약학석사 학위논문

Kinetic difference in the inhibitory activity of statins among hOATP1B1, hOATP1B3 and rOATP1B2 in MDCK cells

Statin계 약물과 human과 rat의 Organic Anion Transporting Polypeptide(OATP) 1B subfamily members의 상호작용에서의 기능 차이에 대한 연구

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약학과 약제과학 전공

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Abstract

Kinetic difference in the inhibitory activity of statins among hOATP1B1, hOATP1B3 and rOATP1B2 in MDCK cells

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Objective. Human organic anion-transporting polypeptide 1B1 (hOATP1B1) and Human organic anion transporting polypeptide 1B3 (hOATP1B3) are both selectively expressed in the liver, where they are localized to the basolateral membrane of hepatocytes. hOATP1B1 and hOATP1B3 have a single rodent orthologue, rOATP1B2. Despite the transporters was classified as the crucial transporter for screening candidates of new drugs, the specie-difference considering transport kinetics has not been systematically studied for the transporter. The objective of this study is to evaluate K_i values for commercially available statins in *in vitro* system expressing the OATP1B transporters.

Methods. The functional expression of the transporters was determined by comparing the uptake ability of radiolabeled

estradiol-17β-D-glucuronide (a standard substrate). Inhibition studies were carried out to determine the IC_{50} for commercially available of statins. The IC_{50} values were converted to absolute inhibition constant K_i using the Cheng-Prusoff equation. One way ANOVA was used to compare K_i among hOATP1B1, hOATP1B3, and rOATP1B2.

Results. Among the eight statins study, four drugs were interacted with human and rat forms to a certain extent. No functional difference was found for type I statins (i.e., pravastatin, mevastatin, simvastatin, lovastatin). The inhibition constants for pravastatin were 24.06 ± 1.88, 40.14 ± 10.48, and 27.61 ± 8.16 μM in hOATP1B1, hOATP1B3 and rOATP1B2 expressing cells. The K_i values for mevastatin were 8.97 ± 4.39, 11.90 ± 2.50, and 12.29 ± 5.51 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. The K_i values for simvastatin were 10.61 ± 4.51, 8.52 ± 3.73 and 13.69 ± 6.04 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. The K_i values for lovastatin were 12.70 ± 6.02, 14.63 ± 3.88, and 11.99 ± 5.06 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. All type II (i.e., fluvastatin, atorvastatin, rosuvastatin) and type III statins (pitavastatin) examined the functional difference. The K_i values for fluvastatin were 2.22 ± 0.35, 2.59 ± 0.42 and 4.15 ± 0.85 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. The K_i values in rOATP1B2 cells was significant different from those with hOATP1B1. The K_i values for atorvastatin were 0.62 ± 0.34, 0.92 ± 0.16 and 2.05 ± 0.16 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. The K_i value in rOATP1B2 cells was significant different from those in hOATP1B1/hOATP1B3 cells. The K_i values for rosuvastatin were 6.99 ± 3.23, 10.14 ± 0.95 and 2.98 ± 1.07 μM in hOATP1B1, hOATP1B3, rOATP1B2 cells. The K_i values in rOATP1B2 cells was significant different from those in hOATP1B3 cells. The K_i values for pitavastatin were 1.07 ± 0.16, 0.91 ± 0.36 and 2.26 ± 0.57 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells. The K_i value in

rOATP1B2 cells was significant different from those in hOATP1B1/hOATP1B3 cells.

Conclusion. The functional differences among hOATP1B1, hOATP1B3, and rOATP1B2 were noted for four of eight statins studied. In particular, no functional difference was found for type I statins, while all type II and III statins examined had the functional difference, suggesting that the kinetic differences among OATP1B transporters are depend on chemical structures of substrates. This aspect of OATP1B transport warrants further research.

Keywords : transporter, hOATP1B1, hOATP1B3, rOATP1B2, statin, interspecies differences, K_i

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Abbreviation and Acronyms

ANOVA: analysis of variance

ATV: atorvastatin

DDI: drug-drug interaction

DMSO: dimethyl sulfoxide

DPBS: Dulbecco's phosphate buffered saline

$E_2$17βG: estradiol-17β-D-glucuronide

E_3S: estrone-3-sulfate

FLV: fluvastatin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC_{50}: half maximal inhibitory concentration

K_i: inhibition constant

K_m: Michaelis-Menten constant

LOV: lovastatin

MDCK: Madin - Darby canine kidney

MEV: mevastatin

OATP: organic anion transporting polypeptide

PRV: pravastatin

PTV: pitavastatin

ROV: rosuvastatin

SMV: simvastatin

1. Introduction

1.1. Drug transporters

Membrane transporters can be key determinants of the pharmacokinetic parameters, safety and efficacy profiles of drugs. Drug absorption and disposition might be influenced by the interactions of drugs with transport processes. The role of uptake and efflux transporters has recently been recognized as a crucial in metabolic clearance of many drugs. In US Food and Drug Administration guidance for drug interaction study, seven major transporters (P-gp, BCRP, hOATP1B1, hOATP1B3, OCT2, OAT1, and OAT3) were requested to assess the effects on pharmacokinetic profiles of drugs for further drug discovery [24].

1.1.1. OATP1B subfamily

The organic anion - transporting polypeptide (OATP/Oatp) 1B family transporters in human and preclinical species mediate cellular uptake of various chemicals into the liver [25]. The human liver specific OATPs are hOATP1B1 and hOATP1B3, and rat ortholog most closely related with human is rOATP1B2 [1]. hOATP1B1 and hOATP1B3 are selectively expressed in the liver, where they are localized to the basolateral membrane of hepatocytes (Fig. 1). rOATP1B2 is also mainly expressed in the liver and is functionally

similar to hOATP1B1 and hOATP1B3 [9]. hOATP1B1 and hOATP1B3 play crucial roles in the hepatic uptake. Their spectrum of uptake substrates includes steroid conjugates, the thyroid hormones T4 and T3, bile salts, BSP, eicosanoids, cyclic peptides, drugs such as benzylpenicillin, methotrexate, rifampicin, telmisartan, all statins and natural toxins microcystin and phalloidin [6, 22, 23]. hOATP1B3 has a similar broad substrate specificity as hOATP1B1. However, hOATP1B3 also exhibits unique transport properties such as, for digoxin and ouabain.

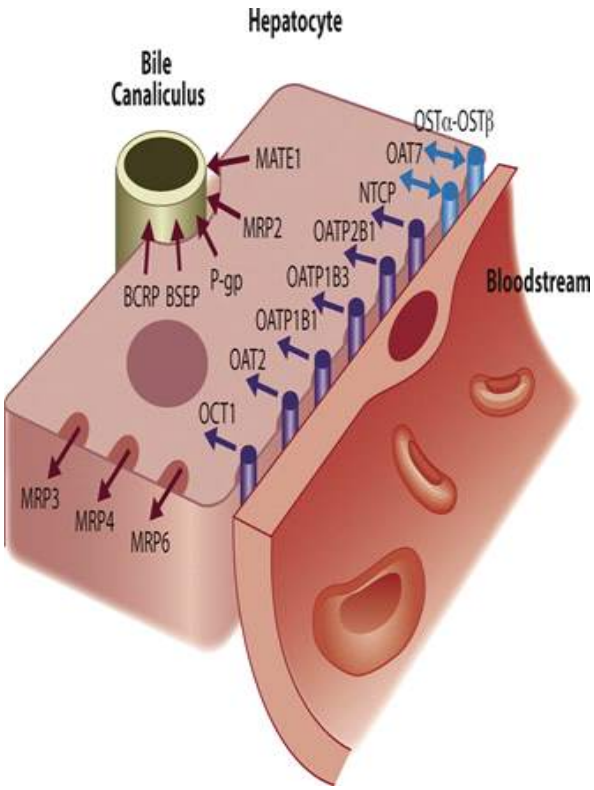

Figure 1. Position of hOATP1B1 and hOATP1B3 in hepatocyte [25]

1.2. Interspecies differences

During developing a new drug, preclinical experimentation using experimental animals are needed and next steps is proceed dependent on *in vivo* data. Transporters in human are different with that of experimental animals in terms of their structure, substrate specificity, affinity and so on. In other words, interpretation of *in vivo* data would followed by kinetic difference between human and an experimental animal. Therefore, understanding about functional differences among OATP1B transporters is essential study to estimate pharmacokinetic parameters in human.

hOATP1B1 encodes a 691-amino-acid glycoprotein. It shares 80% amino acid identity with hOATP1B3, but only 64% identity with its rat ortholog rOATP1B2 [15]. Despite hOATP1B1 and hOATP1B3 were classified as the important transporters for screening candidates of new drugs, the specie-difference considering transport kinetics has not been systematically studied for the transporters.

1.3. *In vitro* system

In vitro study has become a first step in the estimation of drug interactions. The experiments can be used as a screening tool to predict drug interaction potential *in vivo*. Besides *in silico* modeling and simulation may also assist in the prediction of drug interactions. The recent FDA draft drug interaction guidance emphasized the *in vitro* models that may be used to guide further *in vivo* drug interaction studies. *In vitro* methods using cells transfected

transporters are suitable for studying drug interactions with transporters.

1.4. Statins

Statins are drugs to lower blood cholesterol levels, which are the most widely prescribed drugs in the world. They have shown remarkably impact on lowering blood cholesterol, however, severe side effects from drug-drug interactions were reported when patients took multiple drugs for other medical conditions. The uptake mechanism of statins is a combination of passive diffusion and active transporting by OATP transporters. Active transports in hepatocyte are major facilitators of metabolism and elimination of statins. hOATP1B1 is known to transport all statins in commercially use. The current medication guide for rosuvastatin warns that co-administration medications such as cyclosporin which are inhibitors of hOATP1B1 transporters, may lead to increased plasma levels of rosuvastatin and increased risk of myopathy [16].

Statins have sometimes been grouped into two groups of statins according to their structure [12]. Type I group statins are simvastatin, pravastatin, and lovastatin that have substituted decalin-ring structure (Fig. 2). Mevastatin has often been classified as type I statins due to its structural relationship. Type II group statins are fluvastatin, atorvastatin, rosuvastatin (Fig. 3) that are fully synthetic and have fluorophenyl group, the replacement of the butyryl group of type I statins. This group is responsible for additional polar interactions that cause tighter binding to the HMG-CoA reductase.

Type II group statins have larger groups linked to the HMG-like moiety. Pitavastatin has a relationship with type II statins in terms of its structure. Since pitavastatin was added to new kind of statins recently, in this study, pitavastatin was classified as type III statins.

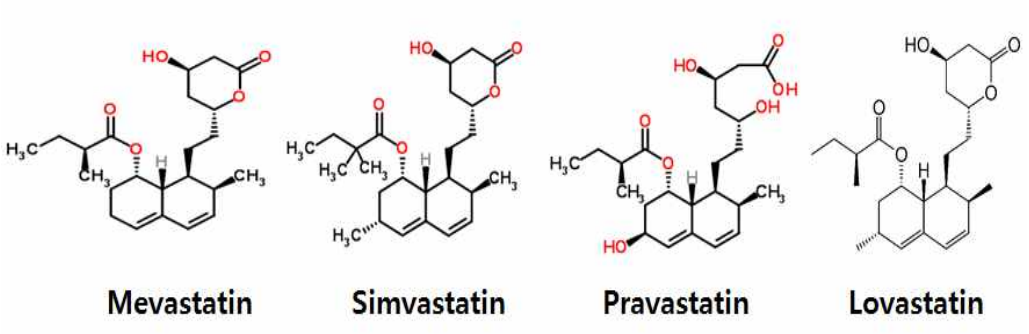

Figure 2. Chemical structures of type I statins

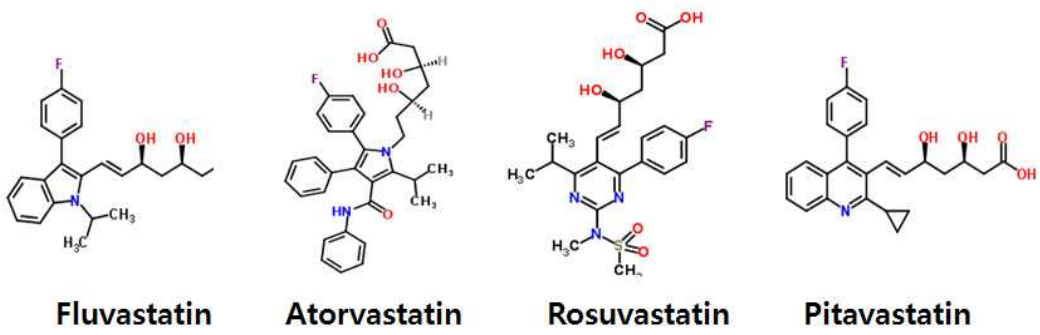

Figure 3. Chemical structures of type II and III statins

2. Materials

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), the non-essential amino acid solution (NEAA), Dulbecco's phosphate buffered saline (DPBS), penicillin/streptomycin, and fatal bovine serum (FBS) were obtained by Welgene (Daegu, Korea). PCR Purification Kit was purchased from Bioneer (Daejeon, Korea). [^{3}H]-estradiol-17β-D-glucuronide ($E_2$17βG) (specific activity, 41.4 Ci/mmol), [^{3}H]-estrone-3-sulfate (E_3S) (specific activity, 45 Ci/mmol), [^{3}H-methotrexate (specific activity, 40.0 Ci/mmol), and [^{3}H-benzylpenicillin (specific activity, 25 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Scintillation cocktail were obtained from PerkinElmer Life Sciences (Boston, MA). Unlabeled $E_2$17βG, pravastatin (98% purity), rosuvastatin (98% purity), bicinchoninic acid (BCA), Hanks' balanced salts (HBSS), sodium bicarbonate, sodium hydroxide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), Corning® Costar® cell culture plate (24 well, 96 well) and poly-L-ornithine solution were purchased from Sigma-Aldrich (St.Louis,MO). Atorvastatin (98% purity), simvastatin (97% purity), lovastatin (98% purity), mevastatin (98% purity) and fluvastatin (98% purity) were obtained by Tokyo Chemical Industry (Tokyo, Japan). Pitavastatin (98% purity) was synthesized by Cayman Chemical (Michigan, USA). All other chemicals were of analytical grade and commercially available.

3. Methods

3.1. Establishment of stable MDCKII cell-lines

hOATP1B1- or hOATP1B3-expressing MDCKII cells and empty vector-transfected MDCKII cells (i.e., Mock cells) were constructed previously [19]. The protein coding region of rOATP1B2 (GenBank accession number NM_031650.3) was cloned from rat total liver mRNA library (Takara, Shuzo, Japan). The RNA (1 μg) was reverse transcribed using Primescript 1st strand cDNA Synthesis Kit (Takara). The experments was consisted in two steps: the first was incubation the mixture (Table 1) at 65°C for 5 min, cooling to 4°C, and the second was adding RNase inhibitor and RTase into reaction mixture (Table 2), incubation at 42°C for 60 min, heating to 95°C for 5 min, and cooling to 4°C. Specific primers for cloning of the rOATP1B2 coding region were 5′-GCTAGCAGTGATTGCAGACGTTCCCA-3′ (sense strand; NheI site under lined) and 5′-AAGCTTGTCCATCCTTGCCCCATTCT-3′ (antisense strand; HindⅢ site underlined). Polymerase chain reaction (PCR) was performed using Ex taq DNA polymerase (Takara) according to the following settings: 30 cycles of denaturation at 94°C for 30 s, a primer-annealing step at 63°C for 30 s, and extension at 72°C for 3 min. The amplicon was cloned into pcDNA5/FRT vector (Invitrogen, Carlsbad, CA) (Fig 4), and the identity of the inserts was confirmed by sequencing. A list of primers used for cloning, mutagenesis, and reverse transcription PCR (i.e., house-keeping gene;

canine GAPDH) is presented in Table 3. The plasmids containing the wild type form of rOATP1B2 were transfected into Madin-Darby Canine Kidney II/FRT (MDCKII/FRT) cells using FuGENE® transfection reagent. The transfected cells were incubated in culture media containing 0.1 mg/mL hygromycin B (Invitrogen) for several weeks for selection. Expression of rOATP1B2 was confirmed by reverse transcription polymerase chain reaction. The functional expression was determined by comparing the uptake ability of radiolabeled Estradiol-17β-D-glucuronide (i.e., $E_2$17βG), a standard substrate of transporters with that in Mock cells (control). Protein amount was determined by a BCA assay [27] and used for correction of cell number used in the function study.

Table 1. Components of RT mixture in first step

Materials	Volume (μL)
RNase free water	6
template RNA	2
dT Primer	1
dNTP	1
	Total 10

Table 2. Components of RT mixture in second step

Materials	Volume (μL)
total mixture in Table 1	10
5x buffer	4
FDDW	4.5
RNase inhibitor	0.5
RTase	1
	Total 20

Figure 4. Map of pcDNA5/FRT vector

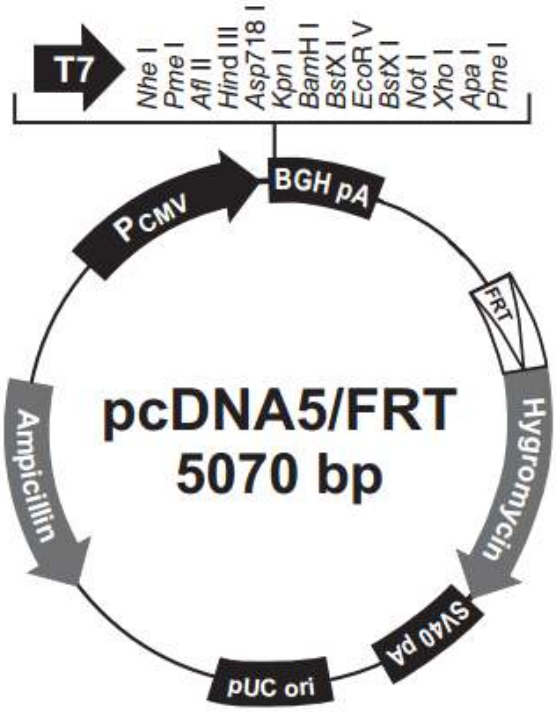

Table 3. A list of primers used cloning, mutagenesis of rOATP1B2 and house-keeping gene of MDCKII cells

Cloning	
Forward	5' GCTAGC AGT GAT TGC AGA CGT TCC CA 3'
Reverse	5' AAGCTT GTC CAT CCT TGC CCC ATT CT 3'
Mutagenesis (To delete T insertion after 2012T)	
Forward	5′ CTC CAC CAC TTT TTT T (T) AT ATG TTG TAT TAA TTT ATT TC 3′
Reverse	5′ GAA ATA AAT TAA TAC AAC ATA T (A) AA AAA AAG TGG TGG AG 3′
Canine GAPDH primer	
Forward	5' TCC ATC TTC CAG GAG CGA GA 3'
Reverse	5' GAT ACA TTG GGG GTG GGG AC 3'

3.2. Cell culture

hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells and Mock cells were grown in DMEM low glucose with 10% (v/v) FBS, 1% NEAA, 100 U/ml penicillin/streptomycin, and 10 mM HEPES at 37°C with 5% CO2 and 95% relative humidity. For the uptake study, cells were seeded in poly-L-ornithine coated 24-well cell-culture plates at a density of 5×10^5 cells/well (added as 0.5 mL) and were grown for 2 days.

3.3. Determination of a probe substrate

[^{3}H]-$E_2$17βG (specific activity, 41.4 Ci/mmol), [^{3}H]-estrone-3-sulfate (E_3S) (specific activity, 45 Ci/mmol), [^{3}H-methotrexate (specific activity, 40.0 Ci/mmol), and [^{3}H- benzylpenicillin (specific activity, 25 Ci/mmol) were reported as substrates of hOATP1B1 and hOATP1B3. Compounds were prepared total 1 μM (1% radiolabeled compound). After washing the cells three times with prewarmed DPBS, TM (200 μL) containing radiolabeled substrates with unlabeled compound to achieve the indicated concentrations (1 μM) were added to initiate transport. The TM consisted of 9.7 g/L Hanks' balanced salts, 2.38 g/L HEPES, and 0.35 g/L sodium bicarbonate, pH adjusted to 7.4. After incubating for 5 min, uptake was terminated by three times washing with ice-cold DPBS. Cells were lysed with 500 μL of 0.1 N sodium hydroxide. Radioactivity was measured by liquid scintillation counter (Tri-Carb 3110TR Liquid Scintillation Analyzer, Perkin-Elmer).

3.4. The uptake study of $E_2$17βG in hOATP1B1-, hOATP1B3-, or rOATP1B2- expressing MDCKII Cells

For the preliminary studies with $E_2$17βG, the time-dependent uptake studies were performed to evaluate the appropriate time when the $E_2$17βG was transported linearly in transporter-expressing MDCKII

cells and Mock cells, respectively (37°C, $E_2$17βG; 1 μM, incubation time; 1, 3, 5, 10, 15, and 30 min). As a result, the 5 min of incubation was applied to study of transporter-mediated uptake of $E_2$17βG in transporters (data not shown). Uptake was initiated by adding TM containing radiolabeled and unlabeled $E_2$17βG after the cells had been washed twice with DPBS and preincubated with TM at 37°C for 15 min. The uptake of $E_2$17βG was measured over a range of eight different concentrations between 0.5 and 200 μM (i.e., 0.2, 0.5, 1, 5, 10, 20, 100 and 200 μM) in hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells and Mock cells. After preincubation for 30 min at 37°C, the TM was removed and replaced with each drug solution (200 μL). Then, the solution was removed and washed three times with ice-cold DPBS (500 μL). After cells were lysed in 0.1 N sodium hydroxide in filtered water (500 μL) at room temperature and shaken for 1h, each sample was transferred to a scintillation vial containing 1 mL of Ultima Gold (Perkin-Elmer Life and Analytical Sciences, Waltham, MA). The total radioactivity was quantified by liquid scintillation counter. The data were normalized by protein concentration determined by a BCA assay [27]

3.5. Estimation IC_{50} of statins as inhibitors of OATP1B-mediated $E_2$17βG uptake

For measurement of $E_2$17βG transport in hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells, the cells were firstly washed twice with pre-warmed DPBS and pre-incubated with TM containing

1% (final concentration) DMSO (i.e., solubilizing agent for $E_2$17βG). When inhibitory effect statins on hOATP1B1, hOATP1B3, and rOATP1B2 uptake of $E_2$17βG was observed, the cells were incubated with 1 μM of $E_2$17βG solution containing various concentrations of statins (i.e., lovastatin, 0-300 μM; mevastatin, 0-100 μM; pravastatin, 0-1000 μM; simvastatin 0-300 μM; atorvastatin, 0-100 μM; fluvastatin, 0-500 μM; pitavastatin, 0-100 μM; and rosuvastatin, 0-500 μM;). Statins were prepared in DMSO and diluted with TM to 1% DMSO solution. After 5 min incubation at 37°C, the uptake was terminated by aspiration the drug solution followed by washing three times with ice-cold DPBS (500 μL). After 0.1 N sodium hydroxide in filtered water (500 μL) was added to each sample, the cells were lysed for 1h with shaking and each sample was transferred to a scintillation vial containing 1 mL of Ultima Gold. The total radioactivity was measured by liquid scintillation counter. The data was normalized by protein concentration determined by a BCA assay [27].

3.6. Data analysis

In vitro uptake study analysis

Kinetic transport parameters (e.g., $K_{m,}$ V_{max} and P_{dif}) were determined with Winnonlin® Professional 5.0.1 software (Pharsight Corporation, Mountain View, CA). using a simple Michaelis - Menten model:

$$v = \frac{V_{max} \times [S]}{(K_m + [S])} + P_{diff} \times [S]$$

where v is the uptake rate (pmol/min/mg protein), V_{max} is the maximal uptake rate, K_m is the Michaelis constant (μM), [S] is the $E_2$17βG concentration as a substrate (μM), and P_{dif} is the non saturable uptake clearance (μL/min/mg protein).

Then inhibition constant (K_i) values were obtained as Cheng-Prusoff equation [4].

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

IC_{50} was determined by fitting the data to the equation using Winnonlin® Professional 5.0.1 software (Pharsight Corporation, Mountain View, CA). K_i is the inhibition constant, [S] is the $E_2$17βG concentration as a substrate and K_m is the Michaelis-Menten constant of $E_2$17βG (μM).

Statistical Analysis

Statistical differences among the K_i of three OATP1B transporters were determined by 1-way ANOVA followed by Tukey's post hoc test, as appropriate (GraphPad Software Inc, San Diego, CA). In this study, data were expressed as the mean ± S.D. and $p < 0.05$ was considered statistically significant.

4. Result

4.1. Confirmation the expression of rOATP1B2 in MDCKII cells

Expression of the transfected slco1b2 gene was verified with reverse transcription polymerase chain reaction (RT-PCR) (Fig 5). Functional expression of rOATP1B2 was verified by evaluating uptake of a positive control substrate, $E_2$17βG (Fig 6). Mean uptake ratio compared to control in rOATP1B2-expressing MDCKII cells was approximately 80 fold higher than in Mock cells. Functional expression was validated every 3 months.

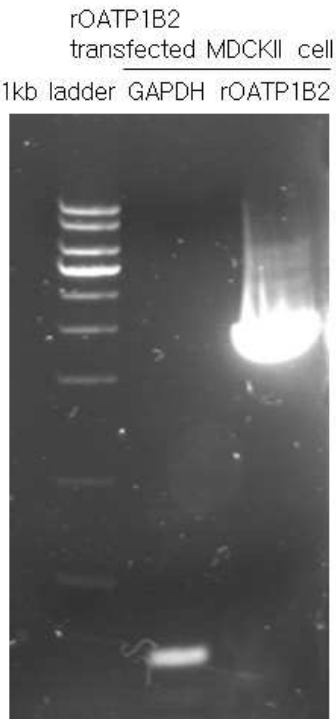

Figure 5. RT-PCR analysis of rOATP1B2 expression in rOATP1B2-expressing MDCKII cells

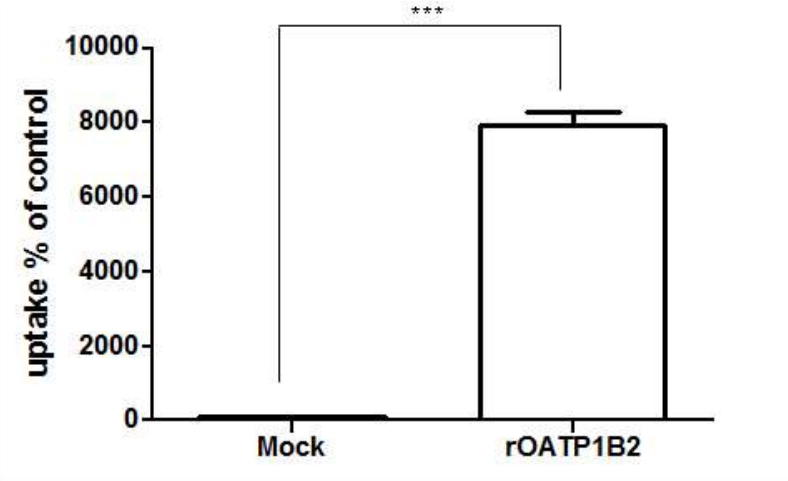

Figure 6. Cellular uptake of standard substrate, $E_2$17βG in rOATP1B2-expressing MDCKII cells compared to Mock cells (***p<0.01)

4.2. Determination of a probe substrate

To determine the common probe substrate in hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells, the screening using four radiolabeled substrates (i.e., $E_2$17βG, E_3S, methotrexate, and benzylpenicillin) were conducted. Figure 7 shows normalized uptake velocities of $E_2$17βG, E_3S, methotrexate, and benzylpenicillin in hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells and Mock cells. Initial transport velocities of $E_2$17βG, E_3S and methotrexate were significantly increased in three kinds of MDCKII cells, compared to Mock cells, whereas no increasing in uptake was

shown for benzylpenicillin. The especially uptake of $E_2$17βG were found that 35-, 5-, 70- fold higher in hOATP1B1-, hOATP1B3-, and rOATP1B2-expressing MDCKII cells, respectively, than control.

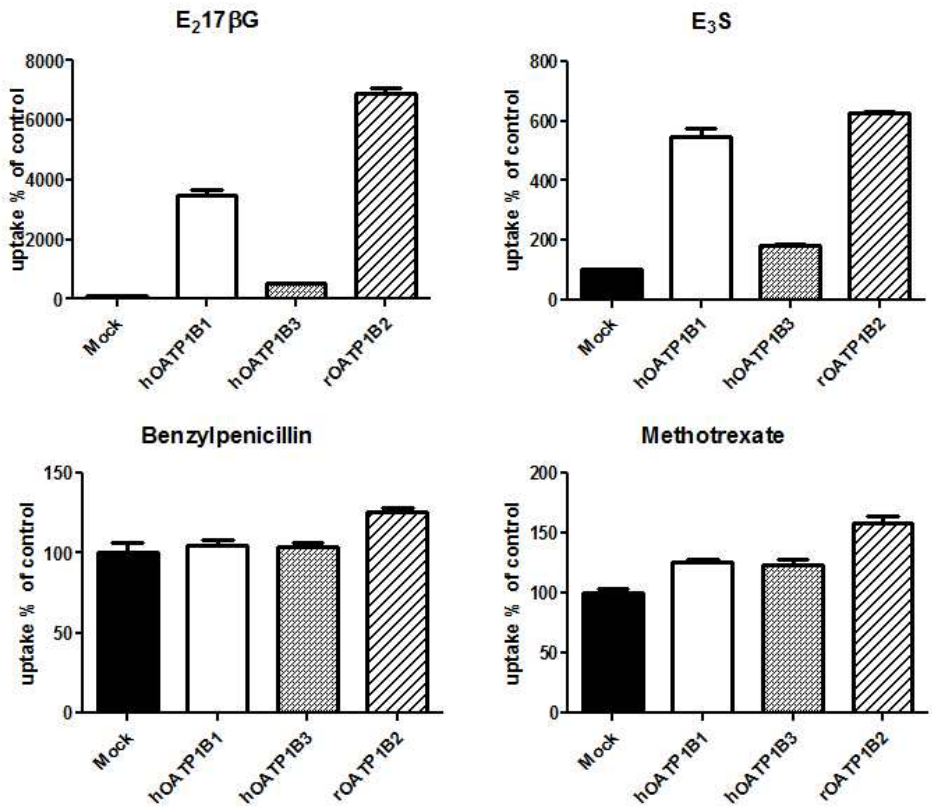

Figure 7. Screening to determine a probe substrate that has highest initial velocity in hOATP1B1-, OAPT1B3- or rOATP1B2-expressing MDCKII cells compared to Mock cells

4.3. Determination of Pharmacokinetic Parameters based on $E_2$17βG Uptake in OATP 1B subfamily-expressing MDCKII cells

Uptakes of $E_2$17βG in hOATP1B1-, hOATP1B3-, or rOATP1B2-expressing MDCKII cells were significantly higher than those in Mock cells (control), and increased linearly up to 10 min (data not shown). The concentration dependences of $E_2$17βG uptake rate are shown in Fig. 8. The uptake rates were saturable, and the K_m values were 10.95 ± 6.38 μM, 6.91 ± 5.23 μM and 11.37 ± 5.41 μM for hOATP1B1, hOATP1B3 and rOATP1B2, respectively. The Vmax values were 23.03 ± 7.02, 4.17 ± 4.88 and 36.02 ± 51.54 pmol/mg protein/min, respectively (Table 4). This result may be affected by the different in structure and active site of the three transporters.

Table 4. Calculation K_m and V_{max} of $E_2$17βG in hOATP1B1-, hOAPT1B3- or rOATP1B2-expressing MDCKII cells

Parameters	hOATP1B1	hOATP1B3	rOATP1B2
K_m (μM)	10.95 ± 6.38	6.91 ± 5.23	11.37 ± 5.41
V_{max} (pmol/mg protein/min)	23.03 ± 7.02	4.17 ± 4.88	36.02 ± 51.54

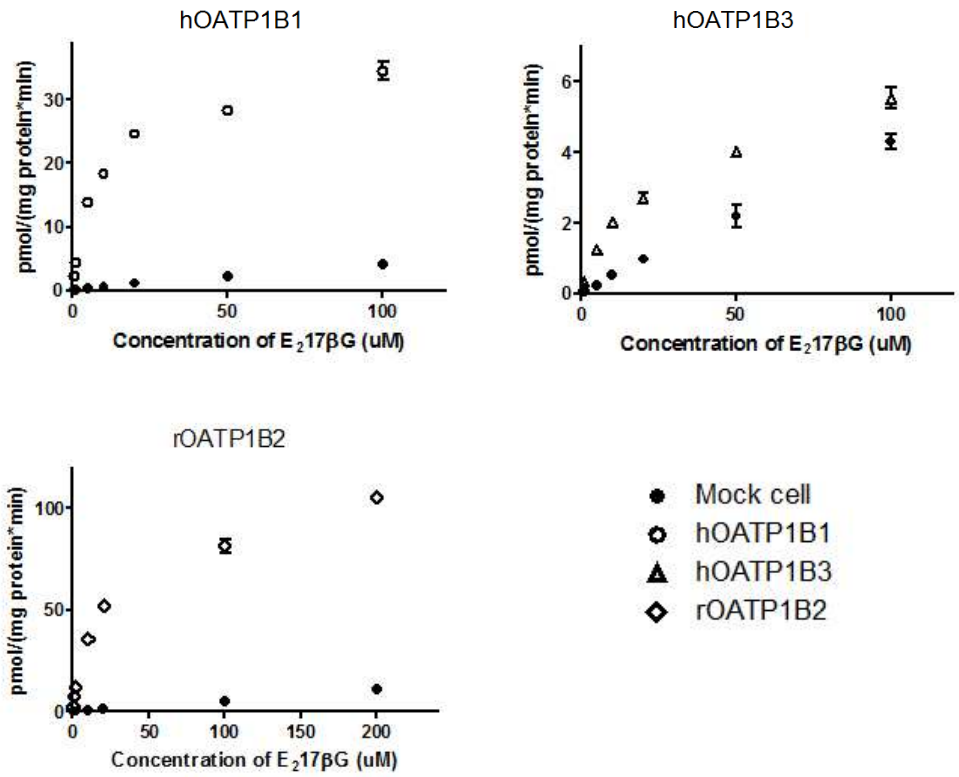

Figure 8. Concentration dependent uptake profile of $E_2$17βG in hOATP1B1-, hOAPT1B3- or rOATP1B2-expressing MDCKII cells

4.4 Statins inhibit transport of OATP1B subfamily probe substrate

Transport of the OATP1B subfamily probe substrate $E_2$17βG into hOATP1B1-, hOATP1B3- or rOATP1B2-expressing MDCKII cells were potently inhibited by statins (i.e., mevasatatin, pravastatin, lovastatin, simvastatin, atorvastatin, rosuvastatin, fluvastatin, pitavastatin) (Fig. 9 and 10). Uptake of $E_2$17βG into hOATP1B1-, hOATP1B3- or rOATP1B2-expressing MDCKII cells were determined in the presence of different statin concentrations. Among the eight statins uptake studies, four drugs were interacted with human and rat to a certain extent (Table 5). No functional difference was found for type I statins (i.e., pravastatin, mevastatin, simvastatin, lovastatin). The inhibition constants (K_i) for pravastatin were 24.06 ± 1.88, 40.14 ± 10.48, and 27.61 ± 8.16 μM in hOATP1B1-, hOATP1B3- and rOATP1B2- expressing cells, respectively. The K_i values for mevastatin were 8.97 ± 4.39, 11.90 ± 2.50, and 12.29 ± 5.51 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells, respectively. The K_i values for simvastatin were 10.61 ± 4.51, 8.52 ± 3.73 and 13.69 ± 6.04 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells, respectively. The K_i values for lovastatin were 12.70 ± 6.02, 14.63 ± 3.88, and 11.99 ± 5.06 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells, respectively. All of type II statins (i.e., fluvastatin, atorvastatin, rosuvastatin) and type III statins (pitavastatin) examined the functional difference. The K_i values for fluvastatin were 2.22 ± 0.35, 2.59 ± 0.42 and 4.15 ± 0.85 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells, respectively. The K_i values of fluvastatin in rOATP1B2 cells was significant

different from those with hOATP1B1. The K_i values for atorvastatin were 0.62 ± 0.34, 0.92 ± 0.16 and 2.05 ± 0.16 μM in hOATP1B1, hOATP1B3 and rOATP1B2 cells, respectively. The K_i value of atorvastatin in rOATP1B2 cells was significant different from those in hOATP1B1/hOATP1B3 cells. The K_i values for rosuvastatin were 6.99 ± 3.23, 10.14 ± 0.95 and 2.98 ± 1.07 μM in hOATP1B1, hOATP1B3, rOATP1B2 cells, respectively. The K_i values of rosuvastatin in rOATP1B2 cells was significant different from those in hOATP1B3 cells. The K_i values for pitavastatin were 1.07 ± 0.16, 0.91 ± 0.36 and 2.26 ± 0.57 μM in hOATP1B1, hOATP1B3, and rOATP1B2 cells, respectively. The K_i value of pitavastatin in rOATP1B2 cells was significant different from those in hOATP1B1/hOATP1B3 cells.

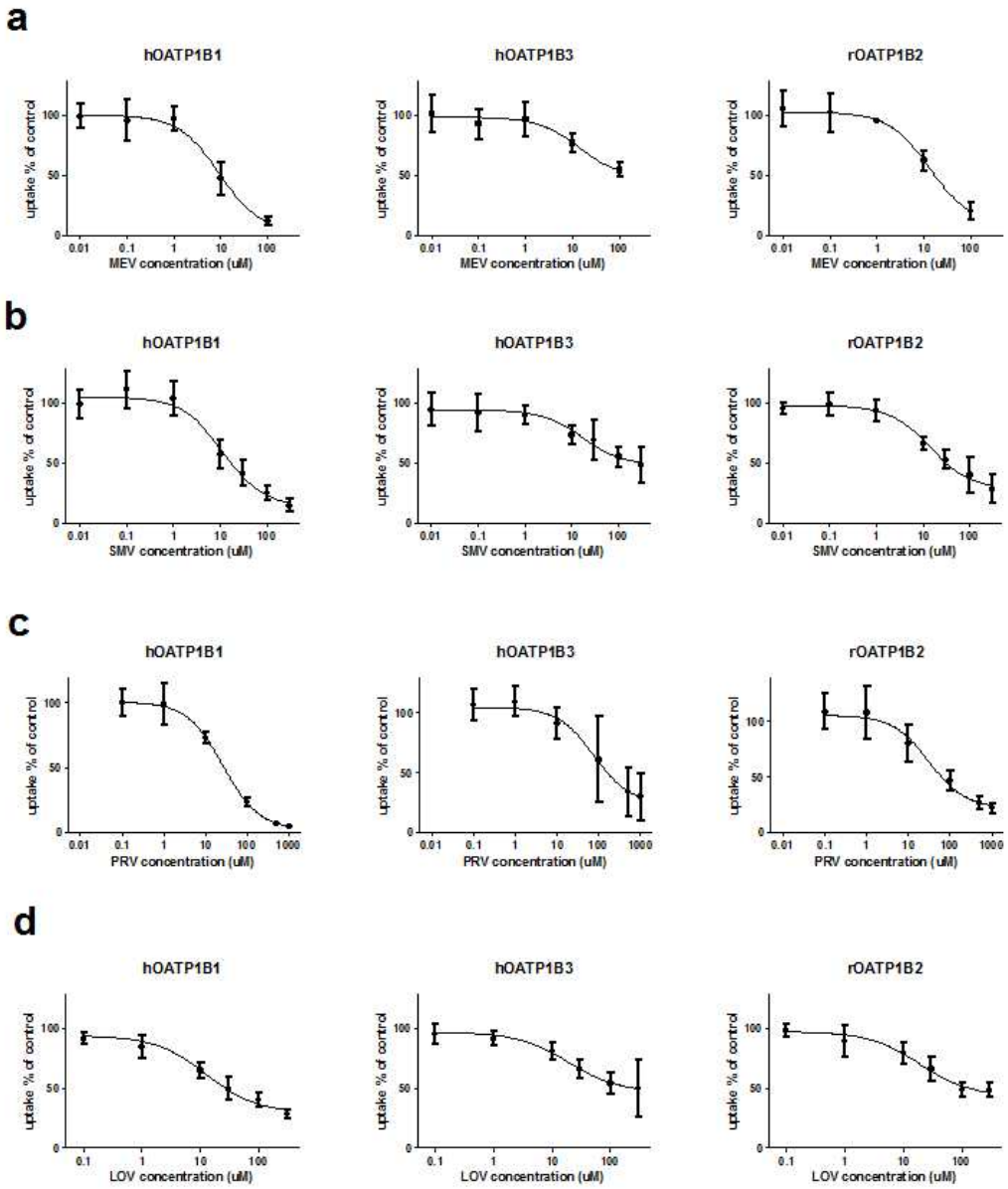

Figure 9. Effect of mevastatin (a), simvastatin (b), pravastatin (c), and lovastatin (d) as inhibitors on OATP1B subfamily-mediated uptake. Data are expressed as the percentage of control uptake measured in the absence of added inhibitors, and each result represents the mean ± SEM (n=7 - 10)

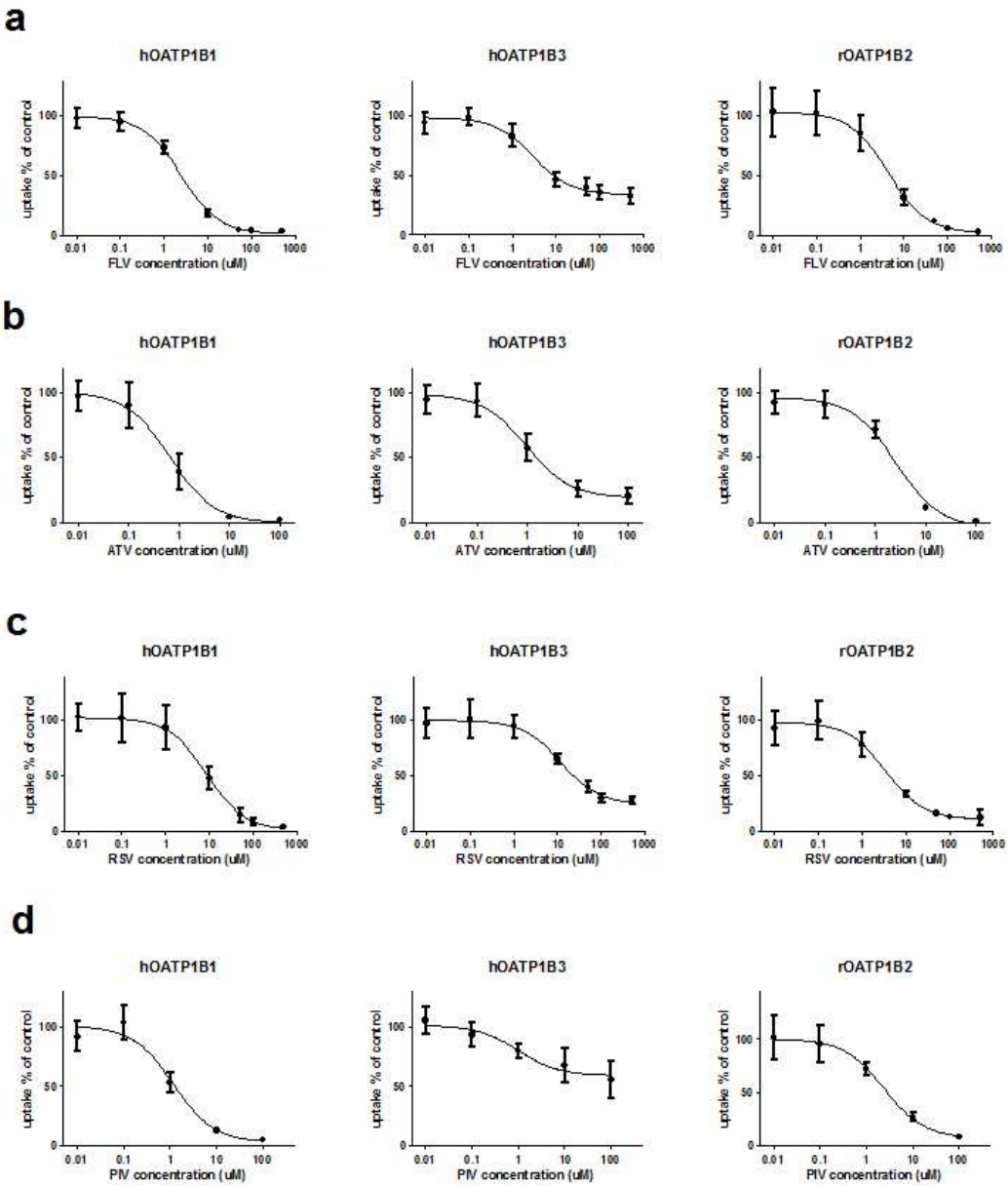

Figure 10. Effect of fluvastatin(a), atorvastatin (b), rosuvastatin (c), and pitavastatin (d) as inhibitors on OATP1B subfamily-mediated uptake

Table 5. K_i values (μM) of eight statins

		hOATP1B1	hOATP1B3	rOATP1B2
Type I	Mevastatin	8.97 ± 4.39	11.90 ± 2.50	12.29 ± 5.51
	Simvastatin	10.61 ± 4.51	8.52 ± 3.73	13.69 ± 6.04
	Pravastatin	24.06 ± 1.88	40.14 ± 10.48	27.61 ± 8.16
	Lovastatin	12.70 ± 6.02	14.63 ± 3.88	11.99 ± 5.06
Type II	Fluvastatin	2.22 ± 0.35*	2.59 ± 0.42	4.15 ± 0.85*
	Rosuvastatin	6.99 ± 3.23	10.14 ± 0.95	2.98 ± 1.07*
	Atorvastatin	0.62 ± 0.34**	0.92 ± 0.16#	2.05 ± 0.16**#
Type III	Pitavastatin	1.07 ± 0.16*	0.91 ± 0.36#	2.26 ± 0.57*#

Statically significant differences (*$p<0.05$ and **,#$p<0.01$)

5. Discussion & Conclusion

The purpose of this study was to express OATP1B transporters in MDCKII cells for the screening in the potential drug-drug-interaction at the level of transporters. In addition, since the literature is not entirely clear on species-difference in the function among OATP1B subfamily transporters, in specific human and rat, the difference in the inhibitory potential was compared for eight of statins, which commonly prescribed, in this study.

hOATP1B1-, hOATP1B3-, and rOATP1B2-expressing MDCKII cells used in this study were sufficient roles to transport the standard substrate, $E_2$17βG, into cells. The *in vitro* system expressing transporters showed being useful in the study of the specie-difference of the function of the OATP1B transporters.

This study showed that there is specie-difference of OATP1B transporters between human and rat and the extent of the specie-difference was summarized by comparing the respective K_i values. The functional differences among hOATP1B1, hOATP1B3, and rOATP1B2 were noted for four of eight statins studied. In particular, no functional difference was found for type I statins, while all type II and III statins examined the functional difference, suggesting that the kinetic differences among OATP1B transporters are depend on chemical structures of compounds. Interestingly, K_i values of type II and III statins are lower than that of type I statins. This result may be related to different affinity of statins with transporters based on structures of active sites.

Interpretation about results in this study can be explained by

different structures among types of statins. Type I statins shares structure of mevastatin. Lovastatin is derived from a fungus source and pravastatin and simvastatin are chemical modifications of lovastatin [11]. All type I statins are partially reduced naphthalene ring structures and have the butyryl group as a side chain. Type II statins have larger groups. Fluvastatin, atorvastatin and rosuvastatin have indole ring, pyrrole and pyrimidine based ring respectively. Also, type II and III statins have fluorophenyl group, which is the replacement of the butyryl group of type I statins. This fluorophenyl group is reported to have a role for additional polar interactions with HMG-CoA reductase, which enhance the effect on lowering blood cholesterol levels.

Interaction with transporters depends on structures of compound. Therefore, from this study, we can estimate that different aspects of OATP1B transporters between human and rat may have correlation with different groups between type I and II statins. Research about structure-based interaction between a drug and a transporter have to be studied further, and it can be useful to investigate basic different structure among OATP1B subfamily transporters.

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국문초록

신약개발에서는 인간을 대상으로 약의 유효성, 안전성을 평가하는 임상시험을 수행하기 전, 다양한 실험동물에 약물을 투여하여 체내 분포, 대사, 배설이 어떻게 진행되는지 알아보는 *in vivo*실험을 반드시 거쳐야 하며 (preclinical experimentation), 여기서 얻은 동물실험 결과를 바탕으로 신약 후보물질을 선정하여 임상시험으로 진행한다. 약물이 특정 조직으로 이동할 때 다양한 수송체가 관여하는데, 사람에서 발현되는 수송체는 실험동물에서 상당히 유사하긴 하지만 엄밀히 말하였을 때 생화학적으로 그 구조가 약간 다르다. 때문에, 종간에는 기질 특이성 / affinity 또는 발현량이 다를 수 있으며, 실제로 일부 문헌에서도 이러한 사실을 확인할 수도 있다. 약물의 체내동태는 수송체의 관여정도에 따라 상당히 좌우되기도 한다. 따라서 사람과 실험동물에서 특정 수송체의 활성이 다를 경우 기질이 되는 약물에 대해서 사람과 동물의 체내동태가 다를 것이다. 그런데 실험동물로 preclinical experimentation하는 이유는 사람과 동물과의 상관성을 상정한 것이므로 수송체 활성의 종차는 신약 개발의 심각한 문제가 된다고 할 것이다. 실제로 임상 시험에 진입한 물질이라고 하더라고 90% 정도의 약물은 개발에 실패한다. 따라서 rat과 human의 *in vitro* 자료의 상관성을 확립할 수 있다면, *in vivo* 상호작용 결과를 예측하는데 큰 도움이 될 것으로 기대된다.

간에 특이적으로 존재하는 hOATP1B1 수송체는 순환혈에서 간 조직내로 수송하는 기능을 담당한다. 일반적으로 사람에서 발현되는 수송체는 rattus norvegicus (이하 rat)의 것과 상당히 유사하다고 보는 것이 보통이다. 흥미롭게도 hOATP1B1과 rat 수송체의 ortholog 연구는 미미하다. 현재 hOATP1B1/1B3의 공통 ortholog로 rat의 rOATP1B2가 알려져 있으나, 몇 가지 기질에 대한 *in vitro* 연구만 수행된 바 있을 뿐이며 체계적이고 구체적인 종차를 연구한 보고는 찾아보기 어렵다.

hOATP1B1이 간으로의 약물 동태에 중요한 역할을 하는 것을 고려할 때, 이 종차 연구는 동물 실험 결과를 인간에게 적용할 때 생길 수 있는 문제점을 극복하는데 일조할 것이다.

이번 연구의 목표는 hOATP1B1, hOATP1B3, rOATP1B2 세 종류의 transporter가 발현된 in vitro system을 갖춘 후, 각각 transporter에 대해 statin계 약물이 표준 기질인 Estradiol-17β-glucuronide (이하 $E_2$17βG)의 수송을 얼마나 저해하는지 비교하는 것이었다. 즉, 각 statin에 대해 산출된 ED의 K_i값 비교를 통해 종차를 표현하는 것이었다.

MDCKII/FRT cell에 hOATP1B1, hOATP1B3이 발현된 cell은 실험실 보유 cell을 사용하였다. rOATP1B2를 같은 cell-line에 발현시키기 위하여 rOATP1B2를 cloning하여 pcDNA5/FRT vector에 삽입한 후, MDCKII/FRT wild type cell에 transfection시켰다. RT-PCR 결과와 표준기질 $E_2$17βG의 uptake가 대조군 대비 69배 높은 것을 통해 안정적으로 기능을 나타냄을 확인하였다. 구축한 cell-system으로 $E_2$17βG의 K_m값을 구하였고, hOATP1B1, hOATP1B3, rOATP1B2에서 각각 10.95 ± 6.38, 6.91 ± 5.23, 11.37 ± 5.41 μM 이었다. 현재 시판되는 총 8가지 statin계 약물 (atorvastatin, rosuvastatin, fluvastatin, pitavastatin, pravastatin, mevastatin, lovastatin, simvastatin)의 여러 농도에 대해 $E_2$17βG 1 μM의 uptake를 억제하는 정도를 % uptake로 나타내었다. 이 curve를 통해 IC_{50}값을 구하였고 Cheng-Prusoff equation으로 K_i값을 산출하였다. 세 번씩 반복한 실험으로 얻은 K_i값을 One way ANOVA 처리하여 transporter간 차이가 있는지 살펴보았다. 8가지 statin 중 type I statin 계열인 pravastatin, mevastatin, lovastatin, simvastatin에서는 종차가 보이지 않았으나, type II인 fluvastatin, atorvastatin, rosuvastatin과 type III statin인 pitavastatin에서는 rat의 rOATP1B2와 human의 hOATP1B1 또는 hOATP1B3간의 종차를 나타냈다. 생체막 약물수송체의 종간차이 결과를 보았을 때, 그 기능 차이가 약물의 구조에 따라서 크게 영향 받는 것으로 보인다. 구조적인 차이가 보다 명확하게 밝혀졌

을 때 종차연구는 더 나아갈 수 있을 것으로 생각되며, 이는 특정 약물 후보물질의 *in vitro in vivo correlation*을 예측할 때도 유용할 것이다.

주요어 : 생체막 약물수송체, hOATP1B1, hOATP1B3, rOATP1B2, statin, 종간차이, K_i
학 번 : 2015-23324