



Bosentan and Rifampin Interactions Modulate Influx Transporter and Cytochrome P450 Expression and Activities in Primary Human Hepatocytes

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Abstract

The incidence of polypharmacy-which can result in drug-drug interactions-has increased in recent years. Drug-metabolizing enzymes and drug transporters are important polypharmacy modulators. In this study, the effects of bosentan and rifampin on the expression and activities of organic anion-transporting peptide (OATP) and cytochrome P450 (CYP450) 2C9 and CYP3A4 were investigated *in vitro*. HEK293 cells and primary human hepatocytes overexpressing the target genes were treated with bosentan and various concentrations of rifampin, which decreased the uptake activities of OATP transporters in a dose-dependent manner. In primary human hepatocytes, *CYP2C9* and *CYP3A4* gene expression and activities decreased upon treatment with 20 μ M bosentan+200 μ M rifampin. Rifampin also reduced gene expression of *OATP1B1*, *OATP1B3*, and *OATP2B1* transporter, and inhibited bosentan influx in human hepatocytes at increasing concentrations. These results confirm rifampin- and bosentan-induced interactions between OATP transporters and CYP450.

Key Words: Drug-drug interaction, CYP450, OATP transporters, Rifampin, Bosentan

INTRODUCTION

Drug metabolism involves CYP450 and drug transporters in the liver. The CYP450 superfamily, which consists of at least 11 enzyme families, is involved in >80% of phase I-dependent drug metabolism (Ootokun, 2005). *CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, and *CYP3A4* are especially important for drug metabolism in humans (Wienkers and Heath, 2005). Drug transporters located in the membrane regulate drug absorption, distribution, and elimination by pumping molecules across cellular barriers (Guo *et al.*, 2011). OATP transporters are drug influx transporters that regulate the cellular uptake of a number of endogenous compounds and clinically important drugs (Kalliokoski and Niemi, 2009).

Combination therapy is a universal practice in patients with more than one disease or condition. However, the administration of two or more drugs can lead to drug-drug interactions when the metabolism of one drug is altered by another. Such

interactions can also occur when CYP450 expression or activity is altered by long-term use of a drug (Lin and Lu, 1998).

Bosentan has mainly been approved for the treatment of pulmonary arterial hypertension, but may also have beneficial effects on other conditions such as essential hypertension, chronic heart failure, and subarachnoid hemorrhage (Dingemans and van Giersbergen, 2004). Bosentan induces and is also metabolized by *CYP2C9* and *CYP3A4*, and is a substrate of drug influx transporters such as *OATP1B1*, *OATP1B3*, and *OATP2B1* (Treiber *et al.*, 2007).

Rifampin (also known as rifampicin) is an antibiotic used to treat various bacterial pathogens such as those causing tuberculosis, leprosy, and legionnaire's disease. Rifampin permeates into tissues and cells, including bacteria; however, the latter develop resistance to the drug at a high frequency (Campbell *et al.*, 2001). Rifampin is a potent inducer of *CYP2C9* and *CYP3A4*; its inhibition potentially affects drug influx transporters such as *OATP1B1*, *OATP1B3*, and *OATP2B1*

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Table 1. Information on cryopreserved human hepatocyte donors

	HMC505	HMC1026	HMC1051
Gender	Male	Male	Male
Age (year)	65	49	51
Race	Caucasian	Caucasian	Caucasian

(Williamson *et al.*, 2013).

The present study investigated the effects of drug-drug interactions on the expression and activity of CYP450 and drug influx transporters (OATP1B1, OATP1B3, and OATP2B1) in cryopreserved human hepatocytes. To evaluate this, bosentan and rifampin were used as a substrate and an inhibitor, respectively, of OATP transporters. The results suggest that bosentan influx by inhibition of OATP transporters alters CYP2C9 and CYP3A4 expression and activities.

MATERIALS AND METHODS

Reagents and chemicals

Rifampin, diphenhydramide hydrochloride (98%), dimethyl sulfoxide (DMSO), ammonium acetate, acetonitrile Chromasolv (ACN), formic acid, dexamethasone, diclofenac, thiazolyl blue tetrazolium bromide (MTT), and methanol Chromasolv were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bosentan was purchased from Actelion Pharmaceuticals (Allschwil, Switzerland). Cryopreserved human hepatocytes, HEK293 cells overexpressing OATP1B1, OATP1B3, and OATP2B1 transporter, HEK293 mock cells, phosphate-buffered saline (PBS), Gentest High Viability CryoHepatocyte Recovery kit, hepatocyte culture medium, ITS+ culture supplement, Dulbecco's Modification Eagle's Medium (DMEM), Minimal Essential Medium (MEM) non-essential amino acid solution, epidermal growth factor (EGF), and Hank's balanced salt solution (HBSS) were purchased from Corning (Corning, NY, USA). Penicillin/streptomycin, William's E medium (without phenol red), fetal bovine serum (FBS), L-glutamine, gentamycin sulfate, SYBR Green ER qPCR Supermix, and Superscript First-strand Synthesis kit were purchased from Life Technologies (Burlington, ON, Canada). Sodium butyrate solution and testosterone were purchased from EMD-Millipore (Darmstadt, Germany) and Acros (Geel, Belgium), respectively. Easy Spin Total RNA extraction kit was purchased from iNtRON (Seongnam, Korea).

Culture of cryopreserved human hepatocytes and OATP transporters-overexpressing cells

Three different cryopreserved human hepatocytes cell lines (Table 1) were thawed using the BD Gentest High Viability CryoHepatocyte Recovery kit. Cells were seeded in 24-well collagen I-coated plates (4×10^5 cells/well) and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 6 h, the medium was replaced with 500 µl of William's E medium supplemented containing 4 mM L-glutamine, 0.05 mg/ml gentamycin, 0.1 mM dexamethasone, ITS+ culture supplement, and 0.25 mg/ml Matrigel matrix, followed by incubation at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. Cells were maintained in hepatocyte culture medium supplemented with 5 µg EGF, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin.

HEK293 cells overexpressing OATP1B1, OATP1B3, and OATP2B1 transporters were thawed in DMEM containing 10% FBS, 5 mM MEM non-essential amino acids, and 100 U/ml penicillin-streptomycin and seeded in 24-well poly-D-lysine plates (4×10^5 cells/well), followed by incubation at 37°C in a low-humidity atmosphere of 5% CO₂. After 4 h, the medium of OATP1B1- and OATP1B3-overexpressing cell cultures was replaced with DMEM with 2 mM sodium butyrate, and the medium of OATP2B1-overexpressing cell cultures was replaced with DMEM (Jeong *et al.*, 2015).

Drug treatment

Hepatocytes were treated with bosentan (2, 10, 20, 100 µM) and rifampin (0, 2, 20, and 200 µM) for 48 h, with a medium change every 24 h. This was followed by treatment with rifampin only (2, 20, 50, 100, and 200 µM) for 48 h, with a medium change every 24 h. Control cells were treated with 0.1% (v/v) DMSO. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Inhibition of OATP transporter uptake

Uptake by OATP transporters was examined in OATP transporter-overexpressing HEK293 cells and hepatocytes. Cells overexpressing OATP1B1, OATP1B3, and OATP2B1 transporters were washed twice with HBSS and incubated in HBSS for 10 min at 37°C, followed by treatment with bosentan (20 µM) and rifampin (0, 0.01, 0.1, 1, 5, 10, 50, 100, and 500 µM for HEK293 cells and 0, 2, 20, 50, 100, and 200 µM for hepatocytes) in HBSS for 10 min at 37°C. Cells were washed three times with ice-cold HBSS, and 80% ACN was added for 20 min at room temperature to lyse the cells. The lysate was collected in 1.5-ml tubes and centrifuged at 14,000 rpm for 3 min at 4°C. A 5-µl volume of supernatant was injected into a 1260 Infinity high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UK-C18 column (2.1×50 mm, 3.0 µm). To confirm the effects of rifampin (inhibitor) on bosentan (substrate), the latter drug was quantified with a 6460 triple quadrupole mass spectrometer (Agilent Technologies). Elution was carried out using a mixture of mobile phases A (0.1% formic acid in distilled water) and B (0.1% formic acid in ACN). Analytes were separated for 10 min under the following gradient conditions: 95% mobile phase A:5% mobile phase B at 0-2 min; 5% mobile phase A:95% mobile phase B at 5-8 min; and 95% mobile phase A:5% mobile phase B at 8.10-10 min. Bosentan was observed for the transition of precursor to product ion at 552.2→202.0 m/z by multiple reaction monitoring (MRM). Half-maximal inhibitory concentrations (IC₅₀) of OATP transporters were calculated using GraphPad Prism v.5.01 software (GraphPad Inc., La Jolla, CA, USA).

Determination of human hepatocyte viability after bosentan and rifampin treatment

Cell viability was evaluated with the MTT assay (Han *et al.*, 2015). MTT (5 mg/ml) added to the cells for 4 h then removed. Cells were lysed in DMSO. Absorbance was measured at 570 nm using A SpectraMAX M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Total RNA extraction and purification

Total RNA was extracted using an RNA extraction kit according to the manufacturer's instructions. Briefly, hepato-

Table 2. Sequences of CYP2C9, 3A4 and OATP transporters primers used for qRT-PCR

Gene	Sequence (5'-3')	Product size (bp)
CYP2C9	F-cccttgggaagtggaccag	200
	R-acggtgccatcccttgactc	
CYP3A4	F-ccactctctcccagtgattgg	353
	R-gcgggcttctcttgaacac	
OATP1B1	F-actgtcttgcattgtctgg	346
	R-ggcaattccaacggtgttca	
OATP1B3	F-gtcacgtcattgcttgc	111
	R-caaccaacgagagtccttagg	
OATP2B1	F-tgggcacagaaaacacacct	265
	R-cggctgcacaaaatagctcac	
GAPDH	F-ccaactgcttagcaccctg	272
	R-gtcaccactgacacgttg	

cytes treated with drug were washed with PBS and 1 ml lysis buffer was added. Lysates were mixed with 200 μ l chloroform and the mixture was centrifuged at 14,000 rpm for 10 min at 4°C. A 400- μ l volume of the supernatant was mixed with 400 μ l binding buffer and centrifuged in a 1.5 ml tube equipped with spin column. After washing, total RNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis by reverse transcription

cDNA was synthesized using the SuperScript First-strand Synthesis kit according to the manufacturer's instructions. Briefly, a mixture consisting of 1 μ g RNA, 1 μ l of 50 μ M oligo(d)T, 1 μ l of 10 mM dNTP, and diethylpyrocarbonate (DEPC)-treated water was incubated at 65°C for 5 min, and 10 μ l of the mixture were combined with 10 μ l of cDNA synthesis mixture and reacted at 50°C for 50 min followed by 85°C for 5 min.

Quantitative real-time (qRT-)PCR analysis of CYP450 and OATP transporter expression

cDNA (1 μ g) was amplified on an IQ5 qRT-PCR detection system (Bio-Rad, Hercules, CA, USA). The reaction mixture consisted of 2 \times SYBR Green ER qPCR Supermix (10 μ l) and 20 pmol forward and reverse primers (1 μ l each) (Table 2). CYP2C9 and CYP3A4 primers were designed using FastPCR software (PrimerDigital, Helsinki, Finland). OATP transporter-specific primers have been previously described (Seki *et al.*, 2009; Fujiwara *et al.*, 2014). The reaction conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s; and a melting curve from 55°C-81°C at 1°C/30 s. Gene expression levels were quantified relative to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR amplification products were qualitatively evaluated by melting curve analysis (Perez *et al.*, 2003).

Determination of CYP450 enzymatic activity

CYP2C9 and CYP3A4 enzymatic activity was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Hepatocyte culture medium containing bosentan and rifampin was removed from plates, which were rinsed with PBS. Hepatocyte culture medium containing CYP450 substrates

(100 μ M diclofenac for CYP2C9 and 200 μ M testosterone for CYP3A4) was added to the hepatocyte monolayers to measure enzymatic activity at 37°C in a humidified atmosphere of 5% CO₂ for 60 min. At the end of the incubation period, 100 μ l of reaction medium were added to 25 μ l of stop solution consisting of 10 mM ammonium acetate in methanol and 5 μ l of the internal standard consisting of 50 ng/ml diphenhydramine.

A 5- μ l volume of reaction mixture was injected into a 1260 Infinity HPLC system equipped with a poroshell SB C18 column (2.1 \times 100 mm, 2.7 μ m). Quantification of 4-hydroxydiclofenac and 6- β -hydroxytestosterone was carried out on a 6460 triple quadrupole mass spectrometer. Elution was carried out with a mixture of mobile phase A (10 mM ammonium acetate in distilled water) and mobile phase B (10 mM ammonium acetate in methanol). Analytes were separated for 10 min under the following gradient conditions: 95% mobile phase A:5% mobile phase B at 0-2 min; 5% mobile phase A:95% mobile phase B at 5-7 min; and 95% mobile phase A:5% mobile phase B at 7.10-10 min. 4-Hydroxydiclofenac, 6- β -hydroxytestosterone, and the internal standard (diphenhydramine) were observed by MRM for the transition of precursor to product ions at m/z 312.0 \rightarrow 230.0, 305.3 \rightarrow 269.2, and 256.2 \rightarrow 167.1, respectively. Enzymatic activity was calculated using the following formula.

$$\text{Enzyme activity} = \frac{\text{Calculated concentration } (\mu\text{M}) \times \text{Incubation volume } (\mu\text{l})}{0.3 \times \text{Confluency } (10^6 \text{ cells per well}) \times \text{Incubation time } (\text{min})}$$

Statistical analysis

Data are expressed as mean \pm SEM and were evaluated by one-way ANOVA analysis of variance with Bonferroni's post hoc test using Sigma Plot v.13.0 software (Systat Software Inc., San Jose, CA, USA). Statistical significance was set as $p < 0.05$. Values were compared to those of the control or to samples treated with 20 μ M bosentan.

RESULTS

Inhibition of OATP transporter uptake of bosentan by rifampin

To evaluate the effects of inhibiting uptake by OATP transporters, we measured uptake in HEK293 cells overexpressing OATP1B1, OATP1B3, and OATP2B1 transporter that were treated with bosentan and rifampin for 10 min. Uptake by all three transporters decreased as a function of rifampin concentration (Fig. 1). Values ranged from 0.370-0.104 pmol/10⁶ cells per well/min for OATP1B1; 0.130-0.034 pmol/10⁶ cells per well/min for OATP1B3; and 0.410-0.142 pmol/10⁶ cells per well/min for OATP2B1. There was no uptake by HEK293 mock-treated cells. The IC₅₀ values of bosentan for OATP1B1, OATP1B3, and OATP2B1 transporter were 3.79, 3.41, and 20.74 μ M, respectively.

Cytotoxicity of bosentan and rifampin

To evaluate cytotoxicity of bosentan and rifampin, human hepatocytes from three donors were treated with bosentan and rifampin for 48 h. These drugs had no effect on cell viability when administered individually (Fig. 2A, 2B) or in combination (Fig. 2C).

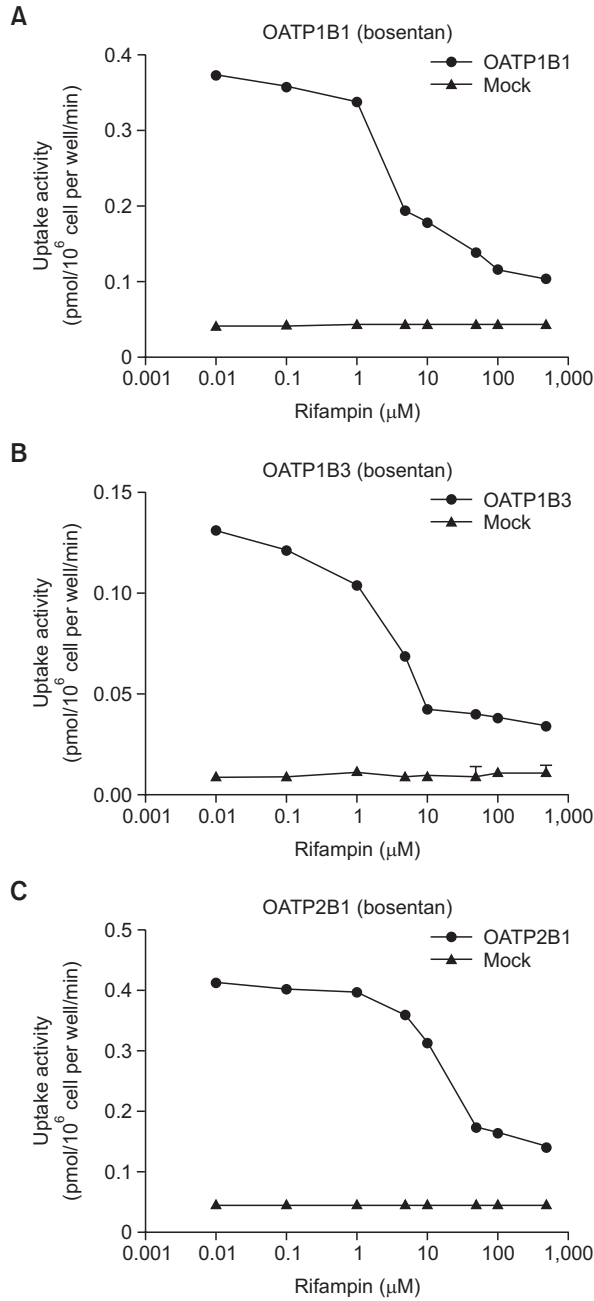


Fig. 1. Inhibition of uptake by OATP transporters in OATP-overexpressing HEK293 cells. Uptake by (A) OATP1B1, (B) OATP1B3, and (C) OATP2B1 was measured in HEK293 cells treated with bosentan (20 μM) and rifampin (0, 0.01, 0.1, 1, 5, 10, 50, 100, and 500 μM). Data are expressed as the mean bosentan uptake activity ± SEM of three replicates.

Effect of bosentan and rifampin on CYP2C9 and CYP3A4 levels

To assess the effects of combined bosentan and rifampin treatment, human hepatocytes from three donors were co-treated with bosentan and rifampin for 48 h. *CYP2C9* gene expression was upregulated by 2.38-, 3.38-, and 3.03-fold in HMC505, HMC1026, and HMC1051 cells, respectively, compared to the control levels upon treatment with 20 μM

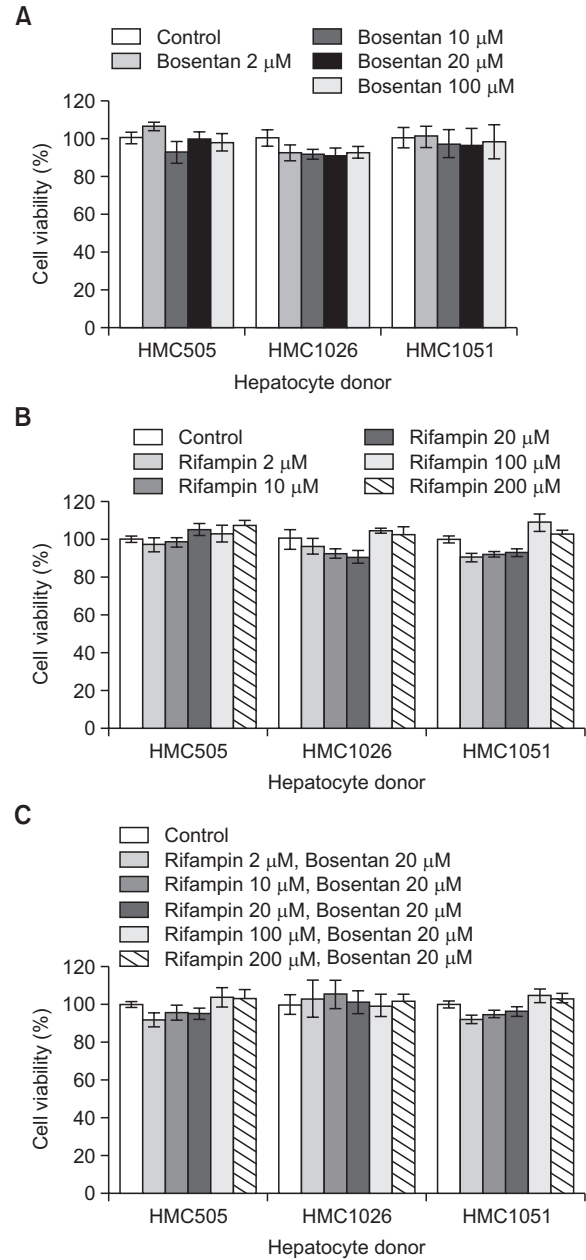


Fig. 2. Cytotoxicity of bosentan and rifampin in human hepatocytes. Cytotoxicity of bosentan (A), rifampin (B) and bosentan+rifampin (C) was evaluated in hepatocytes treated with bosentan (2, 10, 20, and 100 μM), rifampin (0, 2, 10, 20, 100 and 200 μM), or bosentan (20 μM)+rifampin (0, 2, 10, 20, 100 and 200 μM). Data are expressed as the mean cytotoxicity ± SEM of three replicates.

bosentan. The increase was greater in cells co-treated with 20 μM bosentan+2 or 20 μM rifampin than in those treated with bosentan only. For example, *CYP2C9* levels were 3.91-, 4.44-, and 5.54-fold higher in HMC505, HMC1026, and HMC1051 cells, respectively, in the presence of 20 μM bosentan+20 μM rifampin relative to the values reported for the 20 μM bosentan-only group. However, *CYP2C9* levels were only 1.04-, 2.14-, and 1.48-fold higher, respectively, upon co-treatment with 20

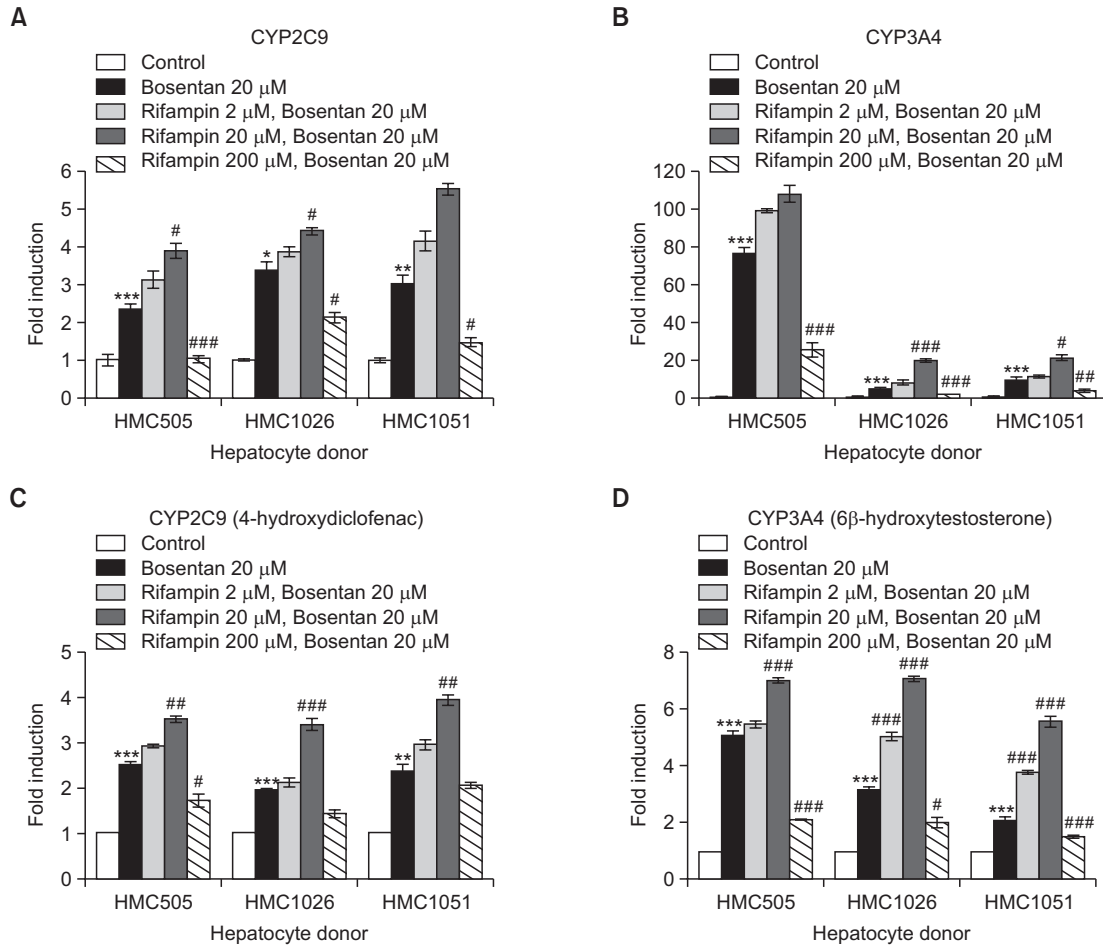


Fig. 3. Effect of bosentan and rifampin on CYP450 expression and activity. The mRNA level of CYP2C9 (A) and CYP3A4 (B) in hepatocytes was determined by qRT-PCR. Enzymatic activity of CYP2C9 (C) and CYP3A4 (D) was evaluated in hepatocytes treated with bosentan (20 μM) and rifampin (0, 2, 20, and 200 μM). Data are expressed as the mean fold induction of mRNA or enzyme activity ± SEM of three replicates. ***p*<0.01, ****p*<0.001 vs. control; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 vs. 20 μM bosentan.

μM bosentan+200 μM rifampin (Fig. 3A). Similar trends were observed for CYP3A4: gene expression was upregulated by 77.00-, 5.75-, and 10.41-fold in HMC505, HMC1026, and HMC1051 cells upon treatment with 20 μM bosentan relative to the values reported for the control group and was increased by 107.73-, 19.96-, and 21.39-fold, respectively, in the presence of 20 μM bosentan+ 20 μM rifampin as compared to that observed in the presence of 20 μM bosentan. However, expression was downregulated by 25.77-, 2.03-, and 4.08-fold, respectively, upon co-treatment with 20 μM bosentan+ 200 μM rifampin (Fig. 3B).

The enzymatic activity of CYP2C9 was increased by 2.51-, 1.94-, and 2.36-fold in HMC505, HMC1026, and HMC1051 cells by treatment with 20 μM bosentan relative to the control values. Compared to treatment with 20 μM bosentan, co-administration of 20 μM bosentan+20 μM rifampin increased the activity in these cells by 3.51-, 3.39-, and 3.93-fold, respectively. However, co-treatment with 20 μM bosentan+200 μM rifampin decreased the activity by 1.72-, 1.44-, and 2.06-fold, respectively, relative to that reported for the 20 μM bosentan group (Fig. 3C). Similar trends were observed for CYP3A4 activity, which was increased by 5.06-, 3.15-, and 2.06-fold

in HMC505, HMC1026, and HMC1051 cells, respectively, by 20 μM bosentan treatment as compared the values reported for the untreated cells. The activity was further increased by 6.97-, 7.01-, and 5.51-fold upon co-administration of 20 μM bosentan+20 μM rifampin relative to that observed for the 20 μM bosentan-only group. However, the activity was decreased by 2.08-, 1.98-, and 1.50-fold, respectively, as compared to that in cells treated with 20 μM bosentan+200 μM rifampin (Fig. 3D).

Effect of rifampin on CYP2C9 and CYP3A4 levels

CYP2C9 and CYP3A4 levels were downregulated by treatment with 20 μM bosentan combined with 200 μM rifampin. The effects of rifampin were investigated in HMC505, HMC1026, and HMC1051 hepatocytes treated with rifampin for 48 h. CYP2C9 expression increased in all cell lines upon treatment with 0, 2, 20, and 50 μM rifampin; for example, at 50 μM rifampin, the expression level was 3.86-, 2.89-, and 3.17-fold higher in HMC505, HMC1026, and HMC1051 cells, respectively, than that in controls. However, rifampin concentrations of 100 and 200 μM inhibited CYP2C9 expression; at 200 μM, the levels were 1.31-, 1.62-, and 1.40-fold lower in

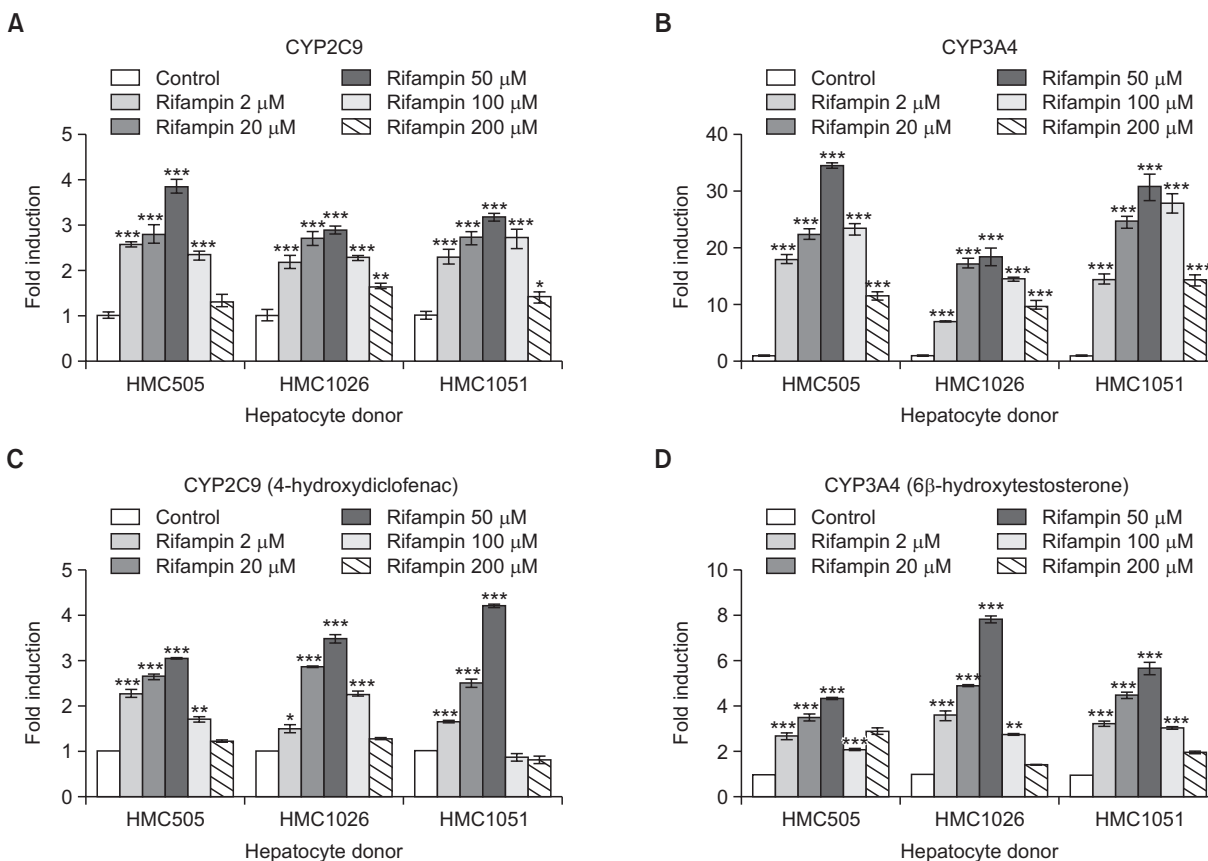


Fig. 4. Effect rifampin on CYP450 expression and activity. The mRNA level of CYP2C9 (A) and CYP3A4 (B) in hepatocytes was determined by qRT-PCR. Enzymatic activity of CYP2C9 (C) and CYP3A4 (D) was evaluated in hepatocytes treated with rifampin (0, 2, 20, 50, 100, and 200 μM). Data are expressed as the mean fold induction of mRNA or enzyme activity \pm SEM of three replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

HMC505, HMC1026, and HMC1051 cells, respectively, than in the control group (Fig. 4A). Similar trends were observed for CYP3A4: for example, at 50 μM rifampin, the expression level was 34.63-, 18.39-, and 30.83-fold lower, respectively, in the three cell lines relative to that observed for the controls, whereas the levels were 11.47-, 9.81-, and 14.30-fold lower, respectively, in the presence of 200 μM rifampin (Fig. 4B).

CYP2C9 activity increased in all donor hepatocytes treated with 0, 2, 20, and 50 μM rifampin. At 50 μM rifampin, the levels were 3.05-, 3.47-, and 4.20-fold higher in HMC505, HMC1026, and HMC1051 cells, respectively, than control levels. However, 100 and 200 μM concentrations suppressed activity; at 200 μM , the levels were 1.21-, 1.27-, and 0.81-fold lower, respectively, than that in control cells (Fig. 4C). CYP3A4 activity showed similar trends; at 50 μM rifampin, activity increased by 4.28-, 7.80-, and 5.66-fold in HMC505, HMC1026, and HMC1051 cells, respectively, compared to control values; however, at 200 μM , activity decreased by 1.14-, 1.40-, and 1.96-fold, respectively (Fig. 4D).

Effect of bosentan and rifampin on OATP transporters

We evaluated the effect of bosentan and rifampin on OATP transporters' gene expression in hepatocytes treated with rifampin for 48 h. *OATP1B1*, *OATP1B3*, and *OATP2B1* transporter gene expression levels were downregulated relative to

that in controls in a rifampin concentration-dependent manner in three donor hepatocytes. At 200 μM rifampin, OATP1B1 gene expression levels were 0.52-, 0.42-, and 0.67-fold lower; OATP1B3 levels were 0.40-, 0.47-, and 0.39-fold lower; and OATP2B1 levels were 0.41-, 0.14-, and 0.50-fold lower in HMC505, HMC1026, and HMC1051 cells, respectively, as compared to the values for the respective control groups (Fig. 5).

We assessed the effects of bosentan and rifampin on drug uptake by OATP transporters in human hepatocytes treated with 20 μM bosentan+200 μM rifampin for 48 h. Bosentan uptake by OATP transporters decreased to 276.62, 68.21, and 86.88 pmol/ 10^6 cell per well/min in HMC505, HMC1026, and HMC1051 cells, respectively, in a rifampin concentration-dependent manner relative to that in cells treated with 20 μM bosentan alone (Fig. 6).

DISCUSSION

We studied drug-drug interactions between CYP450 and OATP transporters in cryopreserved human hepatocytes treated with bosentan and rifampin. Previous studies have reported that the maximum serum concentration (C_{max}) of bosentan is 2.5 μM (Sasayama *et al.*, 2005). Another study showed that the unbound affinity constant (K_{m}) of bosentan is 22.5 μM

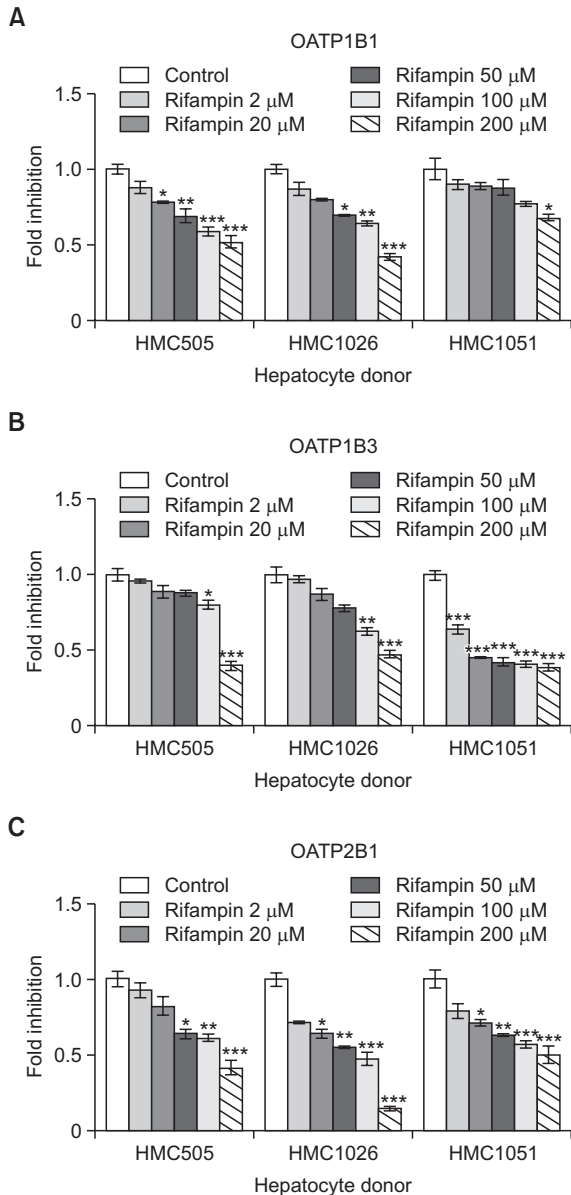


Fig. 5. Effect of rifampicin on OATP transporter gene expression. Expression levels of (A) OATP1B1, (B) OATP1B3, and (C) OATP2B1 were evaluated by qRT-PCR in hepatocytes treated with rifampicin (0, 2, 20, 50, 100, and 200 μM). Data are expressed as the mean fold induction of mRNA \pm SEM of three replicates. * p <0.05, ** p <0.01, *** p <0.001 vs. control.

in human hepatocytes (Menochet *et al.*, 2012). To conclusively establish inhibitor-substrate interaction effects, we used 20 μM bosentan ($10 \times C_{\text{max}}$), which yielded sufficient influx through OATP transporters as endogenous substrates.

Co-treatment with bosentan and rifampicin resulted in a temporary increase in bosentan influx concentrations; rifampicin also inhibited bosentan uptake into cells overexpressing OATP1B1 and OATP1B3 in a concentration-dependent manner, and its IC_{50} values for OATP1B1 and OATP1B3 were 3.2 and 1.6 μM , respectively (van Giersbergen *et al.*, 2007). This is similar to our results, which showed that rifampicin IC_{50} values

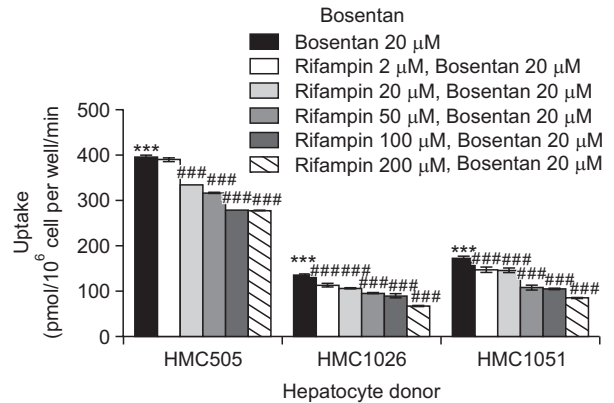


Fig. 6. Inhibition of drug uptake by OATP transporters in hepatocytes. Cells were treated with bosentan (20 μM) and rifampicin (0, 2, 20, 50, 100, and 200 μM). Data are expressed as the mean bosentan uptake activity \pm SEM of three replicates. *** p <0.001 vs. control; ### p <0.001 vs. 20 μM bosentan.

for OATP1B1 and OATP1B3 transporter were 3.79 and 3.41 μM , respectively, in HEK293 cells overexpressing these transporters and treated with bosentan and rifampicin.

CYP2C9 and CYP3A4 enzyme activity was increased by treatment with 20 μM bosentan+2 or 20 μM rifampicin, but was decreased by 20 μM bosentan+200 μM rifampicin in hepatocytes (Fig. 3). However, high concentrations of rifampicin and bosentan+rifampicin were not toxic to human hepatocytes (Fig. 2), consistent with a previous report (Zhang *et al.*, 2012).

Unexpectedly, the effects of treatment with rifampicin alone were similar to those of bosentan and rifampicin co-treatment (Fig. 3, 4). CYP2C9 and CYP3A4 were inhibited by a high concentration of rifampicin, which acts as both OATP transporters substrate and inhibitor. High rifampicin concentrations also inhibited OATP transporters, thereby blocking the influx of both rifampicin and bosentan into cells. This was confirmed by analyzing OATP transporters gene expression and bosentan uptake by the OATP transporters in hepatocytes (Fig. 5, 6).

In conclusion, we found that OATP transporter gene expression as well as bosentan uptake decreased as a function of rifampicin concentration. Our findings suggest that rifampicin and bosentan interact to modulate the expression and activities of CYP450 and OATP transporters, which should be taken into account when prescribing both drugs to patients.

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REFERENCES

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A. and Darst, S. A. (2001) Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell* **104**, 901-912.
 Dingemans, J. and van Giersbergen, P. L. (2004) Clinical pharmacology of bosentan, a dual endothelin receptor antagonist. *Clin. Pharmacokinet.* **43**, 1089-1115.
 Fujiwara, R., Takenaka, S., Hashimoto, M., Narawa, T. and Itoh, T.

- (2014) Expression of human solute carrier family transporters in skin: possible contributor to drug-induced skin disorders. *Sci. Rep.* **4**, 5251.
- Guo, L., Dial, S., Shi, L., Branham, W., Liu, J., Fang, J. L., Green, B., Deng, H., Kaput, J. and Ning, B. (2011) Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. *Drug Metab. Dispos.* **39**, 528-538.
- Han, J. Y., Ahn, S. Y., Yoo, J. H., Nam, S. Y., Hong, J. T. and Oh, K. W. (2015) Alleviation of kainic acid-induced brain barrier dysfunction by 4-o-methylhonokiol in *in vitro* and *in vivo* models. *Biomed Res. Int.* **2015**, 893163.
- Jeong, H. U., Kwon, M., Lee, Y., Yoo, J. S., Shin, D. H., Song, I. S. and Lee, H. S. (2015) Organic anion transporter 3- and organic anion transporting polypeptides 1B1- and 1B3-mediated transport of caltalposide. *Drug Des. Devel. Ther.* **9**, 643-653.
- Kalliokoski, A. and Niemi, M. (2009) Impact of OATP transporters on pharmacokinetics. *British journal of pharmacology* **158**, 693-705.
- Lin, J. H. and Lu, A. Y. (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Br. J. Pharmacol.* **35**, 361-390.
- Menochet, K., Kenworthy, K. E., Houston, J. B. and Galetin, A. (2012) Use of mechanistic modeling to assess interindividual variability and interspecies differences in active uptake in human and rat hepatocytes. *Drug Metab. Dispos.* **40**, 1744-1756.
- Oforokun, I. (2005) Sex differences in the pharmacologic effects of antiretroviral drugs: potential roles of drug transporters and phase 1 and 2 metabolizing enzymes. *Top. HIV Med.* **13**, 79-83.
- Perez, G., Tabares, B., Jover, R., Gomez-Lechon, M. J. and Castell, J. V. (2003) Semi-automatic quantitative RT-PCR to measure CYP induction by drugs in human hepatocytes. *Toxicol. In Vitro* **17**, 643-649.
- Sasayama, S., Kunieda, T., Tomoike, H., Matsuzaki, M., Shirato, K., Kuriyama, T., Izumi, T., Origasa, H., Giersbergen, P. L., Dingemans, J. and Tanaka, S. (2005) Effects of the endothelin receptor antagonist bosentan on hemodynamics, symptoms and functional capacity in Japanese patients with severe pulmonary hypertension. *Circ. J.* **69**, 131-137.
- Seki, S., Kobayashi, M., Itagaki, S., Hirano, T. and Iseki, K. (2009) Contribution of organic anion transporting polypeptide OATP2B1 to amiodarone accumulation in lung epithelial cells. *Biochim. Biophys. Acta* **1788**, 911-917.
- Treiber, A., Schreiber, R., Hausler, S. and Stieger, B. (2007) Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. *Drug Metab. Dispos.* **35**, 1400-1407.
- van Giersbergen, P. L., Treiber, A., Schreiber, R., Dietrich, H. and Dingemans, J. (2007) Inhibitory and inductive effects of rifampin on the pharmacokinetics of bosentan in healthy subjects. *Clin. Pharmacol. Ther.* **81**, 414-419.
- Wienkers, L. C. and Heath, T. G. (2005) Predicting *in vivo* drug interactions from *in vitro* drug discovery data. *Nat. Rev. Drug Discov.* **4**, 825-833.
- Williamson, B., Dooley, K. E., Zhang, Y., Back, D. J. and Owen, A. (2013) Induction of influx and efflux transporters and cytochrome P450 3A4 in primary human hepatocytes by rifampin, rifabutin, and rifapentine. *Antimicrob. Agents Chemother.* **57**, 6366-6369.
- Zhang, Z. H., Tang, J. H., Zhan, Z. L., Zhang, X. L., Wu, H. H. and Hou, Y. N. (2012) Cellular toxicity of isoniazid together with rifampicin and the metabolites of isoniazid on QSG-7701 hepatocytes. *Asian Pac. J. Trop. Med.* **5**, 306-309.