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Influence of sildenafil and tadalafil on the enzyme- and transporter-inducing effects of bosentan and ambrisentan in LS180 cells

Johanna Weiss *, Dirk Theile, Adriana Spalwisz, Jürgen Burhenne, Klaus-Dieter Riedel, Walter Emil Haefeli

Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

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ABSTRACT

The combinations of the endothelin-1 receptor antagonists bosentan or ambrisentan with the phosphodiesterase 5 inhibitors sildenafil or tadalafil are current standard therapies of advanced pulmonary arterial hypertension. However, these drugs have a number of drug interactions. Changes of bosentan pharmacokinetics by sildenafil are attributed to reduced hepatic uptake as a consequence of inhibition of organic anion transporting polypeptides. We therefore tested in vitro the hypothesis that sildenafil and tadalafil reduce the enzyme- and transporter-inducing effects of bosentan or ambrisentan by preventing cellular access. Although intracellular concentrations of bosentan and ambrisentan (measured by high pressure liquid chromatography coupled with tandem mass-spectrometry) after four days of incubation of LS180 cells were lower when sildenafil or tadalafil were present, quantification of mRNA expression in these cells by real-time reverse transcription polymerase chain reaction revealed that bosentan and ambrisentan-mediated induction was stable or even increased in combination with sildenafil or tadalafil. For the drug transporter P-glycoprotein this was confirmed at the protein and functional level with highly significant correlations between P-gp mRNA, protein, and function. Moreover, using a reporter gene assay in LS180 cells, our study demonstrates for the first time that tadalafil is a potent, ambrisentan a weak, and sildenafil no activator of pregnane X receptor. In conclusion, our study demonstrates that although sildenafil and tadalafil indeed reduce intracellular concentrations of bosentan and ambrisentan in LS180 cells, they do not mitigate the inducing effects of these endothelin-1 receptor antagonists.

1. Introduction

The combination of endothelin-1 receptor antagonists (ERAs) and phosphodiesterase 5 inhibitors (PDE5Is) is a standard in current treatment guidelines of advanced pulmonary arterial hypertension (PAH) [1,2]. Currently the ERAs ambrisentan and bosentan and the PDE5Is sildenafil and tadalafil are approved for the treatment of PAH. The kinetics of these compounds share some but not all pathways and thus their drug interaction potential markedly differs. The metabolism of ambrisentan is mainly determined by uridine diphosphate glucuronosyltransferases (UGTs). Only about 20% of ambrisentan undergoes oxidative metabolism mainly by cytochrome P450 (CYP) 3A4 and to a lesser extent by CYP3A5 and CYP2C19 [3]. Hepatic uptake of ambrisentan is presumably mediated by organic anion transporting polypeptides (OATPs/SLCOs) [4]. In contrast, for bosentan the dominant role of OATPs for its hepatic uptake is well defined; systemic exposure depends on the activity of OATP1B1 and OATP1B3 [5] and subsequent oxidative metabolism through CYP3A4 and CYP2C9 [6]. Moreover and in contrast to ambrisentan, which is only a weak activator of, the nuclear pregnane X receptor (PXR) [7], bosentan is a potent PXR ligand that induces CYP3A and CYP2C9 and thus not only its own metabolism [6] but also metabolism and dose requirements of numerous other CYP3A and CYP2C9 substrates [8]. PDE5Is are substrates of CYP3A4 and therefore co-administration of bosentan reduces their exposure by ~50% [9,10], a phenomenon which is not observed with ambrisentan [11,12]. As a perpetrator, sildenafil may inhibit OATP1B transporters and increase bosentan exposure by 50% whereas it only slightly interferes with ambrisentan [11]. In contrast, tadalafil lacks clinically relevant perpetrator properties and thus causes only minor changes of ERA kinetics [10,12].

Taken together, these findings indicate that within the two drug classes drug interactions markedly differ suggesting that exposures of ERAs and PDE5Is will differ depending on the particular combination.

* Corresponding author. Tel.: +49 6221/56 39402; fax: +49 6221/56 4642.
E-mail address: johanna.weiss@med.uni-heidelberg.de (J. Weiss).

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In vitro evidence suggests that the accumulation of bosentan observed in combination with sildenafil in vivo might be caused by reduced hepatic uptake as a consequence of inhibition of OATPs [5]. This type of interaction therefore modifies the distribution of ERAs and may thus increase their access to the site of action (vascular wall) while concurrently reducing their inducing and inhibiting effects in the hepatocyte. We have previously demonstrated in a comprehensive in vitro investigation that bosentan is a moderate to strong inductor of many drug transporters and drug metabolising enzymes, whereas ambrisentan only weakly induces some of the enzymes and transporters investigated [13].

We now hypothesised that reduced uptake of bosentan into the cells could lead to diminished gene induction and investigated whether sildenafil and tadalafil reduced the inducing effects of bosentan in vitro. Although clinical evidence indicates that the interaction between ambrisentan and PDE5Is is less pronounced or even absent [11,12] we also investigated the impact of sildenafil and tadalafil on the inducing properties of ambrisentan.

The results of our study demonstrate that although sildenafil and tadalafil indeed reduce intracellular concentrations of bosentan and ambrisentan in LS180 cells, they do not mitigate the inducing effects of the ERAs.

2. Materials and methods

2.1. Materials

Culture media, medium supplements, dimethyl sulfoxide (DMSO), Hanks' balanced salt solution (HBSS), phosphate-buffered saline, aprotinin, rhodamine123, ammonium acetate, and anti-β-actin (Clone AC-74) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Foetal calf serum (FCS), and G418 were purchased form PAA (Pasching, Austria). Crystal violet, TRIS (2-amino-2-(hydroxymethyl)-propan-1,3-diol), sodium dodecyl sulphate (SDS), glycerol, Tween® 20, dithiothreitol (DTT), ethylene diamine tetraacetic acid (EDTA), Triton® X-100, and rifampicin were from AppliChem (Darmstadt, Germany). 8-Fluorescein-cAMP (8-FcA) was purchased from BIOLOG Life Science Institute (Bremen, Germany). Methanol, acetonitrile, and Rotiphorese® gel 30 were obtained from Roth (Karlsruhe, Germany). Cell culturing bottles and white 96-well plates with clear bottom for luminometry were supplied by Greiner (Frickenhausen, Germany) and 96-well microtiter plates were supplied by Nunc (Wiesbaden, Germany). Collagen R was obtained from Serva (Heidelberg, Germany), RNeasy Mini-Kit from Qiagen (Hilden, Germany), Pefabloc from Serva (Heidelberg, Germany), and leupeptin, pepstatin, and bromphenol blue from Biomol (Hamburg, Germany). The BCA® Protein Assay Kit was obtained from Pierce (Rockford, USA). Nitrocellulose membranes (Optitran BA-S 85) were obtained from Schleicher & Schuell BioScience (Dassel, Germany). Slim-Fast® was obtained from Allpharm (Messel, Germany). The antibody against human P-glycoprotein (P-gp/*ABCB1*) clone C219 and cyclosporine A were obtained from Calbiochem (Darmstadt, Germany). The Dual-Glo™ Luciferase Assay System, the pGL4.21 vector, the pGL4.74 [hRluc/TK] renilla vector, and FuGENE® HD Transfection Reagent were obtained from Promega Corporation (Madison, WI, USA). The NR1I2 (NM_003889) Human cDNA TrueClone® (pCMV6-XL4 vector containing the cDNA of PXR *NR1I2*) was obtained from OriGene (Rockville, MD, USA). Bosentan, sildenafil, and tadalafil were purchased from Sequoia Research Products (Pangbourne, United Kingdom). D10-ambrisentan was purchased from AlsaChim (Strasbourg, France) and D4-bosentan was obtained from Toronto Research Chemicals Inc. (North York, Canada). Ambrisentan was kindly provided by GlaxoSmithKline (Stevenage, United Kingdom) and LY335979 (zosuquidar) was a kind gift of Eli Lilly (Bad Homburg, Germany).

2.2. Stock solutions

Stock solutions of cyclosporine A (10 mM), ambrisentan, bosentan, tadalafil, and rifampicin (100 mM) were prepared in DMSO. Stock solution of sildenafil was prepared in aqua bidest (5 mM). In the induction assays the concentration of DMSO was limited to 0.05% and medium with 0.05% DMSO was used as a negative control.

2.3. LS180 cells

The human colon adenocarcinoma cell line LS180 (available at ATCC, Manassas, VA, USA) was used for induction experiments as a surrogate for the intestine being a major site of drug interactions [13–16]. Moreover, this cell line has already been used for characterising the inducing properties of ambrisentan and bosentan alone [13]. Cells were cultured under standard cell culture conditions with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate, and 0.1 mM non-essential amino acids.

2.4. Determination of the concentrations used for the induction experiments

For bosentan and ambrisentan we used the highest previously tested concentration for induction (50 μM) which also corresponds to the concentration with the maximum effect on mRNA expression [13]. For sildenafil and tadalafil we first defined concentrations devoid of significant modification of LS180 cell proliferation (determination of IC_{20} in a proliferation assay, see also Section 2.5). For sildenafil the IC_{20} was 5.6 μM and for tadalafil 20.5 μM. Therefore and also to ensure OATP1B1 inhibition (IC_{50} of sildenafil for inhibition of OATP1B1 mediated bosentan transport in vitro is 1.5 μM [5]) we chose 5 μM for sildenafil. For tadalafil we used 20 μM ensuring survival of 80% of the cells.

2.5. Growth inhibition assay

Proliferation was quantified by crystal violet staining as described previously with minor modifications [17]. In brief, a 100 μl aliquot of each cell suspension at a concentration of 3×10^5 cells/ml was seeded onto collagen-coated 96-well microtiter plates and incubated for 24 h. Medium was substituted for drug-containing medium and the cells were incubated for further 48 h. After incubation with test compounds, the cells were washed once with PBS and viable adherent cells were stained with 50 μl of 0.5% (w/v) crystal violet in 20% methanol in aqua bidest per well for 15 min. After staining, plates were washed with aqua bidest and dried for 4 h in a drying chamber at 37 °C or at room temperature for 12 h. To dissolve crystal violet 200 μl of methanol were added to each well. Absorption was measured at 555 nm excitation). Proliferation was expressed as proliferation index, which was calculated as the absorption intensity of the test well in percentage points relative to zero proliferation (absorption of wells containing only medium set to 0%) and native proliferation (absorption intensity of untreated control cells set to 100%). Each experiment was performed at least in triplicate with $n = 8$ wells for each concentration. Concentration–response curves and IC_{20} values were calculated by GraphPad Prism version 5.02 (GraphPad Software Inc., La Jolla, CA, USA) according to a sigmoid E_{max} model.

2.6. Induction assay

LS180 cells were seeded in 25 cm^2 (functional analysis) or 75 cm^2 (mRNA and western blot analysis) culturing flasks and