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약학석사 학위논문

Inhibition of Organic Anion Transporting Polypeptides (OATP1B1 and 1B3) by Betulinic Acid:

Effects of Pre-incubation and Albumin in the Media

베툴린산의 유기음이온수송체 1B1과 1B3 (OATP1B1/3)에 대한 저해 작용 연구: Pre-incubation과 media의 알부민에 의한 영향

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서울대학교 대학원

약학과 약제과학 전공

오 윤 석

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이 논문을 약학석사 학위논문으로 제출함

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2018 년 1 월

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Abstract

Inhibition of Organic Anion Transporting Polypeptides (OATP1B1 and 1B3) by Betulinic Acid:

Effects of Pre-incubation and Albumin in the Media

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The transporters of the organic anion transporting polypeptide 1B subfamily (OATP1B1 or OATP1B3, OATP1B1/3) can influence the hepatic uptake and elimination of clinically important drugs such as statins. While it was reported that common plant-derived pentacyclic triterpenoids interact with OATP1B1/3, detailed investigations have been lacking for betulinic acid (BA) including its potential risk for drug-herb interactions. In our current study, we investigated the interactions of BA and its closely related pentacyclic triterpenoids (ursolic acid, UA; oleanolic acid, OA; close similarities in their structures and high-affinity plasma protein binding) with OATP1B1/3 and rat

Oatp1b2 using in vitro and in vivo models. Different experimental conditions were employed including co-incubation and pre-incubation (incubation with inhibitors, followed by washout).

BA, UA and OA effectively inhibited the uptake of fluorescent probes and atorvastatin by OATP1B1/3 or rat Oatp1b2 *in vitro*. In order to examine the *in vivo* relevance of OATP1B1/3 inhibition by BA, the pharmacokinetic profiles were examined in rats that received co-administration of BA (4 or 20 mg/kg) intravenously. The *in vivo* exposure of atorvastatin was altered only in rats that received the BA dose of 20 mg/kg. Additional *in vitro* experiments were carried out to further probe the nature of OATP1B1/3 inhibition by BA, OA or UA. We found that pre-incubation with BA, OA or UA led to a sustained inhibition of the OATP1B3 activity at least up to 2.5 hrs. The inhibited OATP1B3 activity however recovered rapidly in the media containing 10% fetal bovine serum. The addition of albumin to the media was found to decrease intracellular concentrations of BA and to expedite the recovery of OATP1B3 activity following pre-incubation. When asunaprevir and cyclosporin A (previously reported to inhibit OATP1B3 upon pre-incubation) were used, the addition of albumin to the media shortened the recovery time with asunaprevir, but not with cyclosporin A.

Overall, our results show that BA inhibits OATP1B transporters *in vitro*, and has the potential to incur hepatic transporter-mediated drug interactions *in vivo*, especially with high doses of BA. Our results identify BA as another OATP1B3 inhibitor with pre-incubation effect and suggest that the pre-incubation effect and its duration can be impacted by altered equilibrium of

inhibitors between intracellular and extracellular space (e.g. albumin in the media).

Keywords: Natural products; Transporter-mediated drug interactions; Hepatic uptake transporters; Organic anion transporting polypeptides

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1. Introduction

Membrane drug transporters are increasingly recognized as important players in determining the absorption, distribution and elimination of drugs, particularly those with poor passive diffusion across cell membrane. Among several important hepatic transporters, the two members of the OATP1B (Organic Anion Transporting Polypeptide 1B) subfamily, namely OATP1B1 and OATP1B3 (OATP1B1/3), are well-recognized for their roles in influencing the pharmacokinetics and pharmacodynamics (i.e. efficacy and toxicity) of clinically important drugs including lipid-lowering statins. For example, atorvastatin (ATV) is a well-known substrate of OATP1B1/3, which handles the overall rate-determining step in the hepatic elimination.[1,2] Accordingly, several cases of clinically significant drug-drug interactions (DDIs) have been reported when ATV is co-administered with drugs that can inhibit OATP1B1/3.[3] During drug development, the interactions between new drug candidates and hepatic transporters including OATP1B1/3 are thus examined to assess their potential to incur DDIs.[4] Food components and herbal supplements may also interact with transporters, thereby influencing the systemic exposure of drugs.[5] Yet, detailed information is lacking on potential transporter-mediated interactions between clinically used drugs and food/herbal supplements.

Pentacyclic triterpenoids are abundantly present in plants and herbal supplements and their pharmacological activities are currently being explored for the treatment of diabetes, liver diseases and cancer.[6-9] Among pentacyclic triterpenoids, betulinic acid (BA), ursolic acid (UA) and oleanolic acid (OA) are commonly found in fruits (e.g., apple peels,[10] olives[11]) and aromatic herbs (e.g.,

Rosmarinus officinalios, *Lavandula angustifolia*[12]). They are similar not only in their structures, but also in hydrophobic nature and high-affinity plasma protein binding.[13] UA and OA were previously identified as the inhibitors of OATP1B1/3 *in vitro,*[14,15] but no information is available for BA. When the pharmacokinetics of rosuvastatin was examined in rats that received oral administration of UA (80 mg/kg), the systemic exposure of rosuvastatin was substantially enhanced, potentially impacting the efficacy and toxicity of rosuvastatin.[16] It was however unknown whether the observed interactions between rosuvastatin and UA involved transporters in the intestine, the liver or both.

In quantitatively evaluating the inhibitory effects of compounds on transporters, commonly used transport assays involve the measurement of the uptake of a probe substrate for a given transporter using the assay buffer containing both the probe substrate and the inhibitor ("co-incubation"). Recently, it was shown that several inhibitors exert their inhibitory effects upon "pre-incubation" (i.e. pre-incubation with inhibitors, followed by the washout with inhibitor-free media and transport assays with the buffer containing a probe substrate only). The first reported case was cyclosporin A (CysA) using rat hepatocytes,[17] followed by similar findings on the "pre-incubation" effects of several other drugs.[18-22] The pre-incubation effect of CysA received particular attention, given that the use of the previously reported K_i values of CysA under co-incubation conditions often underpredicted the extent of transporter-related DDIs involving CysA.[23] To account for the pre-incubation effect, it was proposed that intracellular CysA concentrations after pre-incubation and subsequent washout remain sufficiently high to occupy intracellular binding site(s) of OATP1B1/3.[24] The authors reported simulated

profiles for intracellular CysA concentrations under pre-incubation conditions, but the experimental measurement of intracellular drug concentrations remains challenging. As additional drugs are identified to have similar inhibitory effects on OATP1B1/3 by pre-incubation (e.g., asunaprevir, simeprevir),[21] it would be important to gain a better mechanistic understanding of the pre-incubation effect by employing different experimental conditions.

In our current study, we investigated whether closely related pentacyclic triterpenoids (i.e. BA, UA, OA and their counterparts lacking a carboxylic group) have the potential to interact with OATP1B1/3 and rat Oatp1b2. Using BA as a model compound, we assessed the impact of intravenously administered BA on *in vivo* exposure of ATV in rats. In separate experiments, we assessed the inhibitory effects and subsequent recovery of BA, asunaprevir or CysA on the OATP1B3 activity under varying experimental conditions (e.g. co-incubation, pre-incubation, the addition of albumin to lower intracellular concentrations of inhibitors following pre-incubation).

2. Materials and methods

2.1. Materials and cell lines

BA, UA, OA, betulin, uvaol, erythrodiol, sodium fluorescein, 2′,7′-dichlorofluorescein (DCF), poly-L-lysine and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). ATV (98% purity) was from Tokyo Chemical Industry (Tokyo, Japan). Simeprevir and asunaprevir were purchased from Shenzhen Wolcase Pharmaceutical Technology Co., Ltd (Guangdong, China). Bovine serum albumin (BSA) was from Promega (Madison, WI). Other chemicals including formic acid and ammonium formate used for analytical assays were obtained from Sigma-Aldrich, unless mentioned otherwise. HEK293 cells were kindly provided by Dr. Markos Leggas (College of Pharmacy, University of Kentucky, USA). MDCKII cells stably expressing rat Oatp1b2 were generated as described in our previous report.[25] The polyclonal antibodies against the C-terminal region of OATP1B3 or OATP1B1 were generated and validated in the previous reports.[26,27] An antibody against β-actin was from Cell Signaling Technology (Danvers, MA).

2.2. Generation and maintenance of HEK293 cells stably expressing OATP1B1 or OATP1B3

HEK293 cells were maintained in Minimum Essential Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Welgene, Gyeongsan, Korea) and 1% non-essential amino acid solution (Sigma-Aldrich) at 37° C in a humidified 5% CO_2 incubator. HEK293 cells were transfected with

the pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA) vector harboring the coding sequences of human OATP1B1 or OATP1B3 as well as the empty vector using Lipofectamine 2000 (Invitrogen). Stably transfected clones were selected using zeocin (150 μg/mL, InvivoGen, San Diego, CA) and individual clones were subsequently screened for protein expression levels and functional activities. Selected clones were maintained in complete media containing zeocin (50 μg/mL) and used for further experiments.

2.3. Immunoblotting analysis

Cell lysates were prepared using modified RIPA buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete®, Roche, Basel, Switzerland) and were mixed with 4X Laemmli buffer (Bio-rad, Richmond, CA). Lysates containing equivalent amounts of total protein were incubated at 37°C for 20 min (to minimize protein aggregation), resolved by 7.5% SDS-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride (Bio-rad) membranes using a semi-dry transfer system (Bio-Rad). After blocking with 5% skim milk in TBST (0.05% Tween-20 in Tris-Buffered Saline) for 1 hr, the blots were incubated with respective primary antibodies diluted in blocking buffer at 4°C overnight. The blots were subsequently incubated with secondary antibodies conjugated with horseradish peroxidase. The immunoreactive bands were visualized using an enhanced chemiluminescence substrate (SuperSignal, Thermo Fisher Scientific, Waltham, MA). β-actin was used as a gel loading control.

2.4. Cellular uptake study using HEK293 cells stably expressing OATP1B1 or OATP1B3

Fluorescein and DCF were used as probe substrates for OATP1B3 and OATP1B1, respectively, based on the previous reports.[28,29] Preliminary experiments were performed to determine the time period where the cellular uptake of probe substrates is proportional to time; 8 and 5 min for fluorescein and DCF, respectively. The effects of BA, UA, OA, rifampicin or CysA on the uptake of probe substrates were assessed by exposing these compounds under co-incubation and pre-incubation conditions (as depicted in Fig. 4). The time course by which the OATP1B3 activity recovers following the pre-incubation was assessed by keeping pre-treated cells in inhibitor-free media (varying compositions in terms of FBS or BSA) for differing wait times (0, 90 or 150 min) prior to the measurement of cellular uptake of probe substrates (as depicted in Fig. 5). These experimental conditions were from the previous reports.[19,21,30]

Briefly, HEK293 cells stably expressing OATP1B1 or OATP1B3 were plated onto poly-L-lysine-pre-coated 24-well plates (1.5×10^5 cells/well, SPL, Pocheon, Korea). When nearly confluent, cells were washed twice using pre-warmed Dulbecco's Phosphate-Buffered Saline (DPBS) and equilibrated with pre-warmed Optimem (ThermoFisher Scientific) at 37°C for 10 min. For co-incubation experiments, the uptake was initiated by replacing the media with Optimem containing both a probe substrate and an inhibitor. The uptake was terminated by removing the media and keeping the plate on ice. After washing three times with 400 μL of ice-cold DPBS, cells were lysed using DPBS

containing 0.5% Triton X-100 (Georgiachem, Norcross, GA). For pre-incubation experiments, the procedures were identical except the following modifications. After cells were washed twice using DPBS, cells were pre-incubated with pre-warmed Optimem with potential inhibitors at 37°C for 30 min, washed twice with ice-cold DPBS and stored in Optimem at 4°C for 30 min. Subsequently, cells were equilibrated in pre-warmed Optimem at 37°C for 10 min and uptake was initiated by replacing the media with Optimem containing a probe substrate only. To assess the duration of the inhibitory effects, cells were incubated in inhibitor-free media with or without 10% FBS or BSA (5 μM for BA, 50 μM for asunaprevir and CysA) for differing waiting times at 37°C followed by 30 min pre-incubation. The rest of experimental procedures were similar to co-incubation conditions. Fluorescence signals in lysates were measured using a plate reader (excitation/emission wavelengths of 460/515 nm, SpectraMAX M5, Molecular Devices). The protein content was quantified using BCA™ Protein Assay (Pierce, Thermo Fisher Scientific) and used for data normalization. The experimental data were fitted to the following equation; Y = bottom + (top - bottom)/(1 +10^((LogIC_{50} - X)*Hillslope)), where X, Y and IC_{50} represent the log-transformed concentration, the response (% control), and the concentration that leads to 50% of the maximal inhibition, respectively. All parameters (IC_{50}, Hillslope, top and bottom) were fitted using GraphPad Prism v.5.01 (GraphPad Software, Inc. La Jolla, CA).

2.5. Bioanalytical assays for ATV, BA and asunaprevir

The levels of ATV in cell lysates or plasma were quantified using the

previously reported liquid chromatography tandem-mass spectrometry (LC-MS/MS) method with slight modifications.[25] Cell lysates were prepared using 50% methanol, subjected to probe-sonication and mixed with an equal volume of acetonitrile containing glipizide (500 ng/mL, an internal standard). Following vortex-mixing for 15 min and centrifugation (1,300 g, 15 min, 4°C), the resulting supernatant was injected onto the LC-MS/MS system equipped with Agilent 1260 HPLC system (Agilent Technologies, Wilmington, DE) and API 3200 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA). The concentration-detector responses were found to be linear from 5 nM to 5 μM in cell lysates for ATV. To quantify BA in cell lysates, media or plasma, samples were prepared in a similar manner to ATV except the following modifications; lysates were prepared in 50% methanol and mixed with an equal volume of methanol containing deoxycholic acid (1 μg/mL, an internal standard). Quantification of BA was using turbo ion spray interface in the negative ionization mode for selected ion monitoring. The concentration-detector responses were found to be linear from 10 nM to 10 μM of BA in cell lysates. To quantify asunaprevir, samples were prepared in 50% methanol, subjected to probe-sonication and mixed with an equal volume of acetonitrile. Prepared samples were analyzed using the previously reported LC-MS/MS assay conditions with slight modifications.[31] The concentration-detector responses were found to be linear from 10 nM to 2 μM of asunaprevir in cell lysates or media. Detailed analytical conditions are also summarized in Table 1.

2.6. Pharmacokinetic profiles of ATV with co-administration of BA in rats and Statistical analyses

In order to assess the impact of BA on the hepatic disposition and systemic exposure of ATV *in vivo*, the pharmacokinetic profiles of ATV were assessed in rats after the intravenous administration of ATV and BA in male Sprague-Dawley rats (body weight, 245-255 g; Orient Bio, Seongnam, Korea). The animal protocols used in our study were reviewed and approved by the Seoul National University Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health *Principles of Laboratory Animal Care* (publication number 85-23, revised in 1985). After anesthetized by intramuscular injection of anesthetics (tiletamine-HCl/zolazepam-HCl, Zoletil®50, 50 mg/kg, Virbac Laboratories, France) and xylazine-HCl (Rompun®, 10 mg/kg, Shawnee, KS), the animals underwent the catheterization of femoral artery and vein for blood sampling and drug administration, respectively. After recovery from anesthesia, rats were divided into the control and co-treatment groups and received the respective intravenous bolus injections. The control group received ATV only (2 mg/kg, dissolved in DMSO/polyethylene glycol 400/saline [0.5:4:5.5 (v/v/v)]) while the co-treatment group received both ATV (2 mg/kg) and BA (4 or 20 mg/kg; for the 4 mg/kg dose, BA was dissolved in N,N-Dimethyl-acetamide/polyethylene glycol 400/saline [2:4:4 (v/v/v)]; for the 20 mg/kg dose, BA was dissolved in chromophore/tween 80/PEG400/ethanol/DMSO [2:1:1:1:2 (v/v/v/v)]). Following the injections, blood samples were collected at 2, 5, 10, 20, 30, 40, 60, 80, 100 and 120 min and immediately centrifuged (at 14,000 g for 10 min at 4°C) to yield plasma

samples. To compensate the loss of body fluid from serial blood sampling, animals received a matching volume of normal saline via venous catheters. At the end of the experiments (120 min after dosing), liver tissues were harvested and used to quantify ATV levels in the liver. Harvested liver tissues were washed four times with ice-cold DPBS and weighed. Liver homogenates were prepared using three parts of DPBS (Ultra Turrax homogenizer; IKA, Staufen, Germany). Three parts of acetonitrile (for ATV) or methanol (for BA) were added to plasma sample or liver homogenates for the deproteinization purpose. After vortexing and centrifugation, 5 μL of the resulting supernatant was subjected to LC-MS/MS analyses.

Pharmacokinetic parameters were calculated using non-compartmental methods (WinNonLin version 5.0.1, Pharsight). The parameters were expressed as the mean with standard deviation. Statistical significance between the groups was determined using Student's t test using GraphPad Prism v.5.01 (GraphPad Software, Inc. La Jolla, CA). P values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Three pentacyclic triterpenoids with a carboxylic group (BA, UA and OA) effectively inhibited the cellular uptake of fluorescein and 2',7'-dichlorofluorescein (DCF) by OATP1B3 and OATP1B1

HEK293 cells stably expressing human OATP1B3 or OATP1B1 were generated and single clones with high expression of the respective proteins were identified (Fig. 1A). In assessing their transport activities, fluorescein and DCF were used as a probe substrate of OATP1B3 and OATP1B1, respectively, as reported previously.[28,29] Cellular retention of fluorescein and DCF was enhanced by approximately 4- and 3- fold in OATP1B3- and OATP1B1-expressing cells compared to the empty vector-transfected cells (Fig. 1B). Co-treatment with rifampicin (50 μM, a general OATP inhibitor) decreased the fluorescence signals to the level comparable to the empty-vector controls, indicating that the enhanced uptake was mediated by OATP1B1/3 (Fig. 1B).

Under co-incubation conditions (Fig. 2B), UA and OA displayed their inhibitory effects on OATP1B3 with the respective IC_{50} value of 365.1 and 304.8 nM, similar to the previous reports.[14,15] BA also inhibited the activity of OATP1B3 with the IC_{50} value of 368.2 nM, comparable to that of OA and UA. The activity of OATP1B1 was also inhibited by all three compounds with a carboxylic group (BA, UA and OA) at sub-micromolar concentrations (Fig. 2C). However, their counterparts lacking a carboxylic group (betulin, uvaol or erythrodiol, with their concentration ranges of 0.1 – 10 μM) showed little to no inhibition of the OATP1B3 and OATP1B1 activities (Figs. 2B and 2C). These

results suggested the importance of a carboxylic group (–COOH) of BA, UA and OA in interacting with OATP1B1/3.

3.2. BA inhibited cellular uptake of atorvastatin (ATV) by OATP1B1, OATP1B3, or rat Oatp1b2, but did not show an increased cellular accumulation by OATP1B1 or OATP1B3.

When tested using the clinically relevant substrate ATV, the co-incubation of BA also led to an effective inhibition of the cellular uptake of ATV by OATP1B1/3 (Fig. 3A, middle and left panels). BA preferentially inhibited OATP1B3 over OATP1B1 with approximately 9-fold difference in IC_{50} values (138.6 nM and 1,235 nM, for OATP1B3 and OATP1B1 inhibition, respectively). The previous report also indicated that UA preferentially inhibited OATP1B3 over OATP1B1 (IC_{50} values of 2.3 μM and 12.5 μM for OATP1B3 and OATP1B1, respectively).[15] As shown in Fig. 3A (right panel), rat Oatp1b2, an ortholog for OATP1B1/3, was also inhibited by BA when tested using MDCKII cells stably expressing rat Oatp1b2 and ATV (IC_{50} value of 855.1 nM).

We further assessed whether BA is a substrate for OATP1B1/3 by quantifying the BA levels in cell lysates following incubation of BA for 5 min at 37° C. The BA levels did not differ between OATP1B1/3-expressing cells and empty vector controls (Fig. 3B). In order to minimize the potential complications arising from non-specific adsorption of BA to cell surface or culture vessels,[32] separate experiments were performed using the uptake assay buffer containing BSA (5 μM). Yet, the BA levels did not increase in lysates from cells expressing OATP1B1/3 regardless of BSA added (Fig. 3C).

3.3. In vivo exposure of ATV in rats was affected by co-administration of BA in a dose-dependent manner.

Next, we examined whether *in vitro* inhibitory effects of BA on the hepatic uptake transporters of OATP1B subfamily have *in vivo* relevance by assessing the impact of BA (4 or 20 mg/kg via intravenous injection) on the systemic exposure of ATV (2 mg/kg via intravenous injection) in rats.

Rats that received intravenous injection of the BA dose of 4 mg/kg (BA solution was prepared at 2.5 mg/ml, the maximal concentration that can be dissolved in DMSO/polyethylene glycol 400/saline [0.5:4:5.5 (v/v/v)]) showed plasma ATV concentration-time profiles, which overlapped with those that received ATV only (Fig. 4A, left panel). The ratios of ATV concentration in the liver to that in plasma at 2 hr post-dosing ($K_{p,liver,2hr}$) were also unaffected by co-administration of BA (4 mg/kg) (Fig. 4A, middle panel). In the rats that received both ATV and BA (4 mg/kg), the plasma BA concentration-time profiles showed a very slow decline with the highest observed BA concentration of approximately 300 nM (137 ng/mL) at 2 min (the earliest sampling point) (Fig. 4A, right panel). Considering the average BA dose administered (1 mg per rat of 250 g) and average blood volume (14-18 mL), these results suggest that BA has a wide tissue distribution, as commonly expected for many hydrophobic drugs. Given that BA is highly protein-bound and only a very small fraction exists as a free drug ($f_{u,\ plasma}$ of 0.0001),[13] it is likely that unbound BA concentrations in plasma were not sufficiently high to inhibit rat Oatp1b2, not affecting the hepatic uptake of ATV *in vivo*.

Considering the possibility that BA may have substantial accumulation

in hepatocytes, the potential interactions between ATV and BA may be observed with the co-administration of higher BA doses. Using the information from the recent report [33], we were able to prepare the BA solution of 25 mg/ml, allowing for BA dosing of 20 mg/kg. The plasma ATV concentration-time profiles were substantially altered in rats that received the BA dose of 20 mg/kg (Fig. 4B, left panel). The ratios of ATV concentration in the liver to that in plasma at 2 hr post-dosing ($K_{p,liver,2\ hr}$) were significantly decreased by co-administration of the BA dose of 20 mg/kg (Fig. 4B, middle panel). Following the administration of the BA dose of 20 mg/kg, the concentrations of BA in plasma were much higher throughout the sampling times than those in rats that received the BA dose of 4 mg/kg (Fig. 4B, right panel). The highest observed BA concentration was approximately 50 μM at 2 min (approximately 160-fold higher than that with the BA dose of 4 mg/kg). When the pharmacokinetic parameters were compared, the overall systemic exposure (the area under the curve of the plasma BA concentration-time profiles up to 2 hr, AUC_{2h}) was also much higher with the BA dose of 20 mg/kg, compared to 4 mg/kg (43.5-fold in AUC_{2h}). Due to the extremely high protein binding affinity of BA, the unbound BA concentrations in plasma observed with the BA dose of 20 mg/kg may still not reach the ranges of the concentrations that were associated with Oatp1b2 inhibition *in vitro*. Yet, the co-administration of the BA dose of 20 mg/kg substantially increased *in vivo* exposure of ATV (~2.2-fold increase in AUC_{2hr}, ~3.0-fold decrease in the observed clearance (CL_{obs})) (Table 2).

3.4. Pre-incubation with BA, UA and OA effectively inhibited the OATP1B3 activity, followed by a rapid recovery with the use of complete media containing FBS.

Several reports exist that the pre-incubation and subsequent washout with compounds can still inhibit OATP1B1/3.[18-22] When the effects of BA, UA and OA (1 μM each) were tested under the pre-incubation conditions, all three compounds effectively inhibited the uptake of fluorescein by OATP1B3 with the potencies comparable to those under co-incubation conditions (Fig. 5).

Next, the time courses by which the OATP1B3 activity recovers following pre-incubation with BA, UA or OA were compared with CysA (a long-lasting inhibitor by pre-incubation [19,21]) and rifampicin (an inhibitor with no pre-incubation effect).[30] The extent of recovery of OATP1B3 activity was assessed with varying lengths of waiting times in media containing 0% or 10% FBS (as depicted in Fig. 6A). For BA, UA and OA, the OATP1B3 activity stayed inhibited even after 150 min of the waiting time in media containing no FBS (Fig. 6B). In contrast, the OATP1B3 activities almost completely recovered within 90 min of the waiting time in media containing 10% FBS (Fig. 6C). For CysA, the OATP1B3 activity did not recover regardless of media compositions, showing a clear difference from the results of BA, UA and OA.

3.5. Addition of bovine serum albumin (BSA) to media expedited the recovery of OATP1B3 activity inhibited by the pre-incubation with BA, asunaprevir or Cyclosporin A (CysA).

The observed difference in the recovery time by FBS (Fig. 6) may arise

from additional protein binding of inhibitors in the media, altering the distribution equilibrium of inhibitors between extracellular and intracellular space. For the validation purpose, we added 5 μM of BSA to the media and measured the BA levels in cell lysates and media after varying lengths of waiting time (Fig. 7A, left). With the addition of BSA, the BA levels decreased in cell lysates, but increased in the media at all three waiting times (30, 90, 150 min) following pre-incubation. As expected with decreased intracellular levels of BA by the addition of BSA, the OATP1B3 activity increased at all three waiting times, compared to the media with no BSA (Fig. 7A, left).

Similar experiments were carried out using asunaprevir, another drug with pre-incubation effect and high-affinity plasma protein binding (unbound fraction in plasma, 0.004).[34] The results obtained with asunaprevir were consistent with those with BA (Fig. 7A, right). With the addition of 50 μM of BSA, the distribution equilibrium of asunaprevir between extracellular and intracellular space was altered and the recovery of OATP1B3 activity was expedited, compared to the media with no BSA. In the case of CysA (0.1 and 1 μM), the addition of BSA led to only slight changes in the OATP1B3 activity at 150 min of waiting time following pre-incubation (Fig. 7B).

4. Discussion

Herbal supplements are typically self-medicated, often with claimed health and therapeutic benefits. However, concerns have been increasingly raised regarding potential herbal-drug interactions. Pentacyclic triterpenoids are commonly found in commercially available supplements, but little was known about their potential to incur hepatic transporter-mediated drug interactions. In our current study, we examined the impact of BA and its closely related analogs (UA and OA) on the activity of hepatic influx transporters of the OATP1B subfamily. Our results showed that BA, UA and OA inhibited the activity of OATP1B1/3 and rat Oatp1b2 *in vitro* (Figs. 2 and 3). In addition, the *in vivo* exposure of ATV was affected in rats that received co-administration of BA (20 mg/kg) intravenously (Fig. 4). Pre-incubation with BA, OA or UA led to a sustained inhibition of the OATP1B3 activity, which recovered rapidly in the media containing 10% fetal bovine serum (Figs. 5 and 6). For BA and asunaprevir, the addition of albumin to the media decreased intracellular levels of these inhibitors and expedited the recovery of OATP1B3 activity following pre-incubation (Fig. 7). Overall, our results showed that BA inhibits OATP1B transporters *in vitro*, and poses a potential risk for hepatic transporter-mediated drug interactions *in vivo* with a high dose of BA. Our results identify BA as another OATP1B3 inhibitor with pre-incubation effect and suggest that the pre-incubation effect and its duration is impacted by altered equilibrium of inhibitors between intracellular and extracellular space (e.g. albumin in the media).

In our current study, we compared the interactions of BA, OA and UA with

the transporters of the OATP1B subfamily using the same models (Fig. 2). We report that BA is an inhibitor for OATP1B transporters with a selectivity toward OATP1B3 (approximately 9-fold differences in IC_{50} values for ATV uptake, Fig. 3A). Yet, our results did not support BA as a substrate of OATP1B1/3 (Fig. 3B and 3C). Similar to BA, UA was also shown to interact preferentially with OATP1B3 over OATP1B1.[15] However, we cannot rule out the possibility that the inhibitory potencies may depend on substrates. For example, OA (100 μM) substantially inhibited OATP1B3 activity when tested using estrone-3-sulfate as a substrate, but only slightly inhibited when tested using estradiol-17β-glucuronide.[14] In the same report, OA inhibited OATP1B1-mediated uptake of estradiol-17β-glucuronide with a potency of approximately 10 times greater than UA. Further investigations will be necessary to obtain structural and mechanistic insights into the observed substrate-dependent effects.

Co-administration of BA (20 mg/kg) altered the pharmacokinetic profiles of ATV in rats: ~2.2-fold increase in AUC_{2hr}, ~3.0-fold decrease in CL_{obs} (Fig. 4, Table 2). These results are consistent with the *in vitro* inhibitory effects of BA on rat Oatp1b2, although we cannot rule out the possibility that other hepatic transporters may be involved. The pharmacokinetic profiles of ATV were altered only by the BA dose of 20 mg/kg, but not 4 mg/kg. When the systemic exposure of BA was compared between the two doses, the overall systemic exposure of BA was also markedly higher with 20 mg/kg compared to 4 mg/kg; with 5-fold higher BA dose, the AUC_{2h} value increased by 43.5-fold. These results indicated that intravenously administered BA displays non-linear pharmacokinetics although the mechanisms underlying the non-linear profiles are currently unknown. When the PK parameters of rats that received ATV only

were compared, a slight difference was noted (although not significantly different by Student's t test). Possible reasons may include differences in solvent systems to prepare dosing solutions.

In our current study, the doses for ATV or BA were chosen based on the literature. The dose of ATV (2 mg/kg) was chosen, based on the previous study reporting a case of hepatic transporter-mediated DDI between ATV and rifampicin.[35] The observed plasma ATV concentration-time profiles in the current study were similar to those reported in the literature.[25,35,36] For the pharmacokinetic and tissue distribution profiles of BA, little information was available especially after intravenous administration. An early study reported the results from the intraperitoneal injection of BA (250 or 500 mg/kg) to mice.[37] In that report, the serum BA concentration-time profiles displayed a rapid decline followed by a terminal phase with a half-life of approximately 12 hrs and BA showed wide tissue distribution, in particular, into the fat and ovary. Although not examined in our current study, BA likely has wide distribution throughout the body and its unbound concentrations in plasma may be extremely low, given its extensive protein binding (>99.9%).[38] Likely, the unbound BA concentrations in plasma were too low to inhibit rat Oatp1b2 and to impact the hepatic uptake of ATV *in vivo* when 4 or 20 mg/kg of BA was used. Further investigations are needed to probe possible reasons for altered ATV exposure only by BA dose of 20 mg/kg. Of note, the previous study reported an increase in the rosuvastatin exposure by co-administration of UA (rosuvastatin, 100 mg/kg and UA 80 mg/kg, both orally).[16] Assuming that UA displays pharmacokinetic profiles similar to BA, the previously observed difference may involve the interactions of orally administered UA with intestinal efflux

transporters.[39,40]

Our results identified BA as another OATP1B3 inhibitor with pre-incubation effect. As more inhibitors are reported to have the pre-incubation effect, it is recommended to include pre-incubation conditions as part of *in vitro* screening assays (discussion regarding the possible update of the EMA guidelines is available, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/04/WC500225363.pdf). Our current results indicate that the recovery times can substantially vary depending on the media compositions and the lengths of waiting times after pre-incubation. It is plausible that the presence of albumin (another binding target) in media alters the distribution equilibrium between extracellular and intracellular space. Albumin is known for its roles in *in vitro* metabolic and transport assays. The enhanced uptake of albumin-bound drugs had been noticed and reported by a number of early investigations.[41] In efforts to improve the prediction accuracy in the *in vitro*-*in vivo* extrapolation, the enhanced uptake of albumin-bound drugs has been again proposed as additional factors to consider *in vivo*, especially for hydrophobic and highly protein-bound drugs.[41-43] For *in vitro* metabolic assays using liver microsomes, albumin has been used to accurately assess intrinsic metabolic clearance of drugs by sequestering intrinsic inhibitors.[44,45] In addition, our current results showed that albumin present in media can influence the extent and duration of the inhibitory effect on transporters caused by pre-incubation.

In conclusion, we report that BA, UA and OA effectively inhibited the activities of OATP1B1/3 by pre-incubation as well as co-incubation. BA may have the potential to incur hepatic OATP1B transporter-mediated drug

interactions *in vivo*, but only at very high dose levels. It remains to be determined whether BA can reach sufficiently high concentrations to incur such drug interactions following the consumption of herbal supplements. Our results identify BA as another OATP1B3 inhibitor with pre-incubation effect and suggest that the pre-incubation effect and its duration are impacted by altered equilibrium of inhibitors between intracellular and extracellular space (e.g. albumin in the media).

5. Future directions

이번 연구를 통해 우리는 BA 가 pre-incubation 만으로도 OATP1B3 에 저해작용을 할 수 있는 inhibitor 라는 것을 실험을 통해 확인하고, OATP1B3 저해효과의 지속 정도는 세포 내외의 inhibitor 의 농도 평형의 변화에 영향을 받을 수 있음을 밝혔다. 이를 통해 세포 내의 inhibitor 의 농도가 pre-incubation 효과에 중요할 수 있다는 점을 간접적으로 보였지만, 아직 OATP1B3 의 세포 내 어떤 binding site 과 interaction 을 하는 것인지에 관해 다루지 못한 한계가 있다. 따라서 이에 대한 추가 연구가 필요하다. 이를 위한 방법으로는 첫째, OATP1B3 의 세포 내 loop 에서 양전하를 띄고 있는 아미노산의 point mutation 을 통해 BA 에 의한 pre-incubation 효과의 차이를 볼 수도 있다. 둘째로는 OATP1B3 의 3 차원적 구조를 밝혀 세포 안쪽 부분에서 특정 저해제의 binding pocket 을 찾는 방법이 있을 수 있다. 이러한 실제적인 binding site 에 대한 정보는 향후 다른 inhibitor 들의 pre-incubation effect 예측과 그로 인한 약물상호작용 가능성을 예측하는 것에 insight 를 제공할 수 있다.

CysA 를 시작으로 하여, OATP1B1/3 에 preincubation effect 가 저해제 특이적이고, dose and time-dependent 하다는 것이 여러 논문을 통해 밝혀지고 있다. 최근 논문에선 세포 내 존재하는 CysA 가 OATP1B1/3 의 internal binding site 를 통한 결합이 가능하고, 이에 의한 preincubation effect 를 simulation approach 를 통해서 증명하고 있다 [24]. 궤를 같이하여 이번 논문에서는 albumin 에 의해 세포내 저해제 농도를 인위적으로 바꿔줬을 때 OATP1B3 의 활성이 그에 맞춰 변화하는 것을 확인하였다. 그렇지만 실제로 저해제의 세포 내 농도를 정량적으로 접근한 것은 아니기에 이에 대한 추가 연구가 필요하다. 향후 연구에서 세포 내 단백결합의 정도, 세포 내 부피 등에 대한 실험적 결과를 얻는 다면, 보다

정확한 $K_{i,in}$ 을 구할 수 있게 되고, 특정 저해제에 의한 pre-incubation 에 의한 영향을 더욱 잘 예측하는 것에 도움이 될 것이다.

In vivo 에서 20 mg/kg 의 BA 를 정맥 투여하여 ATV 의 혈중농도와 간으로의 분포($K_{p,liver,2hr}$)에 차이를 가져왔지만, 이것이 정말 rat Oatp1b2 에 의한 상호작용이었는지에 대한 확인이 추가로 필요하다. 이를 위해 해당 투여 용량에서 간독성을 비롯한 각종 toxicity 수치에 대해 확인할 필요가 있다. 더불어 독성에 의한 결과가 아니라고 하더라도, 이것이 임상적으로 의미가 있는 결과인지에 대한 고찰이 필요하다. BA 는 herbal supplement 로써 주로 경구로 투여가 이루어진다는 것을 고려하면, BA 의 bioavailability 와 plasma protein binding 에 대한 추가적인 실험이 필요하고 이를 통해 경구투여를 통해서도 ATV 의 동태학적 특성에 변화를 가져올 수 있는지 추가 실험이 필요하다. 이러한 실험들은 BA 를 비롯한 구조적으로 유사한 pentacyclic triterpenoid 의 잠재적인 상호작용을 예측하는 것에 중요한 insight 를 제공해 줄 것이다.

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Table 1 Optimized analytical conditions of the LC–MS/MS.

Analyte (Internal standard)	Transition (m/z ratio)	ESI	Column	Mobile phase	Elution [min (A%)] Flow rate (mL/min)	Curtain gas (psi) Ion–spray voltage (V)	source temperature (℃) ion source gas 1–2 (psi)	Declustering Potential (V) Entrance Potential (V) Collision Energy (V)
Atorvastatin (Glipizide)	559.3/440.4 445.8/320.9	+	Luna® 3 μm, C18, 50 x 2 mm	A: 0.1% formic acid in acetonitrile B: 10 mM ammonium formate in water	Gradient: [0 (30%)–0.5 (80%)–1.5 (80%)–2 (30%)–5 (30%)] Flow rate: 0.5	25 4500	550 60–60	45 4 27
Betulinic acid (Deoxycholic acid)	456.0 392.0	–		A: methanol B: 10 mM ammonium formate in water	Isocratic: [0 (90%)–3 (90%)] Flow rate: 0.5	20 –4500	600 40–30	–90 –7 –
Asunaprevir	748.0/648.0 –	+	Eclipse XDB–C18, 3.5 μm, 100 x 2.1 mm	A: 0.1% formic acid in water B: methanol: acetone: formic acid (95:5:0.1; v/v/v)	Gradient: [0 (15%)–0.5(15%)–2.5 (5%)–2.53 (15%)–4 (15%)] Flow rate: 0.3	10 5000	500 20–30	56 6.5 27

Table 2 Pharmacokinetic parameters for ATV and BA from the plasma concentration−time profiles in rats that received the co−adminstration of atorvastatin (ATV, 2 mg/kg) and betulinic acid (BA, 4 or 20 mg/kg) or ATV only (n=3/treatment)

Dosing Regimen	Low dose of BA			High dose of BA		
	ATV only (2 mg/kg)	ATV (2 mg/kg) + BA (4 mg/kg)		ATV only (2 mg/kg)	ATV (2 mg/kg) + BA (20 mg/kg)	
Parameter	ATV	ATV	BA	ATV	ATV	BA
AUC_{2hr} (min*μg/mL)	11.09 ± 2.74	10.24 ± 1.14	7.120 ± 1.56	20.26 ± 10.1	44.62 ± 13.1*	310.0 ± 49.9
CL_{obs} (mL/min/kg)	185.8 ± 49.9	194.1 ± 20.7	227.7 ± 116	108.0 ± 44.3	36.51 ± 17.9*	64.64 ± 10.9
V_{ss_obs} (mL/kg)	2,795 ± 540.5	3,138 ± 486.0	53,960 ± 22460	3,033 ± 1483	3,071 ± 431.5	1,156 ± 87.70

The data are shown as the mean ± S.D (*, $p < 0.05$ by Student's t test).

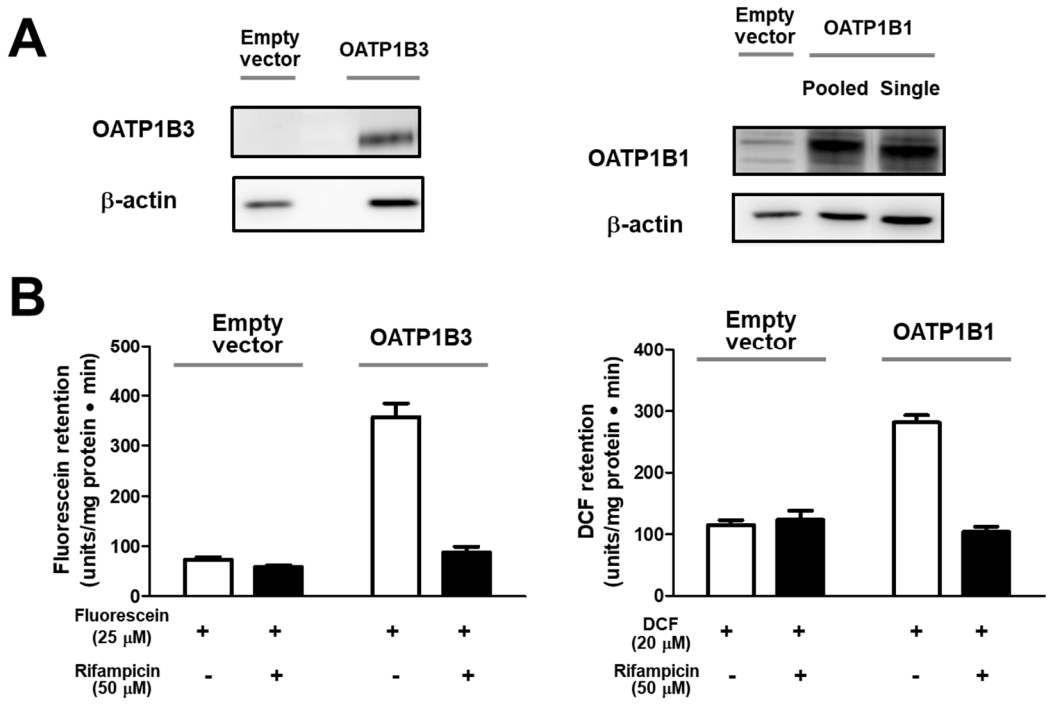

Figure 1. Establishment of HEK293 cells stably expressing OATP1B3 or OATP1B1.

(A) Immunblotting results showing the elevated expression levels of OATP1B3 or OATP1B1. β-actin was used as a gel loading control. (B) The functional activity of OATP1B3 and OATP1B1 was confirmed by increased cellular retention of their respective fluorescent probe substrates (fluorescein, DCF). Cellular uptake by OATP1B3 and OATP1B1 was assessed by incubating cells with fluorescein (25 μM, 8 min) and DCF (20 μM, 5 min) at 37° C, respectively. Enhanced cellular uptake of probe substrates was effectively inhibited by the addition of rifampicin (50 μM, an OATP inhibitor). Data are shown as mean ± SD (n=3).

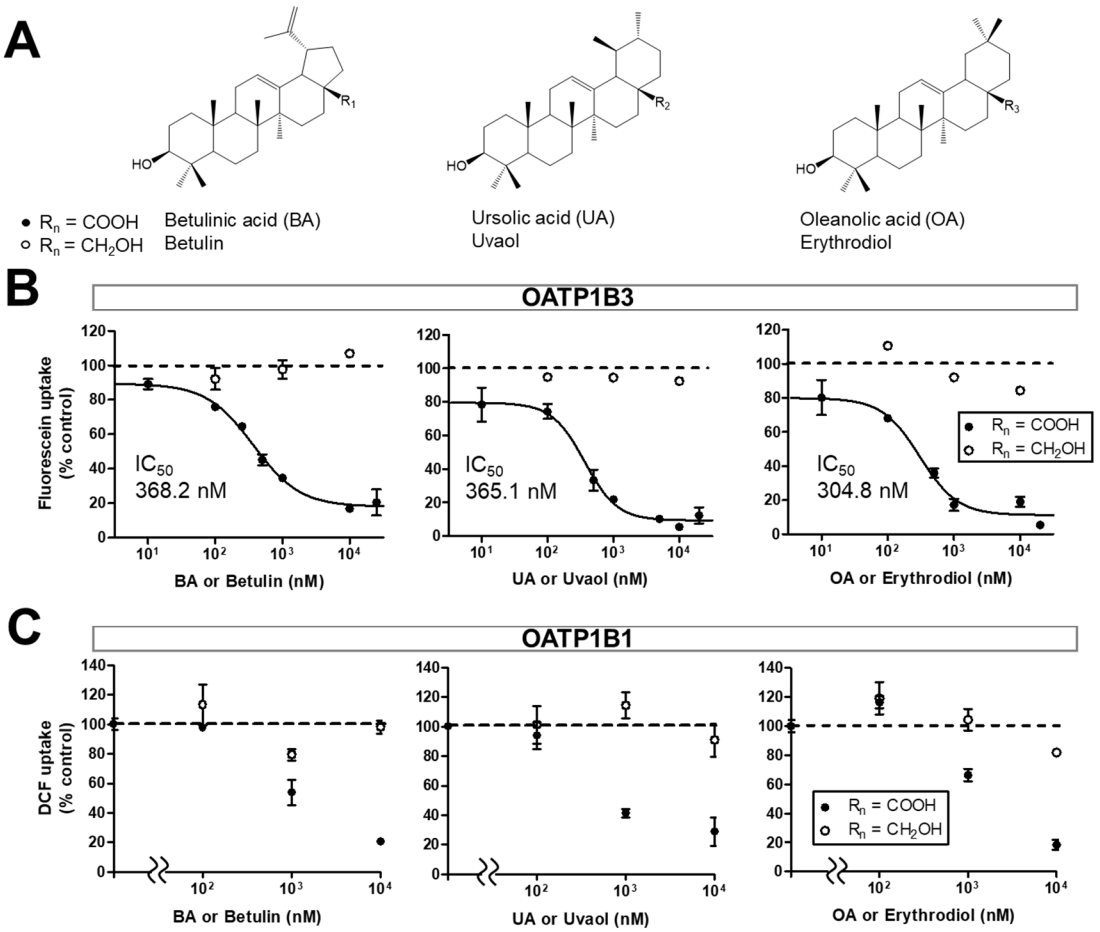

Figure 2. Co-incubation effects of betulinic acid (BA) or its structurally-related pentacyclic triterpenoids on the transport activities of OATP1B1/3 assessed by the uptake of fluorescent probes.

(A) Structures of the selected pentacyclic triterpenoids, betulinic acid (BA), ursolic acid (UA), oleanolic acid (OA) and their counterparts which contain a hydroxymethyl group (-CH2OH) instead of a carboxylic group (-COOH). (B) Co-incubation of BA, UA or OA inhibited the uptake of fluorescein (25 μM) in HEK293 cells stably expressing OATP1B3 in a concentration-dependent manner. In contrast, their counterparts lacking a carboxylic group (betulin, uvaol or erythrodiol) did not lead to concentration-dependent inhibition of the OATP1B3 activity. (C) Similar results were obtained when the effects of BA, UA, OA and their counterparts lacking a carboxylic group were assessed by

measuring the uptake of DCF (20 μM) in HEK293 cells stably expressing OATP1B1. All data are presented as a percentage to the vehicle control (mean ± SD, n=3) after correcting for passive diffusion by subtracting the signals obtained in control HEK293 cells transfected with the empty vector.

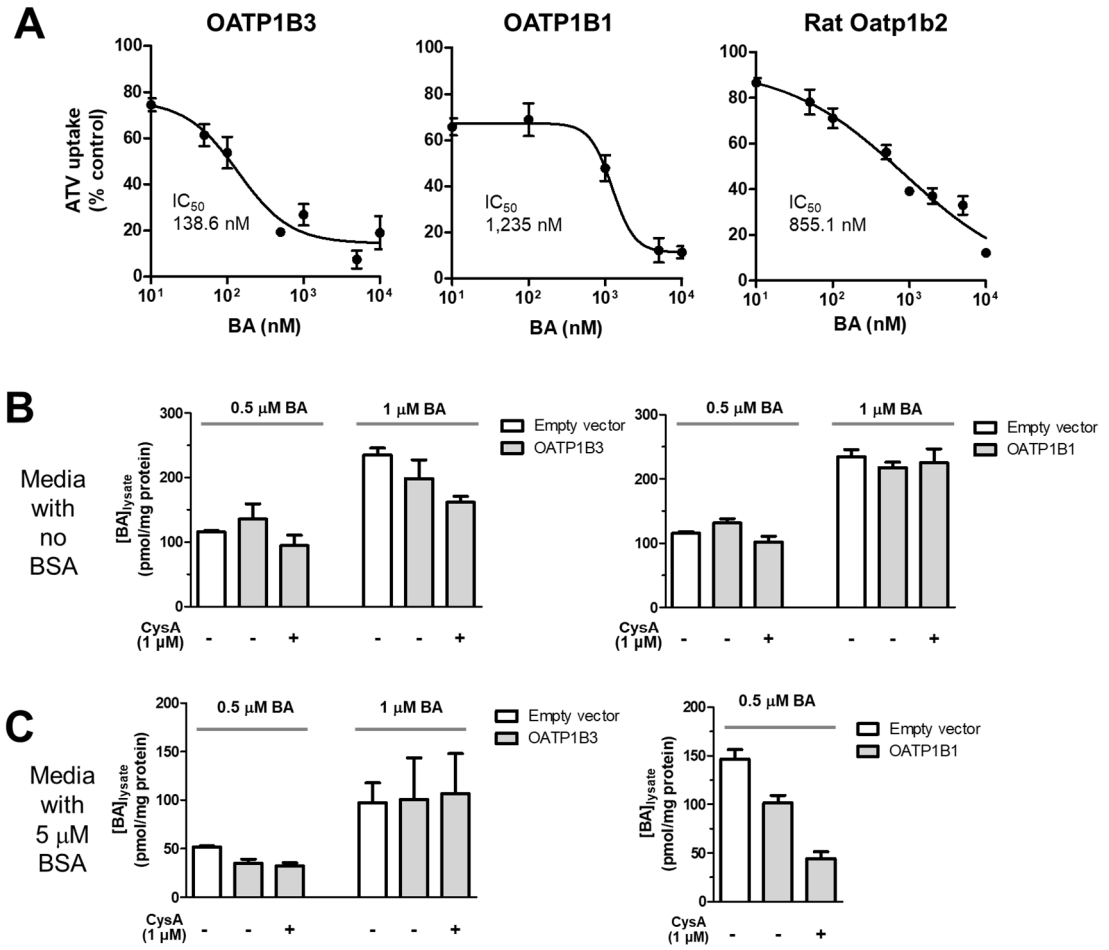

Figure 3. Co-incubation effects of betulinic acid (BA) on cellular retention of atorvastatin (ATV) in HEK293 cells stably expressing OATP1B3, OATP1B1 or rat Oatp1b2.

(A) Co-incubation with BA inhibited the uptake of ATV by OATP1B3, OATP1B1 or rat Oatp1b2 in a concentration-dependent manner. All data are presented as a percentage to the vehicle control after correcting for passive diffusion by subtracting the signals obtained in control HEK293 cells transfected with the empty vector. (B) Intracellular accumulation of BA did not increase by OATP1B3 or OATP1B1. Cell lysates were prepared after incubating HEK293 cells stably expressing OATP1B3 or OATP1B1 or control cells following the incubation with BA (0.5 or 1 μM) for 5 min at 37° C. When the BA levels in cell lysates were quantified, the levels of BA did not differ between control cells

and cells stably expressing OATP1B3 or OATP1B1. Co-incubation with CysA (1 μM, an OATP inhibitor) also had no consistent impact on the BA levels in cell lysates. (C) Although 5 μM of BSA for minimizing non-specific binding was used, the BA levels did not increase in lysates from cells expressing OATP1B1/3. All data are shown as mean ± SD (n=3) and from representative datasets following 2-3 replicate experiments.

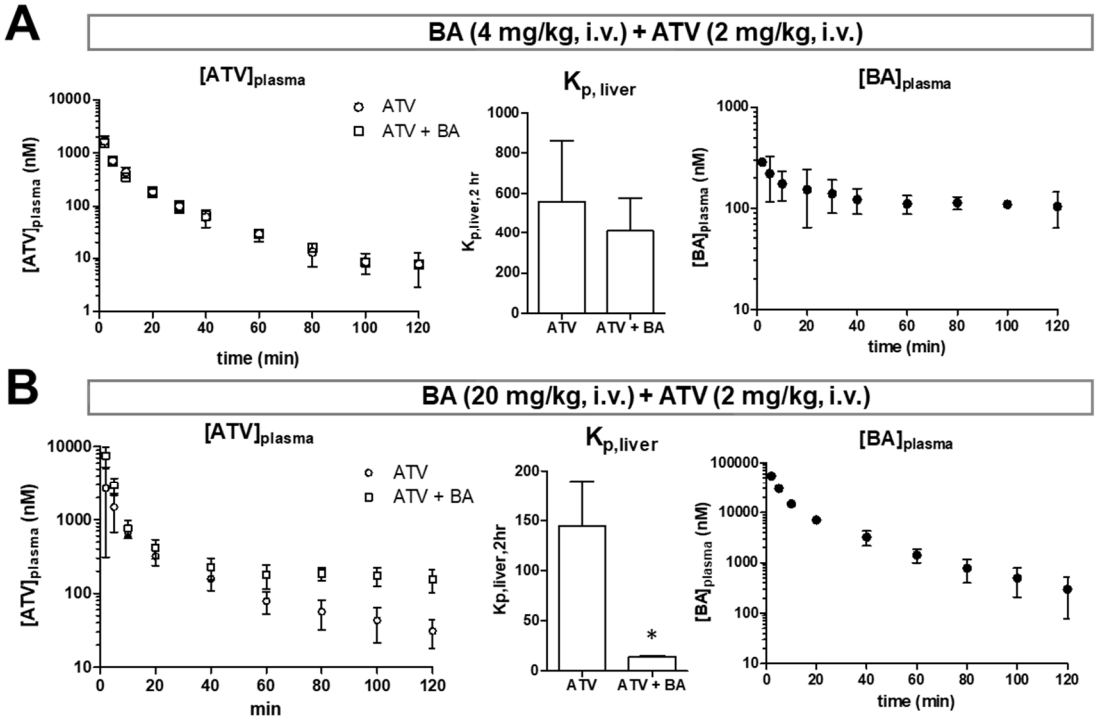

Figure 4. Plasma pharmacokinetic profiles of atorvastatin (ATV) in rats that received intravenous administration of betulinic acid (BA; 4 or 20 mg/kg via intravenous bolus) together with ATV (2 mg/kg via intravenous bolus) in rats.

(A) Plasma ATV concentration-time profiles in rats (n=3) after intravenous bolus dosing of ATV (2 mg/kg) with or without the co-administration of BA (4 mg/kg, intravenous bolus). Open circles represent the data for ATV dosing only and open squares represent the data for co-administration of ATV and BA. The ratios of ATV concentration in the liver to that in plasma after 2 hr post-dosing ($K_{p,liver,\ 2hr}$). Plasma BA concentration-time profiles in rats that received intravenous injection of ATV and BA. (B) Plasma ATV concentration-time profiles in rats (n=3) after intravenous bolus dosing of ATV (2 mg/kg) with or without the co-administration of BA (20 mg/kg, intravenous bolus). All data are shown as mean ± SD (n=3). (*, $p < 0.05$ by Student's t test.)

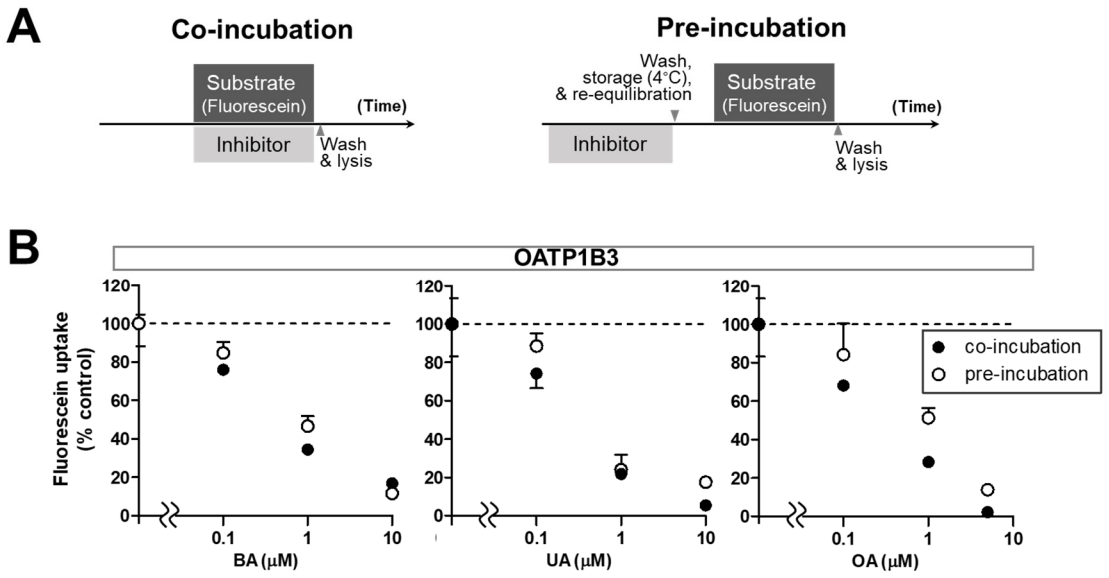

Figure 5. Comparison of co-incubation and pre-incubation effects of betulinic acid (BA), uroslic acid (UA) and oleanolic acid (OA) on the OATP1B3 activity

(A) Graphic presentation of the experimental procedures to examine co-incubation and pre-incubation effects of the tested compounds (detailed information described in the methods). (B) Pre-incubation with BA, UA and OA (1 μM each, 30 min) led to the inhibition of OATP1B3 with a potency comparable to the co-incubation conditions. All data are presented as a percentage to the vehicle control (mean ± SD, n=3) after correcting for passive diffusion by subtracting the signals obtained in control HEK293 cells transfected with the empty vector.

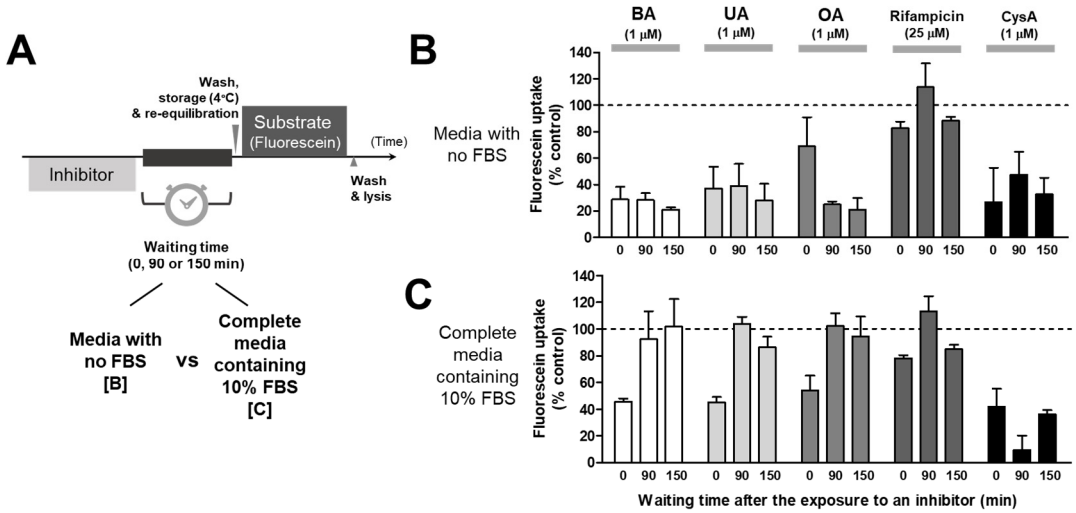

Figure 6. Effect of media compositions on the time courses by which the OATP1B3 activity recovers following the pre-incubation with betulinic acid (BA), uroslic acid (UA) and oleanolic acid (OA)

(A) Graphic presentation of the experimental procedures to examine the time course by which the OATP1B3 activity recovers following the pre-incubation with the tested compounds (detailed information described in the methods). (B, C) The OATP1B3 activity stayed inhibited even with 150 min of the waiting time in the media containing no FBS (B). In contrast, the OATP1B3 activity recovered almost completely within 90 min of the waiting time in the media with 10% FBS (C). Data are shown as a percentage of the vehicle control (mean ± SD, n=3).

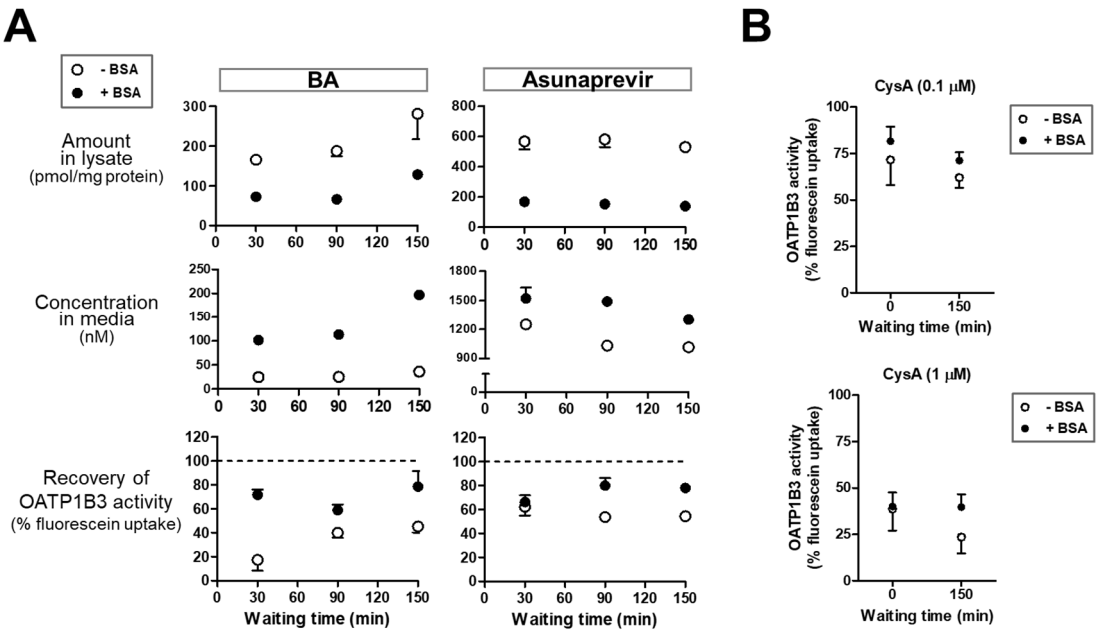

Figure 7. Addition of bovine serum albumin (BSA) to media expedites the recovery of OATP1B3 activity inhibited by the pre-incubation with betulinic acid (BA), asunaprevir or cyclosporin A (CysA).

(A) The levels of BA or asunaprevir in cell lysates and media were measured at different waiting times following the pre-incubation with BA or asunaprevir. With the use of BSA-containing media (closed circles), the levels of BA or asunaprevir decreased in cell lysates, but increased in media compared to those obtained with media lacking BSA (open circles). The recovery of OATP1B3 activity inhibited by BA or asunaprevir was expedited with the media containing BSA. (B) The recovery of OATP1B3 activity inhibited by CysA (0.1 or 1 μM) was only slightly changed by the addition of BSA. Data are shown as mean ± SD (n=3).

국문초록

OATP1B1과 OATP1B3 (OATP1B1/3)는 임상적으로 중요한 statin 계열 약물의 간세포 내 유입 및 배설에 중요한 영향을 미칠 수 있는 약물수송체이다. 선행연구를 통해 몇몇의 식물유래 pentacyclic triterpenoids와 OATP1B1/3의 상호작용에 대해서는 보고가 된 반면, 베툴린산 (betulinic acid, BA)에 의한 잠재적인 상호작용에 대해서는 아직 연구된 바가 없다. 따라서 이번 연구를 통해, BA와 이와 구조적으로 유사한 pentacyclic triterponoids (ursolic acid, UA; oleanolic acid, OA)에 대해 OATP1B1/3 및 rat Oatp1b2와의 상호작용에 관한 연구를 *in vitro*와 *in vivo* 모델을 통해 진행하였다. 이를 위해 co-incubation과 pre-incubation(저해제를 먼저 넣어주고 씻어준 후 기질만 incubation)을 포함한 다양한 실험적 조건을 활용하였다.

이로부터 얻은 결과로, BA, UA, OA는 OATP1B1/3나 rat Oatp1b2에 의한 형광기질과 atorvastatin (ATV)의 세포 내 흡수를 효과적으로 저해한다는 것을 확인하였다. 저용량의 BA (4 mg/kg)를 정맥으로 ATV와 함께 투여 하였을 때는 ATV의 약동학적 양상에 영향을 미치지 못한 반면, 고용량의 BA (20 mg/kg)을 함께 투여 하였을 때는 ATV의 혈중농도와 간으로의 분포에 변화를 가져왔다. BA, UA, OA의 pre-incubation만으로도 2.5시간동안 OATP1B3의 기능 저해를 장기적으로 유지할 수 있었다. 그렇지만, 이러한 저해능력은 배지 속의 10% FBS에 의해 빠르게 회복되었다. 배지에 알부민을 첨가하면 BA의 세포 내 농도가 감소한다는 것을 확인하였고, 이로 인해 OATP1B3의 pre-incubation에 의한 저해가 빠르게 회복된다는 것을 확인하였다. Pre-incubation effect가 있다고 보고된 asunaprevir와 cyclosporinA (CysA)를 이용했을 때, asunaprevir의 경우 배지에 알부민을 첨가하는 것에 의해 회복 시간의 감소하였고, CysA에서는 그렇지 않았다.

끝으로, 우리의 결과는 BA가 *in vitro*에서 효과적으로 OATP1B 수송체를

저해함과 동시에 고용량의 BA에 의해 *in vivo*에서도 잠재적으로 상호작용이 일어날 수도 있음을 제시하였다. 또 BA가 pre-incubation만으로도 저해효과가 있는 OATP1B3 억제제라는 것을 실험적으로 밝히고, pre-incubation효과와 그것의 지속 기간이 세포 안팎의 inhibitor의 농도 평형의 변화(예, 배지에 알부민 첨가)에 영향을 받을 수 있음을 확인하였다.

주요어: 천연 물질; 약물수송체 매개 약물 소실; 유기음이온 흡수수송체 1B1, 1B3(OATP1B1, OATP1B3)

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