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Short communication

Potential of the novel antiretroviral drug rilpivirine to modulate the expression and function of drug transporters and drug-metabolising enzymes in vitro

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ABSTRACT

The objective of this study was to assess the drug–drug interaction potential of the new non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine in vitro. The following were evaluated: P-glycoprotein (P-gp/ABCB1) inhibition by calcein assay; breast cancer resistance protein (BCRP/ABCG2) inhibition by pheophorbide A efflux; and inhibition of organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 by 8-fluorescein–cAMP uptake. Inhibition of cytochrome P450 enzymes was assessed using commercially available kits. Substrate characteristics were evaluated by growth inhibition assays in MDCKII cells overexpressing particular ABC transporters. Induction of drug-metabolising enzymes and transporters was quantified by real-time RT-PCR in LS180 cells, and activation of pregnane X receptor (PXR) by a reporter gene assay. Rilpivirine significantly inhibited P-gp (IC_{50} = 13.1 ± 6.8 μmol/L), BCRP (IC_{50} = 1.5 ± 0.3 μmol/L), OATP1B1 (IC_{50} = 4.1 ± 1.8 μmol/L), OATP1B3 (IC_{50} = 6.1 ± 0.9 μmol/L), CYP3A4 (IC_{50} = 1.3 ± 0.6 μmol/L), CYP2C19 (IC_{50} = 2.7 ± 0.3 μmol/L) and CYP2B6 (IC_{50} = 4.2 ± 1.6 μmol/L). Growth inhibition assays indicate that rilpivirine is not a substrate of P-gp, BCRP, or multidrug resistance-associated proteins 1 and 2. In LS180 cells, rilpivirine induced mRNA expression of *ABCB1*, *CYP3A4* and *UGT1A3*, whereas *ABCC1*, *ABCC2*, *ABCG2*, *OATP1B1* and *UGT1A9* were not induced. Moreover, rilpivirine was a PXR activator. In conclusion, rilpivirine inhibits and induces several relevant drug-metabolising enzymes and drug transporters, but owing to its low plasma concentrations it is most likely less prone to drug–drug interactions than older NNRTIs.

1. Introduction

Rilpivirine is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) with unique resistance and tolerability profiles [1]. It has been licensed since 2011 in the USA and 2012 in Europe for combination therapy in antiretroviral-naïve patients with human immunodeficiency virus 1 (HIV-1) infection. Rilpivirine is mainly metabolised by cytochrome P450 (CYP) 3A, explaining substantial changes in exposure when co-administered with CYP3A4 inducers or inhibitors [1–3]. However, thus far data on its drug–drug interaction potential are insufficient and, except for some data deposited at the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), no published information on the perpetrator characteristics of rilpivirine are available.

We therefore thoroughly characterised the interaction potential of rilpivirine by assessing in vitro: (i) its ability to inhibit P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), organic anion transporting polypeptide (OATP) 1B1 and 1B3, and CYP3A4, CYP2B6, CYP2C19 and CYP2D6; (ii) possible transport by P-gp, BCRP and the multidrug resistance-associated proteins (MRP) 1 and 2; and (iii) its ability to induce the expression of drug-metabolising enzymes and drug transporters and to activate pregnane X receptor (PXR).

2. Materials and methods

2.1. Materials

Culture media, medium supplements, dimethyl sulphoxide (DMSO), phosphate-buffered saline (PBS) and anti-β-actin (Clone AC-74) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum and G418 (Geneticin®) were purchased from PAA (Pasching, Austria). Crystal violet and rifampicin were purchased from AppliChem GmbH (Darmstadt, Germany) and calcein acetoxymethyl ester was purchased from Invitrogen (Karlsruhe, Germany). 8-Fluorescein–cAMP (8-FcA-cAMP) was purchased from BIOLOG Life Science Institute (Bremen, Germany) and pheophorbide A (PhA) was from Frontier Scientific Europe (Carnforth, UK). Methanol was obtained from Roth (Karlsruhe, Germany). Cell culturing bottles and white 96-well plates with a clear bottom and clear lid for luminometry were supplied by Greiner (Frickenhausen, Germany) and 96-well microtitre plates

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by Nunc (Wiesbaden, Germany). The antibody against human P-glycoprotein (P-gp/ABCB1) clone C219 was 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 Corp. (Madison, WI). The NR1I2 (NM_003889) Human cDNA TrueClone® (pCMV6-XL4 vector containing cDNA of the PXR gene *NR1I2*) was obtained from OriGene (Rockville, MD). Rilpivirine was purchased from Sequoia Research Products (Pangbourne, UK).

2.2. Stock solution

Stock solutions (100 μmol/L) of rilpivirine and rifampicin were prepared in DMSO. The maximum DMSO concentration in the inhibition assays was limited to 0.1%, a concentration that has previously been demonstrated to have no effect in these assays.

2.3. Transporter inhibition assays

P-gp inhibition in L-MDR1 cells was assessed using calcein acetoxymethyl ester. Flow cytometric BCRP inhibition was quantified using PhA in MDCKII-BCRP cells as published previously [4]. Inhibition of OATP1B1 and OATP1B3 was analysed by flow cytometric uptake of 8-FcA as described previously [5] but using the human embryonic kidney cell line HEK293 stably transfected with OATP1B1 (HEK-OATP1B1), OATP1B3 (HEK-OATP1B3) or the empty control vector (HEK293-VC-G418) [6,7]. Each experiment was performed at least three times on different days after exclusion of cytotoxic effects of rilpivirine in the respective cell lines (Cytotoxicity Detection Kit; Roche Applied Science, Mannheim, Germany) up to 100 μmol/L. Concentrations leading to 50% inhibition (IC_{50} values) were calculated according to the standard sigmoidal concentration–response equation using GraphPad Prism v5.02 (GraphPad Software Inc., La Jolla, CA).

2.4. Inhibition of CYP3A4, CYP2C19, CYP2B6 and CYP2D6

Inhibition studies were performed with the Vivid® CYP3A4 Green Screening Kit and the Vivid® CYP2D6 Blue Screening Kit (Invitrogen) and the CYP2C19/CEC and CYP2B6/CEC High Throughput Inhibitor Screening Kit (Becton Dickinson Biosciences, Heidelberg, Germany) according to the manufacturers' instructions. The screening kits contain fluorogenic substrates and the respective recombinant CYP. The fluorogenic substrates are blocked dyes yielding minimal fluorescence signal until cleaved by the enzyme. Rilpivirine was analysed for its capacity to inhibit production of the fluorescent signal. Eight concentrations (up to 100 μmol/L) were tested in at least three independent experiments. IC_{50} values were calculated according to the standard sigmoidal concentration–response equation using GraphPad Prism v5.02.

2.5. Growth inhibition assay

Growth inhibition assays with LS180 cells [8] were used to determine optimum concentrations for the induction assay, thus avoiding significant antiproliferative effects. Rilpivirine inhibited the growth of LS180 cells with an IC_{50} of 8.9 ± 1.4 μmol/L ($IC_{20} = 1.4 \pm 0.4$ μmol/L). The maximum concentration for induction was set to 2 μmol/L, thus ensuring ca. 80% viable cells. Growth inhibition assays in MDCKII cells overexpressing human *ABCB1*, *ABCG2*, *ABCC1* and *ABCC2* were used as a surrogate for substrate characteristics of rilpivirine [4]. Each experiment was performed at least in triplicate with eight wells for each concentration. IC_{50} values were calculated according to the standard sigmoidal concentration–response equation using GraphPad Prism v5.02.

2.6. Induction assay

The human colon adenocarcinoma cell line LS180 (available from ATCC, Manassas, VA) was used for induction experiments as a surrogate for the intestine being a major site of drug interactions [9–12]. Following treatment with culture medium containing rilpivirine (0.1–2 μmol/L), rifampicin (20 μmol/L, positive control) or 0.02% DMSO (negative control) in quadruplicate for 4 consecutive days, cells were harvested and split for mRNA and protein isolation.

2.6.1. Quantification of mRNA expression by real-time reverse transcription PCR (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 a LightCycler® 480 (Roche Applied Science) [9]. The most suitable housekeeping gene for normalisation in LS180 cells (β-glucuronidase) was identified using geNorm v.3.4 (Center for Medical Genetics, Ghent, Belgium) [13]. Data were evaluated by calibrator-normalised relative quantification with efficiency correction using the LightCycler® 480 software v.1.5 (Roche Applied Science). All samples were amplified at least in duplicate. The following target genes were quantified: *CYP3A4*, *UGT1A3*, *UGT1A9*, *ABCB1*, *ABCC1*, *ABCC2*, *ABCG2* and *SLCO1B1*.

2.6.2. Western blot analysis of P-glycoprotein

P-gp protein expression was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Cell lysates containing 40 μg of protein were mixed with 5× sample buffer [containing Tris–HCl, SDS, dithiothreitol (DTT), bromophenol blue and glycerol] and then subjected to a 10% SDS-PAGE and electrotransferred to nitrocellulose nitrate membranes. Blots were blocked by incubation for 20–40 min with 5% skim milk (w/v) in PBS containing 0.1% Tween 20. Immunoblot analysis was carried out with murine monoclonal antibodies raised against human P-gp (diluted 1:100 in Tris-buffered saline containing 0.1% Tween 20) or β-actin (Clone AC-74; diluted 1:40 000). The blots were then washed extensively and incubated with horseradish peroxidase-linked secondary anti-mouse antibody (Amersham, Freiburg, Germany). Bands were visualised by enhanced chemiluminescence using the SuperSignal® West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL).

2.7. Pregnane X receptor reporter gene assay

The used reporter plasmid pGL4.21-CYP3A4-Luc was constructed according to Gu et al. [10,14]. For transfection, 2×10^4 LS180 cells were seeded into 96-well microplates, cultured for 24 h and subsequently for a further 4 h in supplement-free Dulbecco's Modified Eagle's Medium (DMEM). Each well was then exposed for 24 h to 20 ng of a PXR expression vector [Human cDNA TrueClone® (pCMV6-XL4 vector containing the cDNA *NR1I2*)], 80 ng of pGL4.21-3A4-Luc and 10 ng of the pGL4.74 [hRluc/TK] Renilla vector using a lipid-based FuGENE® HD Transfection Reagent. After 24 h of exposure to transfection mixture, the medium was replaced by the respective drug-containing cell culture medium. Cells were incubated for 24 h with rifampicin (positive control) or rilpivirine. The Dual-Glo™ Luciferase Assay was conducted according to the manufacturer's instructions (Promega Corp.). Drug-induced increases of PXR activity were calculated by division of firefly luminescence by Renilla luminescence (transfection efficiency control) and were normalised to PXR activity of non-drug-treated controls set to 1 (= 100%).