Chemico-Biological Interactions 296 (2018) 45–56

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

The roles of breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance-associated proteins (MRPs/ABCCs) in the excretion of cycloicaritin-3-*O*-glucoronide in UGT1A1-overexpressing HeLa cells

Shishi Li[a,1], Jinjin Xu[a,1], Zhihong Yao[a,b,*], Liufang Hu[a], Zifei Qin[a,c,**], Hao Gao[a,b], Kristopher W. Krausz[d], Frank J. Gonzalez[d], Xinsheng Yao[a,b]

[a] College of Pharmacy, Jinan University, Guangzhou, 510632, PR China
[b] Guangdong Provincial Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, College of Pharmacy, Jinan University, Guangzhou, 510632, PR China
[c] Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, PR China
[d] Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892, USA

ARTICLE INFO

Keywords:
Cycloicaritin
UGT1A1
Glucuronidation
HeLa cells
Efflux transporters

ABSTRACT

Cycloicaritin is a bioactive natural phenolic compound from *Epimedium* species. However, the glucuronidation and excretion which would influence oral bioavailability and pharmacokinetics of cycloicaritin still remain unknown. Here we aimed to establish UGT1A1 stably transfected HeLa cells, and to determine the contributions of BCRP and MRPs transporters to excretion of cycloicaritin-3-*O*-glucuronide. First, β-estradiol was used to validate the expression of active UGT1A1 protein in engineered HeLa1A1 cells. Furthermore, Ko143 (5 and 20 μM) led to a significant decrease (42.4%–63.8%, $p < 0.01$) in CICT-3-G excretion and obvious accumulation (19.7%–54.2%, $p < 0.05$) of intracellular CICT-3-G, while MK571 (5 and 20 μM) caused a significant reduction (46.8%–64.8%, $p < 0.05$) in the excretion and obvious elevation (50.7%–85.2%, $p < 0.01$) of intracellular level of CICT-3-G. Furthermore, BCRP knocked-down brought marked reduction in excretion rates of CICT-3-G (26.0%–42.2%, $p < 0.01$), whereas MRP1 and MRP4-mediated silencing led to significant decrease in the excretion of CICT-3-G (23.8%–35.4%, $p < 0.05$ for MRP1 and 11.9%–16.0%, $p < 0.05$ for MRP4). By contrast, neither CICT-3-G excretion nor CICT-3-G accumulation altered in MRP3 knocked-down cells as compared to scramble cells. Taken together, BCRP, MRP1 and MRP4 were identified as the most important contributors for CICT-3-G excretion. Meanwhile, the UGT1A1 modified HeLa cells were a simple and practical tool to study UGT1A1-mediated glucuronidation and to characterize BCRP and MRPs-mediated glucuronide transport at a cellular level.

1. Introduction

Glucuronidation mediated by human UDP-glucuronosyltransferases (UGTs) is a principal phase II reaction for the clearance of exogenous drugs (antipsychotics, tricyclic antidepressants, antineoplastic drugs, etc) [1] and endogenous substances (steroids, bilirubin, hormones, etc) [2]. It is well-accepted that glucuronidation is responsible for about 35% drugs metabolized by phase II enzymes [3]. Besides, 15% of the 200 most prescribed drugs in the United States of America (USA) are cleared directly *via* glucuronidation pathway [4]. In addition, as one of the most important UGT enzyme, human UGT1A1 catalyzed approximately 15% of marketed drugs, mainly including irinotecan, SN-38, ethynylestradiol, cyproheptadine and morphine [1]. On the other hand, UGT1A1 plays an important role in maintaining stable balance of bilirubin, bile acids and estrogen levels in human [5]. The abnormality or deficiency of UGT1A1 *in vivo* is highly associated with some diseases (Gilbert syndrome, Crigler-Najjar syndrome, Hyperbilirubinemia) [6], efficacy and toxicity of drugs, and precisely therapeutic personality. Hence, it is of great clinical significance to study the metabolism by UGT1A1 enzyme.

Of note, the excretion of glucuronides from intracellular to extracellular requires two processes, glucuronides formation and excretion

* Corresponding author. College of Pharmacy, Jinan University, Guangzhou, 510632, PR China.
** Corresponding author. College of Pharmacy, Jinan University, Guangzhou, 510632, PR China.
E-mail addresses: yaozhihong_jnu@163.com, yaozhihong.jnu@gmail.com (Z. Yao), qzf1989@163.com (Z. Qin).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cbi.2018.09.001
Received 12 May 2018; Received in revised form 22 August 2018; Accepted 11 September 2018
Available online 17 September 2018
0009-2797/

[7]. Once the glucuronides are formed by UGTs, the soluble metabolites are transported out the cell, in many instances through high affinity efflux transporters, and become sequestered in the water compartments of the tissue that eventually lead to elimination. Drug transporters including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRPs), are characterized to be distributed in the apical cellular membranes of the liver, intestine, kidney and brain, preventing our body from exogenous toxicants [8]. Meanwhile, BCRP and MRPs are members of the C subfamily of ATP-binding cassette (ABC) transporters, which mediate active transport of their substrates across cell membranes using the energy of ATP binding and hydrolysis [9]. Besides, P-gp mostly transports cationic and neutral compounds, while BCRP and MRPs transport anions such as the glucuronides [10]. Meanwhile, the *in vivo* exposure of xenobiotics is driven by phase II metabolic enzymes and efflux transporters in $BCRP^{-/-}$ and $MRP^{-/-}$ mice, which indicated that murine BCRP and/or MRPs contribute significantly to the excretion of glucuronides [11–13]. Therefore, understanding the roles of BCRP and MRPs are important in glucuronide disposition and/or in determining the rate-limiting step (metabolism *vs* excretion) in cellular glucuronide production.

Cycloicaritin (CAS No. 38226-86-7), also called β-anhydroicaritin, was a natural effective flavone occurring in several *Epimedium* species (Berberidaceae family) [14]. It has already exhibited numerous biological activities, including anti-osteoporosis effects, anti-inflammatory effects, antimicrobial and PPAR-γ ligand binding activity, inhibiting P-gp efflux function and significantly down-regulated on the expression of P-gp preotein [15–18]. Meanwhile, cycloicaritin could serve as a leading agent for pharmacological control of metabolic diseases, since cycloicaritin improves diet-induced obesity and alleviates insulin resistance by suppressing sterol regulatory element-binding proteins (SREBPs) maturation which is dependent on LKB1/AMPK/mTOR pathway [15]. Moreover, anti-osteoporosis effects and bioavailability of cycloicaritin-suet oil-sodium deoxycholate self-assembled nanomicelles were significantly increased due to an increase in absorption by reducing the particle sizes of cycloicaritin [19]. The biological properties of flavones are well known to be severely limited by the compound's low bioavailability resulting from extensive metabolism and excretion [20–22]. However, the action mechanism and characteristics of the metabolism and excretion of cycloicaritin still remain unclear.

In this study, we found that cycloicaritin could be efficiently metabolized to cycloicaritin-3-*O*-glucuronide by UGT1A1. To better understand the UGT1A1-catalyzed glucuronidation and efflux transports-mediated excretion of cycloicaritin, UGT1A1 enzyme was stably transfected to HeLa cells to catalyze the glucuronidation of cycloicaritin. Furthermore, chemical inhibitors (Ko143, MK571, dipyridamole and leukotriene C4) and biological knock-down assays [short hairpin RNA (shRNA)-mediated silencing of a target transporter] were performed to investigate the roles of efflux transporters. The results would be valuable in achieving better prediction of cycloicaritin disposition, which could be the main factors affecting its bioavailability and biological activities. In addition, the findings could also add our general understanding of the action mechanisms of cycloicaritin *in vivo*. Moreover, this cell model provided a practical and feasible approach to evaluate the UGT-catalyzed glucuronidation and efflux transports-mediated excretion of clinical drugs and xenobiotics.

2. Materials and methods

2.1. Materials

Alamethicin, β-estradiol, dipyridamole (DIPY), D-saccharic-1, 4-lactone, magnesium chloride ($MgCl_2$), MK-571, Ko143, leukotriene C4 (LTC4) and uridine diphosphate glucuronic acid (UDPGA) were all obtained from Sigma-Aldrich (St Louis, MO). Apigenin, chrysin, cycloicaritin, genistein, and wushanicaritin with purity over 98% was purchased from Aladdin Reagents (Shanghai, China). Apigenin-7-*O*-glucuronide, chrysin-7-*O*-glucuronide, genistein-7-*O*-glucuronide, and β-estradiol-3-*O*-glucuronide was purchased from Toronto Research Chemicals (North York, ON, Canada). Wushanicaritin-3-*O*-glucuronide was prepared in our laboratory as described previously [23]. 293T cells, HeLa cells, pLVX-mCMV-ZsGreen-PGK-Puro vector [9371 base pairs (bp)] and pLVX-shRNA2-Neo vector (9070 bp) were all provided from BioWit Technologies (Shenzhen, China). The pGEM-T plasmid which carried the UGT1A1 cDNA clone was purchased from Sino Biological Inc. (Beijing, China), while recombinant human UGT1A1 was purchased from Corning Biosciences (New York, USA). The anti-BCRP, anti-GAPDH, anti-MRP1, anti-MRP2, anti-MRP3, anti-MRP4 and anti-UGT1A1 antibodies were all obtained from OriGene Technologies (Rockville, MD). All other chemicals and reagents were the highest grade commercially available.

2.2. Establishment of UGT1A1-overexpressing HeLa cells

As published previously [23,24], the development of UGT1A1-overexpressing HeLa cells mainly underwent three steps. Firstly, the purified fragment of UGT1A1 cDNA (1602 bp) was obtained after the restriction of BamHI and MluI were introduced in pGEM-T-UGT1A1 plasmid. The cloned genes were sequenced within the vector construct and were found to be identical to the known genomic sequence (NM_000463.2). Secondly, based on the third-generation packaging system from BioWit technologies, lentiviral vectors were produced by transient transfection into 293T cells. Finally, HeLa cells were transfected by incubation with the lentivirus. The stably transfected HeLa cells were cryopreserved for future use. The multiplicity of infection (MOI) value was 10 in stable transfection of HeLa cells. The UGT1A1 modified HeLa cells are named HeLa1A1 cells.

2.3. Transient transfection of shRNA plasmids

The shRNA plasmids for efflux transports including BCRP, MRP1, MRP2 and MRP4 were constructed as described in previous studies [23,24]. Each pair of shRNA was ligated into the pLVX-ShRNA2-Neo plasmid. The shRNA fragments within the vector construct were sequenced using the primer U6—F (5′-TACGATACAAGGCTGTTAGA GAG-3′) by Invitrogen (Carlsbad, CA). Further, HeLa1A1 cells were transiently transfected with the corresponding shRNA plasmids. Briefly, the HeLa1A1 cells were cultured at a density of 2.0×10^5 cells/well in a 6-well plate. After 48 h, the control scramble, shRNA-BCRP, shRNA-MRP1, shRNA-MRP2 and shRNA-MRP4 plasmids (4 μg) were individually transfected into HeLa1A1 cells using the Polyfectine transfection reagent (Biowit Technologies, Shenzhen, China). The HeLa1A1 cells silencing BCRP, MRP1, MRP3 or MRP4 proteins could be ready for excretion experiments after transfection for 2 days. The established cells were named as HeLa1A1-BCRP-shRNA cells, HeLa1A1-MRP1-shRNA, HeLa1A1-MRP3-shRNA, and HeLa1A1-MRP4-shRNA cells, respectively.

2.4. Preparation of HeLa1A1 cell lysate

HeLa1A1 cells were collected in 50 mM Tris-HCl buffer (pH 7.4), following disrupting by sonication for 1 min. Protein concentration was determined by the Bio-Rad protein assay kit using bovine serum albumin as a standard.

2.5. Cycloicaritin-3-O-glucuronidation assay

Glucuronidation activities of cycloicaritin by recombinant UGT1A1 and HeLa1A1 cell lysate were measured as mentioned previously [23–26]. The mixture (200 μL) was incubated at 37 °C. After incubation for 2 h, the reaction was terminated using ice-cold acetonitrile (200 μL). The samples were mixed and centrifuged at 13800 g for 10 min. The supernatant was subjected to ultra-high-performance liquid