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 μM bosentan+200 μM 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 (Ofotokun, 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 (Kallikokski 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 (Dingemanse and van Giersbergen, 2004). Bosentan induces and is also metabolized by CYP2C9 and CYP3A4; its inhibition potentially affects 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.
(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 CYP-P2C9 and CYP3A4 expression and activities.

**MATERIALS AND METHODS**

**Reagents and chemicals**

Rifampin, diphenhydramide hydrochloride (98%), dimethyl sulfoxide (DMSO), ammonium acetate, acetoni trile Chloromosol (ACN), formic acid, dexamethasone, diclofenac, thiazolyl blue tetrazolium bromide (MTT), and methanol Chloromosol 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 hepatocyte 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% CO<sub>2</sub> in a low-humidity atmosphere of 5% CO<sub>2</sub>. 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% CO<sub>2</sub>.

**Inhibition of OATP transporter uptake**

Uptake by OATP transporters was examined in OATP transport-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 (IC<sub>50</sub>) 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, hepa-
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 μM diclofenac for CYP2C9 and 200 μM 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 μl of reaction medium were added to 25 μl of stop solution consisting of 10 mM ammonium acetate in methanol and 5 μl of the internal standard consisting of 50 ng/ml diphenhydramine. A 5-μl volume of reaction mixture was injected into a 1260 Infinity HPLC system equipped with a poroshell SB C18 column (2.1×100 mm, 2.7 μm). 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). Analyses 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→230.0, 305.3→269.2, and 256.2→167.1, respectively. Enzymatic activity was calculated using the following formula:

\[
\text{Enzyme activity} = \frac{\text{Calculated concentration (μM) × Incubation volume (μl)}}{0.3 \times \text{Conflueny (10⁶ cells per well) × Incubation time (min)}}
\]

Statistical analysis

Data are expressed as mean ± 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 μM 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 OATP1B3; and 0.410-0.142 pmol/10⁶ cells per well/min 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 μM, 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).

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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 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 μM bosentan and rifampin.
The enzymatic activity of CYP2C9 was increased by 2.51-, 1.94-, and 2.36-fold in HMC505, HMC1026, and HMC1051 cells, respectively, upon co-treatment 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 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).

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).

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.
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 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).

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 (Cmax) of bosentan is 2.5 μM (Sasayama et al., 2005). Another study showed that the unbound affinity constant (Ku) of bosentan is 22.5 μM.
in human hepatocytes (Menochet et al., 2012). To conclusively establish inhibitor-substrate interaction effects, we used 20 μM bosentan (10×C_{max}), which yielded sufficient influx through OATP transporters as endogenous substrates.

Co-treatment with bosentan and rifampin resulted in a temporary increase in bosentan influx concentrations; rifampin 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 rifampin IC_{50} values for OATP1B1 and OATP1B3 transporter were 3.79 and 3.41 μM, respectively, in HEK293 cells overexpressing these transporters and treated with bosentan and rifampin.

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

Unexpectedly, the effects of treatment with rifampin alone were similar to those of bosentan and rifampin co-treatment (Fig. 3, 4). CYP2C9 and CYP3A4 were inhibited by a high concentration of rifampin, which acts as both OATP transporters substrate and inhibitor. High rifampin concentrations also inhibited OATP transporters, thereby blocking the influx of both rifampin 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 rifampin concentration. Our findings suggest that rifampin 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


Fujiwara, R., Takenaka, S., Hashimoto, M., Narawa, T. and Itoh, T.


