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SHORT COMMUNICATION

Interaction potential of the endothelin-A receptor antagonist atrasentan with drug transporters and drug-metabolising enzymes assessed in vitro

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Abstract
Purpose Atrasentan is a highly potent and selective endothelin receptor A (ET_A) antagonist under development for the treatment of prostate cancer. Only little data exist on its interaction with drug-metabolising enzymes and drug transporters possibly influencing its safety and effectiveness. Our study evaluated whether atrasentan can induce the expression of relevant human drug transporters and cytochrome P450 isozymes (CYPs), whether it retains its efficiency in multidrug resistant cell lines, and whether it inhibits P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP).
Methods Induction of transporters and enzymes was quantified at the mRNA level by real-time RT-PCR in LS180 cells and for P-gp also at the protein level by Western blot. P-gp inhibition was evaluated by calcein assay in P388/dx and L-MDR1 cells and BCRP inhibition in MDCKII-BCRP cells by pheophorbide A efflux. Substrate characteristics were evaluated by growth inhibition assays in MDCKII cells overexpressing particular ABC-transporters.
Results Atrasentan profoundly induced several CYPs and drug transporters (e.g. 12-fold induction of CYP3A4 at 50 μM). It was a moderate P-gp inhibitor (IC_{50} in P388/dx cells = 15.1 ± 1.6 μM) and a weak BCRP inhibitor (IC_{50} in MDCKII-BCRP cells = 59.8 ± 11 μM). BCRP or P-gp overexpressing cells were slightly more resistant towards antiproliferative effects of atrasentan.
Conclusions Our data provide a comprehensive analysis of the induction profile of atrasentan and its interaction with P-gp and BCRP. The profound induction effects stress the need for thorough assessment of its interaction potential in vivo.

Keywords Atrasentan · Endothelin receptor A antagonist · CYPs · Drug transporters · Induction · Drug-drug interaction

Introduction

Atrasentan is a highly potent and selective endothelin receptor A (ET_A) antagonist being developed for chemotherapy of hormone-refractory prostate cancer and other malignancies [1, 2]. Efficacy of chemotherapeutic drugs is often limited in tumours with multidrug-resistance (MDR), for which one major cause is the overexpression of ATP-binding cassette (ABC-) transporters like P-glycoprotein (P-gp, encoded by *ABCB1*) or breast cancer resistance protein (BCRP, encoded by *ABCG2*) [3]. Moreover, safety and effectiveness of cytostatic drugs might be influenced by drug–drug interactions. Inhibition and induction of enzymes and drug transporters involved in the clearance and distribution of drugs may critically reduce or increase exposure with their substrates and thus lead to nonresponse or toxic side effects.

So far there are only little data on the interaction of atrasentan with drug-metabolising enzymes and drug transporters, and there are no data at all whether atrasentan retains its efficacy in MDR.

Preclinical data demonstrate that atrasentan is extensively metabolised by glucuronidation and oxidation mainly by cytochrome P450 3A4 (CYP3A4) [1, 4]. Moreover in vitro data indicate that atrasentan is a substrate

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of P-gp and the organic anion transporting polypeptides 1B1 (OATP1B1) and OATP1B3 [4, 5] and the relevance of OATP1B1 for atrasentan kinetics has been assessed and confirmed in a clinical study [5]. However, atrasentan's interaction potential is only partially elucidated, and potential transporter and enzyme inducing properties relevant for anticancer therapy are unknown.

We therefore investigated (1) whether atrasentan can induce the expression of relevant human drug transporters, CYPs, and the induction mediating transcription factor pregnane X receptor (PXR), (2) whether it retains its efficiency in MDR cell lines, and (3) whether it can inhibit P-gp and BCRP.

Materials and methods

Materials

The sources of supply of all materials were published previously [6, 7].

Cytotoxicity assay

Atrasentan was tested for cytotoxic effects prior to P-gp and BCRP inhibition assays with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). Inhibition assays were conducted with maximum concentrations that have not more than 30% cytotoxicity in the respective cell line.

P-gp inhibition assay (calcein uptake assay)

The calcein assay was used to assess P-gp inhibition in L-MDR1 cells and P388/dx cells. The cell lines were obtained and cultivated, and the assay was conducted and validated as described previously [8–10]. Each concentration was tested in octuplet, and each experiment was performed at least in triplicate.

BCRP inhibition assay (pheophorbide A flow cytometry efflux assay)

Flow cytometric ABCG2 inhibition assays with MDCKII and overexpressing MDCKII-BCRP cells were conducted as described and validated previously [11] and performed in triplicate.

Induction assay

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 [6, 12]. Cells were cultured as described previously [6]. For the induction experiments, LS180 cells were seeded in 75-cm^2 culturing flasks and incubated for 3 days. Cells were then treated with culture medium containing atrasentan (1–50 μM) in quadruplicate for 4 consecutive days. Rifampicin (10 μM) and bosentan (50 μM) served as positive controls and culture medium with 0.05% DMSO as a negative control. Cells were harvested; the cell pellet was split for mRNA and Western blot analysis.

Growth inhibition assay

Growth inhibition assays in LS180 cells were conducted to determine suitable maximum concentrations for the induction assay without profound antiproliferative effects. Proliferation was quantified by crystal violet staining as described previously [13]. Each experiment was performed at least in triplicate with $n = 8$ wells for each concentration.

Growth inhibition assays in MDCKII, LLC-PK1, and PA317 cells and their ABC-transporter overexpressing counterparts (MDCKII-MDR1, MDCKII-BCRP, L-MDR1, and PA317-BCRP) were conducted to evaluate whether atrasentan sustains its efficacy in multidrug resistant cell lines. Overexpressing cell lines were kindly provided by Dr. A. H. Schinkel and Dr. P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands; MDCK and LLC-PK1) and by Dr. T. Efferth (University of Mainz, Germany; PA317-BCRP) and cultured as published previously [6, 8, 11].

Quantification of mRNA expression by real-time RT-PCR

RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). cDNA was synthesised with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany) according to the manufacturer's instructions. mRNA expression was quantified by real-time RT-PCR with the LightCycler® 480 (Roche Applied Science, Mannheim, Germany), as described previously [6, 7]. All samples were amplified in duplicate, and the following genes were quantified: *CYP3A4, CYP3A5, CYP2C19, CYP2C9, ABCB1, ABCB11, ABCC2, ABCG2, SLCO1B1, SLCO1B3, SLCO2B1*, and *PXR*.

Western blot analysis

Protein expression of P-gp was analysed in triplicate by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting as described previously [6]. Blots were semi quantified by ImageJ 1.43u (NIH, Bethesda, Maryland, USA).

Statistical analysis

Data were analysed using GraphPad Prism Version 5.02 and InStat Version 3.06 (GraphPad Software, San Diego, CA, USA). The differences in mRNA expression following incubation with the compounds investigated compared with the respective vehicle controls were tested using ANOVA with Dunnett's post hoc test. The differences of the IC_{50} values obtained in the growth inhibition assays were tested using two-tailed t test. $P \leq 0.05$ was considered significant.

Results

Induction of CYPs, drug transporters, and PXR

Growth inhibition assays in LS180 cells demonstrated that atrasentan does not have any anti-proliferative effects up to 50 μM. Thus, induction experiments were performed with 1, 5, 10, and 50 μM. Atrasentan profoundly induced *CYP3A4, CYP3A5, ABCB1, ABCB11*, and *SLCO1B3* (Fig. 1). Smaller inducing effects were observed also for *ABCG2, SLCO1B1*, and *PXR*. *SLCO2B1* was slightly repressed and no change in mRNA expression was observed for *CYP2C9*, *CYP2C19,* and *ABCC2* (data not shown).

P-gp induction by atrasentan was also confirmed at the protein level by Western blot analysis. Semiquantitative analysis revealed a 1.9-fold induction of P-gp by 50 μM atrasentan (compared to 2.7-fold induction by 10 μM rifampicin).

Inhibition of P-gp

Atrasentan moderately inhibited P-gp in P388/dx cells with an IC_{50} of 15.1 ± 1.6 μM (for comparison: IC_{50} for the strong P-gp inhibitor quinidine in this cell system is 3.9 μM [14]). In the LLC system, IC_{50} could not be calculated, because atrasentan not only increased calcein fluorescence in L-MDR1 but also in LLC-PK1 cells indicating unspecific effects or MRP inhibition [15].

Inhibition of BCRP

Atrasentan concentration-dependently increased pheophorbide A fluorescence in MDCKII-BCRP but not in MDCKII cells, indicating BCRP inhibition. The IC_{50} for BCRP inhibition was 59.8 ± 11.0 μM, which is only weak compared with the strong inhibitor fumitremorgin C (IC_{50} = 0.7 μM [16]).

Efficacy in MDR cell lines

Growth inhibition assays in MDCKII, MDCKII-BCRP, and MDCKII-MDR1 cells consistently demonstrated that BCRP and P-gp overexpressing cells are more resistant towards antiproliferative effects of atrasentan than the native cell line (IC_{50}-values: MDCKII cells = 85.4 ± 4.2 μM, MDCKII-BCRP = 105.7 ± 11 μM ($P < 0.05$), MDCKII-MDR1 = 132.3 ± 6.5 μM ($P < 0.001$); LLC-PK1 = 109.5 ± 10.6 μM, L-MDR1 = 258.5 ± 5.3 μM ($P < 0.001$); PA317 = 151.4 ± 32.9 μM, PA317-BCRP = 224.7 ± 7.9 μM ($P < 0.01$)).

Discussion

For the ET_A receptor antagonist atrasentan, which is currently in phase III of clinical development, the safety profile concerning its potential for drug–drug interactions and its efficiency in tumours with MDR have not been defined in detail yet. We therefore investigated the impact of atrasentan on the expression of efflux and uptake transporters and CYPs relevant for antineoplastic activity and drug interactions and studied its interaction with the two most relevant efflux transporters contributing to MDR.

Analysis of atrasentan's inducing properties revealed that it significantly induced several drug transporters and CYP3A4/CYP3A5 but for most genes only at the highest concentration tested (50 μM). Therapeutic plasma concentrations of atrasentan are very low (C_{max} at steady state = 190 nM–1.6 μM) and protein binding of atrasentan is high (98.8%) [1]. However, experimental and clinical evidence clearly indicates that atrasentan is an OATP1B1 and OATP1B3 substrate [4, 5] likely leading to accumulation in hepatocytes. Therefore, concentrations inside liver cells might be high enough to trigger induction. Moreover, concentrations in the intestine estimated according to the formula published by the FDA [17] are between 73 μM (after intake of 10 mg) and 548 μM (after intake of 75 mg) substantially exceeding effective in vitro concentrations and thus suggesting that at two major sites of drug interactions relevant concentrations will accumulate. So far there is only one clinical study investigating the impact of atrasentan on the pharmacokinetics of the CYP3A substrate midazolam demonstrating no effect of atrasentan on midazolam pharmacokinetics [18]. These data suggest that induction of CYP3A4 and CYP3A5 does either not occur in vivo or that it is compensated by inhibitory effects, which in vitro already occurred at lower concentrations than induction (IC_{50} about 3 μM) [18]. However, this earlier study assessed midazolam kinetics after 4–6 days exposure to atrasentan which is likely not sufficiently long to allow full development of CYP induction [19]. More

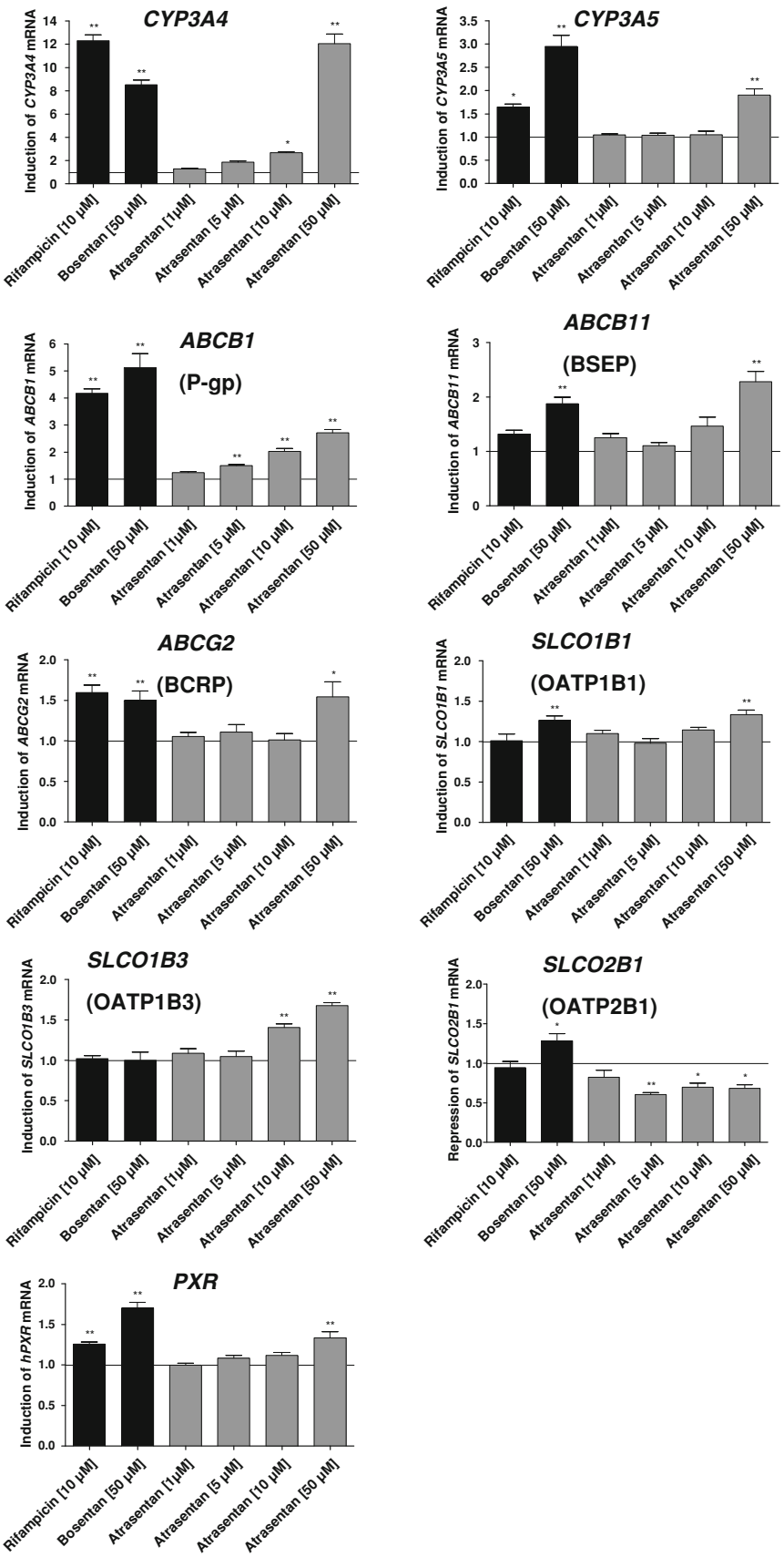

Fig. 1 Concentration-dependent effect of atrasentan (1–50 µM), 10 µM rifampicin and 50 µM bosentan (positive controls) after 4 days on mRNA expression in human colon adenocarcinoma cells (LS180) compared to untreated medium control. Expression data were normalised to the housekeeping gene *villin*. Data are expressed as mean ± SEM for $n = 12$ (4 biological replicates and 3 PCR runs for every sample). $^{*}P < 0.05$, $^{**}P < 0.01$. Bosentan responses have been reported previously [7]

importantly, the inducing effect of a combined inhibitor/inducer of a common target can only be correctly quantified immediately after its discontinuation when the longer-lasting CYP induction is unmasked by the withdrawal of the inhibitor [20]. Thus, our studies stress the need to perform dedicated interaction studies.

At present, the mechanism of induction by atrasentan is not elucidated. However, induction of *CYP3A4* and *ABCB1* suggests that atrasentan is a PXR ligand like the ET-receptor antagonist bosentan [21]. Moreover, our data demonstrate that atrasentan also induces the mRNA expression of PXR. Similarly, induction of *ABCB11* and *SLCO1B1* suggests that atrasentan is a farnesoid X receptor (FXR) ligand [22, 23].

Aside from induction, we also demonstrated that atrasentan is a moderate inhibitor of P-gp and a weak inhibitor of BCRP. Whereas for BCRP clinical interaction data are lacking, atrasentan acted as a moderate inhibitor of intestinal P-gp also in vivo as shown during co-administration of fexofenadine and its IC_{50} has been reported to be about 12 μM thus supporting our data [24]. Moreover, this clinical study demonstrated that P-gp inhibition appears to be more relevant in vivo than P-gp induction, because atrasentan significantly increased fexofenadine bioavailability by ~50% arguing for inhibition of intestinal P-gp.

Our results for the first time demonstrate that atrasentan might not retain its full efficacy in patients with established MDR. P-gp or BCRP overexpressing cell lines were slightly more resistant towards the antiproliferative effects of atrasentan, indicating that it is a weak substrate for both efflux transporters. In contrast, a previous study reported atrasentan to be no P-gp substrate, but this discrepancy might be explained by differences in the applied methods, which have not yet been reported in detail [24].

Limitations: (1) We only investigated induction in LS180 cells, which are well established for this purpose [6, 7, 12]. However, the extent of induction might be different in other cell lines and cannot be transferred one-to-one to the in vivo situation. (2) In the majority of cases, variations in transporter mRNA levels are indeed translated into changes of the corresponding protein or altered function [6, 25–27]. We therefore chose to prove the association of mRNA and protein expression only for P-gp, i.e. the transporter with the most significant induction by atrasentan.

In conclusion, our data provide a comprehensive analysis of the induction profile of atrasentan and its interaction with P-gp and BCRP. At the highest concentration tested, atrasentan exerted similar or even greater induction effects compared with rifampicin, stressing the need for thorough assessment of its interaction potential in vivo.

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Conflict of interest None.

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