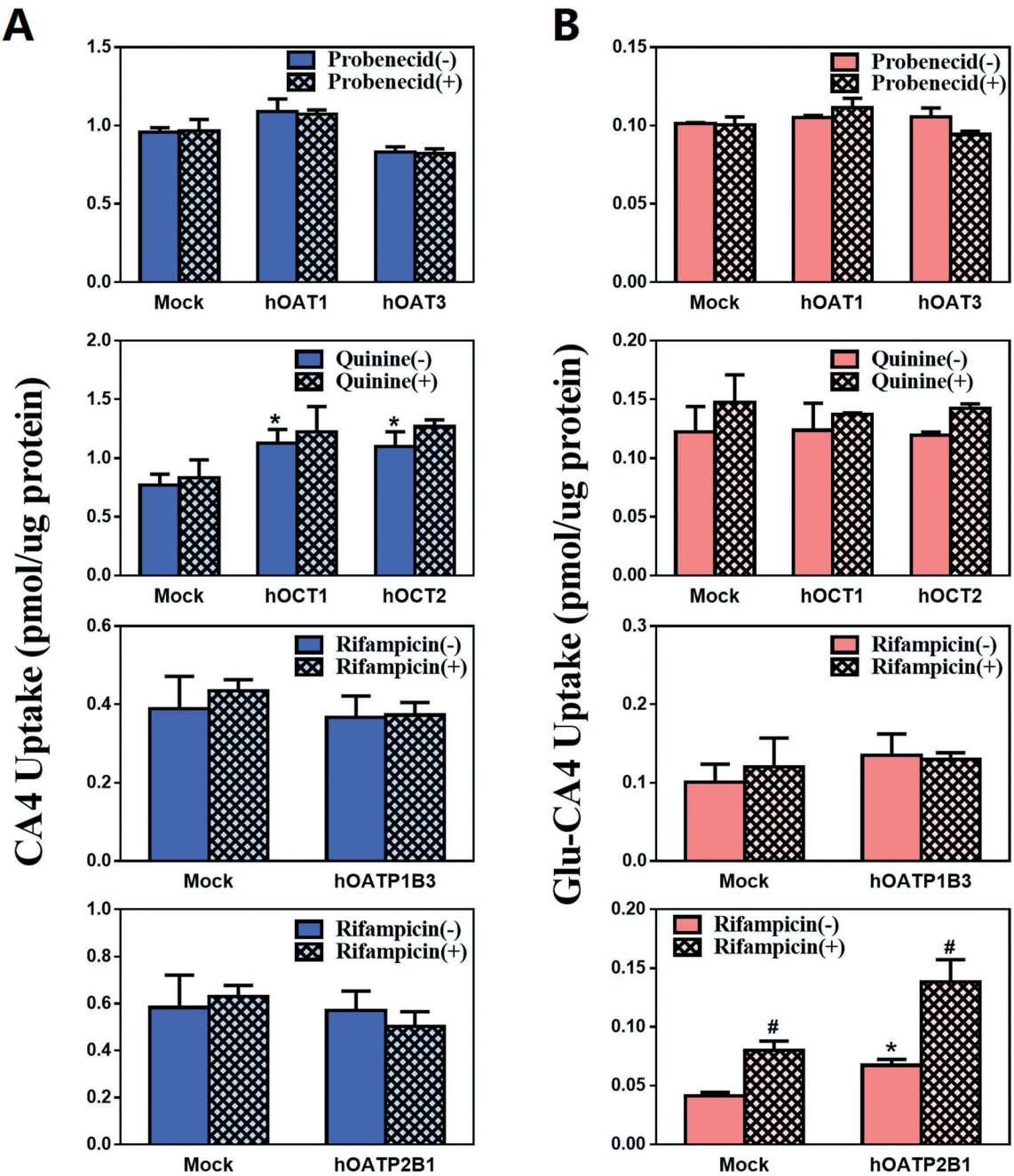


Fig. 7 Cellular uptake of CA4 (A) and **Glu-CA4** (B) in transporter-overexpressing cells and their mock cells.

Since OAT1 and OAT3 are almost exclusively expressed in the kidney and responsible for the renal excretion of a broad range of drugs, including anticancer drugs, such as methotrexate, 6-mercaptopurine, azathioprine, cisplatin, imatinib, cytarabine and vinblastine,[27] in addition, both OCT2 and OATP2B1 as renal and hepatic transporters play key roles in disposition and clearance of endogenous molecules and drug compounds, therefore, CA4 has great potential for causing DDIs through inhibitions of OATs and OATPs. In contrast, the inhibitory effect of **Glu-CA4** on the six transporters was minimal and thus the DDI potency of **Glu-CA4** is negligible.

3.6. *In vivo* toxicity of CA4 and its sugar conjugates

Since toxicity is a major concern for CA4 due to its narrow therapeutic margin, maximal tolerated dose (MTD) studies were conducted in order to evaluate the *in vivo* toxicity of the synthesized conjugates. Adult DBA/2 male mice were treated with either CA4 or its sugar conjugates by i.p. injection at daily intervals for 3 days, and the MTD was defined as the allowance of a median body weight loss of 15% of the body weight before treatment. As summarized in Table 2, the MTD values for CA4, **Glu-CA4**, **Man-CA4**, and **Gal-CA4** were 25, 400, 400, and 550 mg kg^{-1}, respectively. Thus, the present study confirmed that sugar conjugates of CA4 have a superior safety profile with their MTD values exhibiting a 16–34-fold increase compared to CA4.

4. Conclusion

With regard to drug efficacy, the pharmaceutical industry has great interest in drug transporters, particularly those with broad substrate specificities.[11] In the present study, we demonstrated for the first time the relationship between CA4 and six key drug transporters, namely, hOAT1, hOAT3, hOCT1, hOCT2, hOATP1B3 and hOATP2B1, which as an origin, might directly contribute to the safety profiles and affect the potential DDI properties of CA4 and its derivatives. CA4 was found to be a highly potent inhibitor of hOAT3 and hOATP2B1 and showed moderate inhibition of hOAT1 and hOCT2, whereas its inhibitory effects on hOCT1 and hOATP1B3 were very weak. With the synthesis of CA4 sugar conjugates using glucose, mannose, and galactose as sugar motifs, we systematically investigated the effects of sugar conjugation on the water solubility, *in vitro* cytotoxicity and *in vivo* safety, as well as their transport mechanisms. The results revealed that sugar conjugation greatly improved the water solubility and *in vivo* safety profile of CA4. Additionally, we demonstrated that the sugar conjugated CA4 analogs have no interactions with the six key transporters that were tested and thus may circumvent the interaction risk associated with the most important drug transporters, therefore significantly reducing the DDI potency of CA4. Given the drawbacks of CA4, the enhanced solubility and safety profiles of CA4 sugar conjugates augur well for further investigation into these intriguing candidates' *in vivo* efficacy.

Conflict of interest

The authors declare no competing interests.

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