Characterization of chrysin glucuronidation in UGT1A1-overexpressing HeLa cells:

Elucidating the transporters responsible for efflux of glucuronide

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Non-standard abbreviations
BCRP/Bcrp, breast cancer resistance protein; CG, chrysin glucuronide; CYP, cytochrome P450; DIPY, dipyridamole; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; K_m, Michaelis-Menten constant; K_si, substrate inhibition constant; LTC4, leukotriene C4; MRP/Mrp, multidrug resistance-associated protein; MS, mass spectroscopy; P-gp, P-glycoprotein; QTOF, Quadrupole time-of-flight; shRNA, short hairpin RNA; UDPGA, uridine diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra performance liquid chromatography; V_max, maximal velocity.
Abstract

Active transport of glucuronide out of cells is a critical process in elimination of drugs via the glucuronidation pathway. Here HeLa cells were stably transfected with UGT1A1 and the contributions of BCRP and MRP family transporters to cellular efflux of chrysin glucuronide (CG) were determined. The cDNA of UGT1A1 was introduced into HeLa cells using the lentiviral transfection method. The modified cells were functional in generation of the glucuronide from chrysin. Ko143 at 10-20 μM (a dual inhibitor of BCRP and UGT1A1) caused a marked decrease (51.3-59.7%, p < 0.01) in the excretion rate and efflux clearance of CG. Likewise, MK-571 at 5-20 μM (an inhibitor of MRPs but an activator of UGT1A1) resulted in a significant reduction in the excretion rate (18.2-64.0%, p < 0.01) and efflux clearance (37.0-90.2%, p < 0.001). By contrast, dipyridamole and LTC4 showed no inhibitory effects on CG excretion. The chemical inhibition indicated that excretion of CG was contributed by the MRP family transporters, whereas the role of BCRP was unclear. Further, short hairpin RNA (shRNA) mediated silencing of a target transporter led to a marked reduction (38.6% for BCRP, 39.3% for MRP1, 36.4% for MRP3 and 28.7% for MRP4; p < 0.01) in the excretion rate of CG. Transporter silencing also led to substantial decreases (44.7% for BCRP, 60.4% for MRP1, 36.7% for MRP3 and 28.7% for MRP4; p < 0.01) in the efflux clearance. The gene silencing results suggested that BCRP, MRP1, MRP3 and MRP4 were significant contributors to excretion of CG.
Introduction

Investigations of drug-like properties such as solubility, permeability and pharmacokinetics have become an indispensable part of drug discovery and development programs because poor properties are associated with a high rate of drug attrition (Kola and Landis, 2004). Drug metabolism, a component of pharmacokinetics, is a major determinant to the drug exposure at the target site(s) as well as the toxicity profiles (Costa et al., 2014). Drug-metabolizing enzymes are historically classified into phase I and phase II enzymes. Phase I enzymes such as cytochrome P450s (CYPs) catalyze the oxidation, reduction and hydrolysis reactions (Ortiz de Montellano, 2005). Phase II enzymes catalyze the conjugation of a polar moiety (e.g., glutathione, sulfate, and glucuronic acid) to the substrates (Jancova et al., 2010).

Glucuronidation is an important phase II metabolic reaction that is responsible for clearance of 35% drugs metabolized by phase II enzymes (Evans and Relling, 1999). In the glucuronidation reaction, the glucuronic acid derived from the cofactor UDPGA is transferred to the substrates via the action of the UDP-glucuronosyltransferase (UGT) enzymes (Wells et al., 2004). In addition to conjugating substrates that have undergone phase I metabolism, UGTs can also directly metabolize many drugs such as raloxifene and gemfibrozil (Wienkers and Heath, 2005). In humans, UGTs constitute a number of enzymes that are divided into five families, UGT1A, UGT2A, UGT2B, UGT3A and UGT8A (Mackenzie et al., 2005; Rowland et al., 2013). Enzymes from UGT1A (with nine members) and 2B families (with seven members) are the main
contributors to xenobiotic/drug metabolism (Rowland et al., 2013). Of all UGT enzymes, UGT1A1 perhaps is the most important one due to its role in detoxification of bilirubin. Genetic deficiency of UGT1A1 leads to various forms of hyperbilirubinemia (Tukey and Strassburg, 2000).

Drug transporters are membrane-bound proteins expressed in various tissues such as liver, intestine and kidney (Giacomini et al., 2010; DeGorter et al., 2012). By controlling the movement of drug molecules across cell membranes, these transporters play an important role in drug absorption, distribution and excretion (Giacomini et al., 2010; DeGorter et al., 2012). Efflux transporters mediate the transport of drug/metabolite molecules out of cells. The members of efflux transporters with well-defined roles in drug pharmacokinetics include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) (DeGorter et al., 2012). P-gp transports mostly cationic and neutral compounds, whereas BCRP and MRPs transport anions such as the UGT metabolite glucuronide (Yang et al., 2009).

Drug elimination via the glucuronidation pathway involves at least two distinct and sequential processes, namely, glucuronide formation and excretion (Siissalo et al., 2010; Wu, 2012). The glucuronide formation process refers to cellular production of the glucuronide by UGT enzymes, whereas the glucuronide excretion refers to the transport of produced glucuronide out of cells by efflux transporters. Active transport of glucuronide by efflux transporters is necessary because the glucuronide is impermeable to cell membranes due to a high hydrophilicity (Jeong et al., 2005; Liu and Hu, 2007; Siissalo et al., 2010). A number of studies have shown that murine Bcrp
and/or Mrp2 contribute significantly to excretion of glucuronides (Zamek-Gliszczynski et al., 2006; Lee et al., 2009; Yang et al., 2012). Also, human BCRP may be involved in cellular efflux of glucuronides based on chemical inhibition experiments (Jiang et al., 2012; Tang et al., 2014). The efflux transporters appear to work in concert with UGT enzymes in efficient removal of drugs from the body, a phenomenon termed “glucuronidation-transport interplay” (Jeong et al., 2005; Liu and Hu, 2007).

The efflux transporters are a critical determinant to the pharmacokinetics of many xenobiotics (including drugs) and their glucuronides (Zamek-Gliszczynski et al., 2006; Wu, 2012). Hence, it assumes great importance to determine the glucuronide transporters and further to elucidate the mechanisms underlying the “glucuronidation-transport interplay”. Toward the goal, HeLa cells (lacking in expression of drug-metabolizing enzymes) were stably transfected with UGT1A1 to generate the cells that were metabolically active at the glucuronidation pathway only. Identification of glucuronide transporters in the cells were performed employing both the chemical inhibition (with chemical inhibitors of transporters) and biological inhibition (shRNA mediated silencing of a target transporter) methods. Chrysin (a natural flavonoid) was selected as the model compound for a comprehensive evaluation because it is a good substrate of the UGT1A1 enzyme.
**Materials and Methods**

**Materials**

Expressed or recombinant human UGT1A1 and the anti-UGT1A1 antibody were purchased from BD Biosciences (Woburn, MA). pGEM-T plasmid carrying UGT1A1 cDNA clone was purchased from Sino biological Inc. (Beijing, China). HeLa cells, 293T cells, the pLVX-mCMV-ZsGreen-PGK-Puro vector (9371 bp) and the pLVX-shRNA2-Neo vector (9070 bp) were purchased from BioWit Technologies (Shenzhen, China). The anti-BCRP, anti-MRP1, anti-MRP2, anti-MRP3 and anti-MRP4 antibodies were purchased from OriGene Technologies (Rockville, MD). Uridine diphosphogluconic acid (UDPGA), alamethicin, D-saccharic-1,4-lactone monohydrate, leukotriene C4 (LTC4), MK-571, dipyridamole (DIPY) and Ko143 were purchased from Sigma-Aldrich (St Louis, MO). Chrysin was purchased from Aladdin chemicals (Shanghai, China). Chrysin-7-O-glucuronide (chrysin glucuronide or CG) was synthesized using rat liver microsomes as described (Liu et al., 2014). All other materials (typically analytical grade or better) were used as received.

**Development of stably transfected HeLa cells**

**Cloning:**

Full-length cDNA for human UGT1A1 was PCR-amplified from the pGEM-T-UGT1A1 plasmid. Forward and reverse primers were 5’-CGCGGATCCGCCACCATGGCTGTGGAGTCCCGG and 5’-CGACGCGTTCCATGGGTCTTGGGATTTGTGGGCTTTCTTAACTC, respectively. In the
primers, the restriction sites of BamHI and MluI were introduced (underlined in the sequences). The PCR conditions were as follows: 3-min denaturation at 95 °C followed by 35 cycles of 20 s at 95 °C, 30 s at 55 °C and 2 min at 72 °C, and a final step of 72 °C for 5 min using Pfu polymerase (Stratagene, La Jolla, CA). The PCR products were separated by electrophoresis on a 1.0% agarose gel, and the fragment of 1602 bp (UGT1A1 cDNA) was purified. The purified UGT1A1 cDNA was subcloned to the pLVX-mCMV-ZsGreen-PGK-Puro vector through BamHI and MluI restriction. The cloned genes were sequenced within the vector construct and were found to be identical to the published genomic sequence (GenBank accession no NM_000463.2).

**Lentiviral vector production:** lentiviral vectors were produced by transient transfection into 293T cells using a modified third-generation packaging system from BioWit technologies (Shenzhen, China). In brief, 6–8 × 10⁶ 293T cells were seeded into 10-cm dishes and maintained at 37°C under 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Plasmid DNAs were introduced to the cells using the modified calcium precipitation method on day 2. The culture medium was changed to DMEM containing 10% FBS and 1% penicillin/streptomycin on day 3. The lentiviral particles in the supernatant were collected by filtration (using 0.45 μm PVDF filter) and high-speed centrifugation (50,000 g, 2 h). The viral titers (= 5.0 × 10⁷ TU/ml) were determined using the Lentiviral Rapid Titer Kit (Biowit Technologies, China) according to the manufacturer’s instructions.

**Cell transfection:** HeLa cells were seeded at a density of 6 × 10⁵ cells/well in a six-well plate
and maintained at 37°C under 5% CO₂ in DMEM with 10% FBS. The cells were transfected by incubation with the lentivirus. After transfection, the cells were maintained at 37°C under 5% CO₂ in DMEM containing 10% FBS and puromycin (8 μg/ml). Media were changed every 2 or 3 days. After one week, the medium was changed to DMEM containing 10% FBS and 2 μg/ml puromycin. Once 100% confluence was reached, the cells were collected and processed for DNA identification. The transfection efficiency was evaluated by a fluorescence microscopy (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan) and a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). The stably transfected cells were cryopreserved for future uses. Each vial of cryopreserved cells was used for 15 passages before a new one was initiated for continued use.

**Transient transfection of shRNA plasmid**

**Plasmid construction:** The shRNAs containing the restriction site of *MluI* enzyme (Table 1) were synthesized by Biowit Technologies (Shenzhen, Chia). The shRNAs were ligated into the pLVX-ShRNA2-Neo plasmid through BamHI and XhoI restriction. The recombinant plasmids were transformed into *E. coli* JM109 and the resultant cells were cultured in LB medium. Colonies containing cloned plasmids were recovered. The recombinant plasmid in the selected colonies was prepared. The plasmid was purified by a Plasmid Maxi Preparation Kit (QIAGEN, Germany). The integrity of plasmid was confirmed by agarose gel electrophoresis.

**Cell transfection:** The UGT1A1-overexpressing HeLa cells (HeLa1A1 cells) were seeded at a
density of 2.0 × 10^5 cells/well in a six-well plate and maintained at 37°C under 5% CO₂ in DMEM containing 10% FBS. On the next day, the plasmid construct carrying the shRNA or scramble (4 μg) was transfected into the cells using Polyfectine following the manufacturer’s protocol (Biowit Technologies, Shenzhen, Chia). Cells were ready for excretion experiment 2 days after transfection. Following an identical procedure, shRNA plasmid of MRP1 or MRP3 or MRP4 was transfected into the HeLa1A1 cells.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Cells were collected, and total RNA isolation was performed using the TRIzol extraction method. The RNA purity of each sample was checked by absorbance ratios of 260 nm/280 nm and 260 nm/230 nm (> 1.8). The total RNA was converted to cDNA using the iScript cDNA synthesis kit according to the manufacturer’s protocol (Bio-Rad, Hercules, CA). The PCR conditions were as follows: 3-min denaturation at 95 °C followed by 30 cycles of 20 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final step of 72 °C for 5 min using Taq DNA polymerase (TransGen Biotech, Beijing, China). Information on the primer sequence is summarized in Table 2. Primer specificity was checked by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). After RT-PCR, agarose gel electrophoresis and UV visualization were used to determine the relative amounts of PCR products.

**Preparation of cell lysate**

Membranes of HeLa1A1 cells (from 75 ml flask) cultured for 3 days were disrupted in pH 7.4
HBSS buffer, by sonication for 15 min in an ice-cold water bath. The protein content of the lysate was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

**Glucuronide excretion experiment using HeLa1A1 and shRNA transfected cells**

The excretion experiment was performed as described (Jiang et al., 2012). In brief, all culture wells were washed three times with 37°C HBSS (pH 7.4) prior to experimentation. HeLa1A1 cells were incubated at 37°C in HBSS (2 ml) containing 10 μM chrysin. Inhibitors, when used, were co-incubated with chrysin. At each specified time points (i.e., 0.5, 1, 1.5 and 2 h), a 200-μl aliquot of incubation medium was taken and then replaced with the same volume of dosing solution (containing chrysin). The concentrations of chrysin glucuronide in incubation medium were measured by UPLC analysis. After sampling at the last time point, the medium was rapidly removed by suction and the cells were washed twice with ice-cold HBSS. The intracellular amounts of chrysin and CG were obtained by solubilizing each culture well with 400-μl of ice-cold MeOH:H2O (5:5, v/v) and sonicating the mixture (in an ice-cold ultrasonic bath) for 15 min. The homogenate was centrifuged for 15 min at 18,000 g, and the resulting supernatant was analyzed by UPLC. The intracellular concentration of chrysin (or CG) was estimated as the intracellular amount of chrysin (or CG) divided by the intracellular volume of the cells. The intracellular volume of the cells was assumed to be 4 μl/mg protein (Jiang et al., 2012). The excretion rate (ER) of intracellular glucuronide was calculated based on equation 1. The apparent efflux clearance (CL_app) for glucuronide was derived by ER/Ci. Ci was the intracellular concentration of
glucuronide.

\[ \text{ER} = V \frac{dC}{dt} \quad \text{eq.1} \]

Where \( V \) was the volume of the incubation medium; \( C \) was the cumulative concentration of glucuronide and \( t \) is the incubation time. \( dC/dt \) described the changes of the glucuronide levels with the time.

**Glucuronidation assay**

Chrysin was incubated with expressed UGT1A1 or HeLa1A1 cell lysate to determine the rates of glucuronidation as described in our publication (Liu et al., 2014). In brief, the incubation medium contained the UGT1A1 enzyme (or cell lysate at 26.5 \( \mu \)g/ml), MgCl\(_2\) (0.88 mM), saccharolactone (4.4 mM), alamethicin (22 \( \mu \)g/ml), UDPGA (3.5 mM) and chrysin in 50 mM potassium phosphate (pH 7.4). The reaction was terminated by adding ice-cold acetonitrile, followed by vortex and centrifugation (15 min; 18,000g). The supernatant was subjected to UPLC analysis. Preliminary experiments were performed to ensure that the rates of glucuronidation were determined under linear conditions with respect to the incubation time and protein concentration. All experiments were performed in triplicate. Glucuronidation rates were calculated as nmol glucuronide formed per reaction time per protein amount (or nmol/min/mg).

**Glucuronide hydrolysis experiment**

The HeLa cell lysate was incubated with chrysin glucuronide to determine the rates of glucuronide hydrolysis. In brief, the incubation medium maintained at 37°C contained the cell
lysate (0.5 mg/ml) and chrysin (11.2-170 μM) in 50 mM potassium phosphate (pH 7.4). The reaction was terminated by adding ice-cold acetonitrile, followed by vortex and centrifugation (15 min; 18,000g). The supernatant was subjected to UPLC analysis. The intrinsic clearance (CL_{int}) was determined from the profile of hydrolysis rate vs. substrate concentration using linear regression (CL_{int} equaled to the slope).

Quantification of chrysin and its glucuronide by UPLC analysis

The concentrations of chrysin glucuronide were determined by Waters ACQUITY UPLC system equipped with a photodiode array (PDA) detector (Milford, MA). Chromatographic separation was performed on a BEH column (2.1 × 50 mm, 1.7 μm; Waters). Elution was performed using a gradient of 0.1% formic acid in water (mobile phase A) versus acetonitrile (mobile phase B) at a flow rate of 0.45 ml/min. The gradient program was 10% B at 0 to 0.5 min, 10 to 90% B at 0.5 to 2.8 min, 90% B at 2.8 to 3.3 min, and 90 to 10% B at 3.3 to 4 min. The detection wavelength was 267 nm. At least six standard curve samples (covering the range of 39-2500 nM) were prepared using the vehicle solution (i.e., 50 mM phosphate buffer, HBSS buffer or 50%MeOH).

Kinetic evaluation

The rates of glucuronidation were determined for chrysin at a series of concentrations (0.078-10 μM) according to the glucuronidation assay protocol. The substrate inhibition model (eq.2) was fitted to the data of glucuronidation rates versus substrate concentrations because the corresponding Eadie–Hofstee plot was featured with a hook in the upper panel (Hutzler and
Tracy, 2002). Model fitting and parameter estimation were performed using the Graphpad Prism V5 software (San Diego, CA).

\[
V = \frac{V_{\text{max}} \cdot [S]}{K_s + [S] \left(1 + \frac{[S]}{K_{si}}\right)} 
\]

**Immunoblotting**

The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in the RIPA buffer (Sigma-Aldrich). The resulting cell lysate (40 μg total protein) was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8% acrylamide gels) and transferred onto PVDF membranes (Millipore, Bedford, MA). Blots were probed with the UGT1A1 or transporter antibody (i.e., anti-BCRP, anti-MRP1, anti-MRP2, anti-MRP3 and anti-MRP4) followed by horseradish peroxidase-conjugated rabbit antigoat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were detected by enhanced chemiluminescence and imaged by autoradiography.

**Statistical analysis**

Data are expressed as mean ± SD. Data were analyzed by one-way analysis of variance or Student’s t test as appropriate, and the level of significance was set at \( p < 0.05 \) (*) or \( p < 0.01 \) (**) or \( p < 0.001 \) (***).
Results

Construction of stably transfected HeLa cells

Digestion of the lentiviral vector by restriction enzymes produced a fragment of 1602 bp that corresponded well to the cDNA of UGT1A1 (Figure 1A). PCR analysis of the transfected cells showed a clear band of UGT1A1, indicating that the gene was integrated into the cell genome (Figure 1B). The cells were visualized under a fluorescence microscope. The intensive fluorescence was an indicator of a high efficiency of transfection (Figure 1C). The transfection efficiency was further quantified by flow cytometry. The results showed that 99% of cells were transfected with the target gene (Figure 1D). The UGT1A1-overexpressing HeLa cells were abbreviated as HeLa1A1 cells for ease of reading.

Functional characterization of HeLa1A1 cells

Western blotting showed that the UGT1A1 protein was found significantly in HeLa1A1 cells, whereas UGT1A1 was not expressed in wild-type HeLa cells (Figure 2A). Further, HeLa1A1 cells were rather active in generation of the glucuronide after incubation with chrysin (Figure 2B). By contrast, the wild-type HeLa cells were inactive in glucuronidation of chrysin (Figure 2B). In addition to chrysin, the HeLa1A1 cells were able to generate glucuronides from many other types of aglycones including genistein, emodin, estradiol, SN-38 and raloxifene (data not shown). The results indicated that UGT1A1 expressed in the engineered cells are functional in catalyzing the glucuronidation reaction.
Expression of efflux transporters in HeLa and HeLa1A1 cells

Expression of the BCRP, MRP1, MRP2, MRP3 and MRP4 transporters in HeLa and HeLa1A1 cells was determined at both mRNA and protein levels. Both HeLa and HeLa1A1 cells expressed the mRNAs of BCRP, MRP1, MRP3 and MRP4, but did not express MRP2 mRNA (Figure 3A). Further, the proteins of BCRP, MRP1, MRP3 and MRP4 were detected in both HeLa and HeLa1A1 cells, whereas the MRP2 protein was not found (Figure 3B). It was noted that the wild-type and engineered cells showed an identical pattern in transporter expression (Figure 3).

Glucuronidation of chrysin by recombinant UGT1A1 enzyme and HeLa1A1 cell lysate

The reaction kinetics for glucuronidation of chrysin by recombinant UGT1A1 (rUGT1A1) and HeLa1A1 cell lysate were determined, modelled and compared. Chrysin glucuronidation by both rUGT1A1 and cell lysate followed the substrate inhibition kinetics (Figure 4). Comparing the derived kinetic parameters showed that there were no significant differences (p > 0.05) in the $K_m$ or $K_{si}$ value (Figure 4). However, the $V_{max}$ value of rUGT1A1 was significant higher (p < 0.05) than that of cell lysate (Figure 4).

Effects of chemical inhibitors on disposition of chrysin in HeLa1A1 cells

Ko143 is a well-recognized BCRP inhibitor (Burger et al., 2004; Hori et al., 2004; Pan et al., 2007; Giri et al., 2009). Use of Ko143 (10 & 20 μM) caused a significant reduction (51.3-54.0%, p < 0.001) in excretion of CG (Figure 5A). Although changes in the intracellular level of CG were not significant (p > 0.05) (Figure 5B), the apparent efflux clearance ($CL_{app}$) was markedly decreased
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(52.9-59.7%, p < 0.01) by Ko143 (Figure 5C). Dipyridamole (DIPY) had been also used as a BCRP inhibitor in the literature (Zhang et al., 2005; Wang et al., 2008; Liu et al., 2012). Although DIPY at 10 μM did not alter the rate of CG excretion, DIPY at 20 μM resulted in a significant increase (110%, p < 0.05) in excretion of CG (Figure 5D). There were no significant changes (p > 0.05) in either intracellular level or efflux clearance of CG (Figure 5E/F).

The pan-MRP inhibitor MK-571 (5 & 20 μM) caused a significant reduction (18.2-64.0%, p < 0.01) in excretion rate of CG but an elevation (130-369%, p < 0.05) in the intracellular level of CG (Figure 6A/B). The CL_app value was significantly decreased (37.0-90.2%, p < 0.001) (Figure 6C). LTC4 is a high-affinity substrate of MRP1/MRP2 that may be used to inhibit the activity of MRPs (Loe et al., 1996; Cui et al., 1999; Miller et al., 2000; Hu et al., 2003). However, LTC4 (0.1 & 0.4 μM) did not alter the disposition of chrysin in HeLa1A1 cells (Figure 6D-F). No changes were observed with the rate of CG excretion, the intracellular level of CG or efflux clearance (Figure 6D-F).

**Effects of chemical inhibitors on glucuronidation of chrysin**

Effects of the transporter inhibitors on glucuronidation of chrysin were determined using HeLa1A1 cell lysate and recombinant UGT1A1 (rUGT1A1). Ko143 showed significant inhibitory effects (p < 0.05) on chrysin glucuronidation mediated by both cell lysate and rUGT1A1 (Figure 7). DIPY showed an activation effect on glucuronidation at a high concentration of 20 μM (Figure 7). However, it did not alter the glucuronidation activity at a lower concentration of 5 μM (Figure
7). LTC4 (0.1 & 0.4 μM) did not cause any changes in the glucuronidation rate (Figure 7). By contrast, MK-571 showed potent activation effects (p < 0.001) on glucuronidation (Figure 7). It was noted that a stronger activation effect was observed at a lower concentration of MK-571 (Figure 7).

**Effects of transporter silencing on disposition of chrys in in HeLa1A1 cells**

BCRP silencing caused a significant reduction in the excretion rate of CG (38.6%, p < 0.01) as well as in the efflux clearance (44.7%, p < 0.01) (Figure 8A/C). However, the intracellular level of CG was not changed (Figure 8B). The shRNA mediated silencing of BCRP gene resulted in obvious decreases in the protein (49%, p < 0.01) and mRNA levels (83%, p < 0.001) of BCRP (Figures 8D & Supplemental Figure 1). The results suggested that BCRP played a role in excretion of CG in HeLa cells.

Silencing of MRP1 resulted in a significant reduction in the excretion rate but an elevation in the intracellular level of CG (Figure 9A/B). As a result, a marked increase in the value of CL_app was observed (Figure 9C). The altered glucuronide excretion can be ascribed to the reduced expression of MRP1 (Figures 9D & Supplemental Figure 1). MRP3 silencing caused a significant reduction in excretion of CG (36.4%, p < 0.01) and in the efflux clearance (36.6%, p < 0.01) (Figure 10). Likewise, MRP4 silencing caused a moderate reduction in excretion of CG (28.7%, p < 0.01) and in the efflux clearance (29.0%, p < 0.01) (Figure 11). The compromised CG excretion was accounted for by reduced expression of these two proteins (Figures 10-11 & Supplemental
Figure 1). Taken together, the results suggested that MRP1, MRP3 and MRP4 were responsible for excretion of CG in HeLa cells.

Hydrolysis of CG by HeLa1A1 cell lysate

The cell lysate was utilized to determine whether conversion of the glucuronide back to the parent compound (chrysin) can occur within the cells. The results showed that the hydrolysis reaction occurred at a wide range of glucuronide concentrations with an intrinsic clearance of 3.98 ul/h/mg. Hence, there was a possibility that the intracellular CG can be hydrolyzed back to the parent compound.
Discussion

In this study, HeLa cells were stably transfected with the UGT1A1 gene. The engineered cells were fully functional in generation of glucuronides from the UGT substrates. It was shown that the glucuronidation ability of the cells arose from the UGT1A1 enzyme produced within the cells. Hence, UGT1A1-overexpressing HeLa cells were a useful tool for studying UGT1A1 functions at a cellular level. We also demonstrated that cellular excretion of CG was contributed by multiple efflux transporters (including BCRP, MRP1, MRP3 and MRP4) using a combined approach of chemical inhibition and shRNA mediated silencing. Our findings were consistent with the study of Jiang et al (2012) in which BCRP was involved in excretion of flavonoid glucuronides in HeLa cells. However, Jiang et al (2012) showed that contributions of MRP family proteins (e.g., MRP2/MRP3) to glucuronide excretion were negligible. In our study, we provided strong evidence that MRP family proteins contributed significantly to excretion of CG. First, the pan-MRP inhibitor MK-571 caused a significant reduction in CG excretion, suggestive of efflux contribution from one or more MRP transporters (Figure 6). Second, decreased expression of any of the three MRP proteins (i.e., MRP1, MRP3 and MRP4) led to reduced CG excretion (Figures 9-11). The inconsistent role of MRPs in glucuronide excretion between the present study and that of Jiang et al (2012) may be explained by the HeLa cell heterogeneity (HeLa cell line may vary significantly between laboratories) (Carson and Pirruccello, 2013). It was thus noted that the results presented here about the expression levels of efflux transporters may not necessarily be valid in another batch of HeLa cells.
It was a novel discovery that the transporter inhibitors Ko143 and MK-571 can markedly modify the glucuronidation activity (Figure 5). Ko143 was an effective inhibitor of UGT1A1 and the inhibitory effects on chrysin glucuronidation were concentration-dependent (Figure 5). Further, inhibition by Ko143 followed the competitive mechanism with a Ki value of 3.3 μM (Supplemental Figure 2). By contrast, MK-571 was an apparent ‘inducer’ of UGT1A1 as it showed activation effects at both concentrations of 5 and 20 μM (Figure 6). It was noteworthy that the activation effect was much weaker at the high concentration of 20 μM. Inhibition kinetics revealed that MK-571 was a ‘mixed’ modifier of UGT1A1 rather than a pure inducer (Supplemental Figure 3). A ‘mixed’ modifier shows activation effects at low concentrations but inhibition effects at high concentrations (Zhou et al., 2010). The activation effect was attained through increased catalytic efficiency (γ > 1) after binding to the allosteric site (Figure S3).

Identification of efflux transporters responsible for glucuronide excretion by chemical inhibitors should be treated with caution due to their potential in modification of the glucuronidation activity. Ko143 is a potent and selective BCRP inhibitor (Allen et al., 2002). It has gained a widespread use in inhibiting the transport activity of BCRP in the literature (Burger et al., 2004; Hori et al., 2004; Pan et al., 2007; Giri et al., 2009). However, Ko143 also showed significant inhibitory effects on the glucuronidation activity (Figure 7). Thus, the altered glucuronide excretion cannot be simply ascribed to a reduced BCRP activity. This was because suppressed metabolism would also lead to a reduction in glucuronide excretion. Clearly, the altered glucuronidation activity was a compounding factor to determining the role of BCRP in
efflux of glucuronide with the inhibitor Ko143.

Compared to Ko143, use of dipyridamole (DIPY) as a BCRP inhibitor is relatively limited (Zhang et al., 2005; Wang et al., 2008; Liu et al., 2012). This may be because little is known about its inhibitory selectivity toward other transporters. Our results showed that DIPY did not alter the efflux clearance of CG. In addition, DIPY at a high concentration of 20 μM led to a slight increase in the excretion rate of CG (Figure 5B). This increase most likely was contributed by the enhanced glucuronidation activity (Figure 7). The lack of inhibition by DIPY was contrasted by the strong effect of BCRP silencing on CG efflux (Figure 8). The exact reasons were unknown as to why the transport of CG was not inhibited by DIPY. However, a plausible explanation was provided here. DYPY was a much weaker inhibitor of BCRP compared to Ko143 (Allen et al., 2002; Zhang et al., 2005). The IC$_{50}$ values were 6.4 μM (for DYPY) and 23 nM (for Ko143) in inhibiting transport of mitoxantrone by BCRP (Allen et al., 2002; Zhang et al., 2005).

MK-571 is a pan-MRP inhibitor that effectively inhibits transport of drugs by MRP family members (Williams et al., 2002; Lüders et al., 2009; Takeuchi et al., 2012; Ferslew et al., 2014). It was found that MK-571 increased the rates of glucuronidation (Figure 7). Even so, use of MK-571 resulted in a reduction in both the excretion rate and efflux clearance (Figure 6A/B). The results collectively indicated that MRP transporters played an important role in transporting CG out of cells. A reduced activity of MRPs blocked the glucuronide transport, leading to intracellular accumulation of glucuronide (Figure 6C). It was noteworthy that MK-571 may also inhibit the
transport activity of BCRP (Matsson et al., 2009). However, MK-571 was a much potent inhibitor for MRPs compared to BCRP because the IC$_{50}$ value for inhibition of BCRP by MK-571 was five times of that for inhibition of MRP2 (Matsson et al., 2009).

LTC4 is a high-affinity substrate of MRP1/MRP2 ($K_m = 0.1/1\, \mu M$) that has been used to inhibit the transport of drugs/metabolites by MRPs (Loe et al., 1996; Cui et al., 1999; Miller et al., 2000; Hu et al., 2003; Jiang et al., 2012). However, the inhibition selectivity of LTC4 toward MRP family members was unknown. Our results showed that LTC4 did not cause any changes in either glucuronidation activity or CG excretion (Figures 6 & 7). Similar observations have been also noted in a previous study (Jiang et al., 2012). Hence, it remained to clarify whether LTC4 is an effective inhibitor of MRPs.

Glucuronidation of chrysin followed the substrate inhibition kinetics (Figure 4). This was not surprising because UGT1A1-mediated glucuronidation of flavonoids (e.g., genistein and flavonols) often times showed substrate inhibition (Tang et al., 2009; Wu et al., 2011). The $V_{\text{max}}$ value of rUGT1A1 was much larger than that of cell lysate (Figure 4). This was reasonable because the UGT1A1 enzyme was much more concentrated in rUGT1A1 than in cell lysate. The derived kinetic parameters were not corrected using protein binding in microsomal incubations. This was because binding of chrysin (LogP = 2.1) to microsomal proteins was negligible (fu > 0.99) according to the Halifax and Houston model (Zhou et al., 2010). The Halifax and Houston model provides accurate predictions on fu values particularly for the compounds with
intermediate lipophilicity (Gao et al., 2010). Further, adequate modeling of the kinetic profiles also indicated that correction of protein binding was unnecessary (Figure 4).

It was an interesting finding that CG can be efficiently hydrolyzed back to the parent compound (aglycone) within the cells (Figure 12). It helped us to understand why inhibition of transporter activity would lead to a reduction in excreted glucuronide or glucuronide production. The deglucuronidation (i.e., conversion of glucuronide back to the aglycone) process appeared to be a feedback mechanism (Wu, 2012). A compromised efflux of glucuronide would lead to glucuronide accumulation within cells. The latter triggered an elevation in the rate of deglucuronidation reaction according to a first-order reaction mechanism (the conversion rate = the intrinsic clearance × the glucuronide concentration) (Figure 12). As a result, the net production of CG was reduced in the cell system.

Use of HeLa1A1 cells for glucuronide transport studies was more advantageous compared to the membrane vesicles overexpressing a transporter. This was because synthesis of the glucuronide (usually lacks a commercial availability) was not required as the glucuronide was produced within cells from the aglycone via the action of UGT1A1. The HeLa1A1 cells were also more advantageous than Caco-2 cells in studying cellular glucuronidation as well as glucuronide transport. Caco-2 cells express various types of drug-metabolizing enzymes including CYPs, UGTs, and SULTs. Drug molecules often times undergo multiple metabolic pathways in Caco-2 cells. Evaluation of glucuronide transport would be seriously complicated by simultaneous
transport of other types of metabolites (e.g., sulfates). It was noteworthy that in addition to the transfected HeLa cells, evaluation of glucuronide transport can be well performed using the monolayer cells co-overexpressing UGT enzyme and transporters (e.g., triple-transfected MDCK-OATP1B1-UGT1A1-MRP2 cells) (Fahrmayr et al., 2012).

In summary, HeLa cells have been stably transfected with the UGT1A1 gene. The engineered cells were fully functional in generation of glucuronides from the UGT substrates. It was shown that the glucuronidation ability of the cells arose from the UGT1A1 enzyme produced within the cells. Hence, the UGT1A1-overexpressing HeLa cells were a useful tool for studying UGT1A1 functions at a cellular level. Further, we showed that cellular excretion of CG was potentially contributed by multiple efflux transporters (including BCRP, MRP1, MRP3 and MRP4) using combined approaches of chemical inhibition and shRNA mediated silencing.
Authorship Contributions

Participated in research design: Qaun, Wang, Dong and Wu.

Conducted experiments: Quan and Wang.

Contributed new reagents or analytic tools: Dong.

Performed data analysis: Quan, Wang, Zhang and Wu.

Wrote or contributed to the writing of the manuscript: Quan, Wang and Wu.
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Footnotes

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EQ and HW contributed equally to this work.
Legends for Figures

**Figure 1** Development of stably transfected HeLa cells. (A) Digestion of the lentiviral vector by restriction enzymes, showing a fragment of 1602 bp that corresponded well to the cDNA of UGT1A1. (B) PCR analyses of the transfected and wild-type cells. Lane 1, the PCR result of the UGT1A1 transfected cells; lane 2, the PCR result of the blank plasmid transfected cells; lane 3, the PCR result of the wild-type cells. (C) Visualization of the cells under a fluorescence microscope (left panel). The right panel was the corresponding white-light image. (D) Flow cytometric analyses of the transfected and wild-type cells (control).

**Figure 2** Functional characterization of HeLa1A1 cells. (A) Western blotting of the transfected and wild-type (WT) cells against human UGT1A1. (B) Comparisons of UPLC chromatograms, showing that HeLa1A1 cells were rather active in generation of the glucuronide after incubation with chrysin.

**Figure 3** Expression of efflux transporters in HeLa and HeLa1A1 cells. (A) mRNA expression of BCRP and four MRP family transporters in HeLa and HeLa1A1 cells. (B) Protein expression of BCRP and four MRP family transporters in HeLa and HeLa1A1 cells.

**Figure 4** Kinetic profiles for chrysin glucuronidation by HeLa1A1 cell lysate and recombinant UGT1A1 enzymes. (A) Kinetic profile for glucuronidation of chrysin by HeLa1A1 cell lysate. (B) Kinetic profile for glucuronidation of chrysin by recombinant UGT1A1 enzyme. In each panel, the insert shows the corresponding Eadie-Hofstee plot.

**Figure 5** Effects of chemical inhibitors on disposition of chrysin in HeLa1A1 cells. (A) Effects of Ko143 on the excretion profile of glucuronide; (B) Effects of Ko143 on the intracellular level of glucuronide; (C) Effects of Ko143 on the efflux clearance of glucuronide; (D) Effects of dipyridamole (DIPY) on the excretion profile of glucuronide; (E) Effects of DIPY on the intracellular level of glucuronide; (F) Effects of DIPY on the efflux clearance of glucuronide. Each data point is the average of three determinations with error bar representing the S.D. (n = 3). **p < 0.01; ***p < 0.001.

**Figure 6** Effects of chemical inhibitors on disposition of chrysin in HeLa1A1 cells. (A) Effects of MK-571 on the excretion profile of glucuronide; (B) Effects of MK-571 on the intracellular level of glucuronide; (C) Effects of MK-571 on the efflux clearance of glucuronide; (D) Effects of leukotriene C4 (LTC4) on the excretion profile of glucuronide; (E) Effects of LTC4 on the intracellular level of glucuronide; (F) Effects
of LTC4 on the efflux clearance of glucuronide. Each data point is the average of three determinations with error bar representing the S.D. (n = 3). *p < 0.05; ***p < 0.001.

Figure 7 Effects of chemical inhibitors on glucuronidation of chrysin. (A) Effects of chemical inhibitors (Ko143, DIPY, LTC4 and MK-571) on glucuronidation of chrysin mediated by HeLa1A1 cell lysate. (B) Effects of chemical inhibitors (Ko143, DIPY, LTC4 and MK-571) on glucuronidation of chrysin mediated by recombinant UGT1A1 enzyme (rUGT1A1). *p < 0.05; ***p < 0.001.

Figure 8 Effects of shRNA-mediated BCRP silencing on the excretion of chrysin glucuronide. (A) Effects of BCRP silencing on the excretion profile of glucuronide; (B) Effects of BCRP silencing on the intracellular level of glucuronide; (C) Effects of BCRP silencing on the efflux clearance of glucuronide; (D) Effects of gene silencing on the protein level of BCRP. Each data point was the average of three determinations with error bar representing the standard deviation (n = 3). **p < 0.01.

Figure 9 Effects of shRNA-mediated MRP1 silencing on the excretion of chrysin glucuronide. (A) Effects of MRP1 silencing on the excretion profile of glucuronide; (B) Effects of MRP1 silencing on the intracellular level of glucuronide; (C) Effects of MRP1 silencing on the efflux clearance of glucuronide; (D) Effects of gene silencing on the protein level of MRP1. Each data point was the average of three determinations with error bar representing the standard deviation (n = 3). *p < 0.05; **p < 0.01.

Figure 10 Effects of shRNA-mediated MRP3 silencing on the excretion of chrysin glucuronide. (A) Effects of MRP3 silencing on the excretion profile of glucuronide; (B) Effects of MRP3 silencing on the intracellular level of glucuronide; (C) Effects of MRP3 silencing on the efflux clearance of glucuronide; (D) Effects of gene silencing on the protein level of MRP3. Each data point was the average of three determinations with error bar representing the standard deviation (n = 3). **p < 0.01.

Figure 11 Effects of shRNA-mediated MRP4 silencing on the excretion of chrysin glucuronide. (A) Effects of MRP4 silencing on the excretion profile of glucuronide; (B) Effects of MRP4 silencing on the intracellular level of glucuronide; (C) Effects of MRP4 silencing on the efflux clearance of glucuronide; (D) Effects of gene silencing on the protein level of MRP4. Each data point was the average of three determinations with error bar representing the standard deviation (n = 3). **p < 0.01.

Figure 12 Hydrolysis of chrysin glucuronide by HeLa1A1 cell lysate. Each data point was the average of three determinations with error bar representing the standard deviation (n = 3).
### Table 1

**Design of shRNAs targeting the transporter genes**

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<th>shRNA</th>
<th>Forward Nucleotide sequence</th>
<th>Reverse Nucleotide sequence</th>
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<td>BCRP</td>
<td><strong>5’-GATCCGGATACACCCATACAGAGTGCTCATCTTCAAGAGAGTAGCACACTCTGTAGTGATATCCCTTTTTTACGCGTC-3’</strong></td>
<td><strong>5’-TCGAGACGCCTGGAAAAAGGATACACAGAGTGCTCATCTTTCATGAAAGTAGACACTCTGTGATATCCCTTTTTACGCGTC-3’</strong></td>
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<td>MRP1</td>
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Table 2

Primer sets for reverse transcription-polymerase chain reaction (RT-PCR)

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<td>AGGGGTCTACAGGCAACTG</td>
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</table>
Figure 3

A

Marker (272bp)  MRP1 (300bp)  MRP2 (328bp)  MRP3 (309bp)  MRP4

(bp)  (bp)  (bp)  (bp)  (bp)

3000  3000  3000  3000  3000

2500  2500  2500  2500  2500


1500  1500  1500  1500  1500

1000  1000  1000  1000  1000

500  500  500  500  500

250  250  250  250  250

WT  WT  WT  WT  WT  HeLa1A1  HeLa1A1

B

BCRP  MRP1  MRP2  MRP3  MRP4  GAPDH

WT  HeLa1A1  WT  HeLa1A1  WT  HeLa1A1

Marker (593bp)  Marker

WT  HeLa1A1
Figure 4

A. **HeLa1A1**

- $V$, nmol/min/mg vs. Chrysin, µM
- $K_m = 2.97 \pm 0.944$
- $K_s = 2.13 \pm 0.701$
- $V_{max} = 0.710 \pm 0.173$

B. **rUGT1A1**

- $V$, nmol/min/mg vs. Chrysin, µM
- $K_m = 2.52 \pm 0.998$
- $K_s = 3.70 \pm 1.55$
- $V_{max} = 2.74 \pm 0.747$
Figure 5

A. No inhibitor
- O Ko143 (10 μM)
- △ Ko143 (20 μM)

B. Intracellular glucuronide pmol/mg protein

No inhibitor
- No inhibitor
- Ko143_10μM
- Ko143_20μM

C. Efflux CL_app (μl/h/mg protein)
- **
- ***

D. No inhibitor
- O DIPY (5 μM)
- △ DIPY (20 μM)

E. Intracellular glucuronide pmol/mg protein

No inhibitor
- DIPY_5μM
- DIPY_20μM

F. Efflux CL_app (μl/h/mg protein)

No inhibitor
- DIPY_5μM
- DIPY_20μM
Figure 12

Hydrolysis in Cell Lysate

\[ V, \text{ pmol/min/mg} \]

\[ CL_{\text{int}} = 3.98 \mu l/h/mg \]

Chrysin glucuronide, \( \mu M \)