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In vitro assessment of the interactions of dopamine β-hydroxylase inhibitors with human P-glycoprotein and Breast Cancer Resistance Protein

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ABSTRACT

Inhibition of the biosynthesis of noradrenaline is a currently explored strategy for the treatment of hypertension, congestive heart failure and pulmonary arterial hypertension. While some dopamine β-hydroxylase (DBH) inhibitors cross the blood-brain barrier (BBB) and cause central as well as peripheral effects (nepicastat), others have limited access to the brain (etamicastat, zamicastat). In this context, peripheral selectivity is clinically advantageous, in order to prevent alterations of noradrenaline levels in the CNS and the occurrence of adverse central effects. A limited brain exposure results from the combination of several factors, such as a reduced passive permeability or affinity for efflux transporters, but efflux liabilities may also lead to unwanted drug-drug interactions (DDIs) in the presence of co-administered substrates or inhibitors.

Thus, the purpose of the study herein presented was to explore the interaction of P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP), the two major efflux transporters of the BBB that hamper the entry of several drugs to the brain, with the DBH inhibitors, etamicastat, nepicastat and zamicastat. Madin-Darby canine kidney cells (MDCK II) and transfected lines with human *MDR1* (MDCK-MDR1) and *ABCG2* (MDCK-BCRP) genes were used as a BBB surrogate model. P-gp and BCRP substrates and/or inhibitors were identified through intracellular accumulation and bidirectional permeability assays. The obtained data revealed that zamicastat is a concentration-dependent dual P-gp and BCRP inhibitor with IC_{50} values of 73.8 ± 7.2 μM and 17.0 ± 2.7 μM, while etamicastat and nepicastat inhibited BCRP to greater extent than P-gp, with IC_{50} values of 47.7 ± 1.8 μM and 59.2 ± 9.4 μM, respectively. Additionally, etamicastat was identified as P-gp and BCRP dual substrate, as demonstrated by net flux ratios of 5.84 and 3.87 and decreased > 50% by verapamil and Ko143. Conversely, nepicastat revealed to be a P-gp-only substrate, with a net flux ratio of 2.01, reduced to 0.92 in the presence of verapamil. Furthermore, nepicastat displayed a consistently higher apparent permeability ($> 8.49 \times 10^{-6}$ cm s^{-1}) than etamicastat ($< 0.58 \times 10^{-6}$ cm s^{-1}).

The identification of etamicastat as a dual efflux substrate suggests that P-gp and BCRP may be partially responsible for the limited central exposure of this compound, in association with its low passive permeability. Moreover, the weak efflux inhibitory potencies of etamicastat and nepicastat revealed a low DDI risk, while the dual P-gp/BCRP inhibition of zamicastat could be studied in the future with synergically effluxed compounds, for which BBB penetration is severely impaired.

1. Introduction

P-glycoprotein (P-gp; *ABCB1*) and Breast Cancer Resistance Protein (BCRP; *ABCG2*) are efflux transporters from the ATP-binding cassette family with broad and partly overlapping substrate specificity (Xia et al., 2005). Their widespread and privileged location in several tissues greatly influences the absorption, distribution and elimination of endogenous compounds and structurally diverse drugs (Chen et al., 2016).

Abbreviations: BBB, blood-brain barrier; BCRP, Breast Cancer Resistance Protein; BSA, bovine serum albumin; DAD, diode-array detection; DBH, dopamine β-hydroxylase; DDIs, drug-drug interactions; DMSO, dimethyl sulfoxide; EMA, European Medicines Agency; HBSS, Hanks balanced salt solution; HPLC, high performance liquid chromatography; MDCK, Madin-Darby canine kidney; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; Na-F, sodium fluorescein; Papp, apparent permeability; P-gp, P-glycoprotein; SD, standard deviation; TEER, transepithelial electrical resistance

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At the blood-brain barrier (BBB) of rodents and humans, P-gp and BCRP cooperatively contribute to efflux xenobiotics from cerebral endothelial cells, and when one is inhibited, the other compensates its function (Agarwal et al., 2011; Bauer et al., 2016; Kodaira et al., 2010). Hence, identifying P-gp and BCRP substrates and/or inhibitors is not only important to comprehend brain exposure, but also to investigate the occurrence of drug-drug interactions (DDIs) that may compromise clinical efficacy and safety. Thus, in vitro assays are recommended by the Food and Drug Administration and European Medicines Agency (EMA) to evaluate P-gp and BCRP-mediated efflux (European Medicines Agency, 2012; Food and Drug Administration, 2012).

In this context, the main objective of the present work was to investigate the interaction of three dopamine β-hydroxylase (DBH) inhibitors with P-gp and BCRP. DBH catalyses the conversion of dopamine into noradrenaline in the catecholamine biosynthetic pathway and its inhibitors have been developed for the treatment of resistant hypertension, congestive heart failure and pulmonary arterial hypertension, associated with an overactivity of the sympathetic nervous system (Bonifácio et al., 2015; Igreja et al., 2017). First and second generation DBH inhibitors did not reach the market due to low potency, poor selectivity and severe adverse effects (Bonifácio et al., 2015). Despite having demonstrated greater potency, nepicastat, a third-generation inhibitor, altered noradrenaline and dopamine levels in the cerebral cortex, indicating that it crosses the BBB, which could lead to undesired central effects (Beliaev et al., 2009). Thus, peripheral selectivity is an important clinical requirement for the development of DBH inhibitors, as the decrease of noradrenaline levels should occur at the periphery in sympathetically enervated tissues, thereby avoiding potentially serious side-effects in the CNS (Almeida et al., 2013). In contrast, etamicastat is a reversible DBH inhibitor with limited access to the brain. It decreases peripheral noradrenaline levels in sympathetically innervated tissues, without affecting noradrenaline levels in parietal and frontal cortex (Beliaev et al., 2006). Similarly, zamicastat also reversibly inhibits DBH without effect in brain tissue (Igreja et al., 2012).

Herein, the interactions of etamicastat, nepicastat and zamicastat with P-gp and BCRP were evaluated. Although recent investigations have been performed to assess the involvement of P-gp in the brain exposure of nepicastat and etamicastat (Loureiro et al., 2015), the contribution of BCRP remains undetermined. Moreover, there is yet no available information in literature concerning the interaction of zamicastat with these transporters. Intracellular accumulation and bidirectional permeability assays were performed using Madin-Darby canine kidney cells (MDCK II) and transfected lines with human *MDR1* (MDCK-MDR1) and *ABCG2* (MDCK-BCRP) genes as surrogate BBB models. In addition to fast growth and low metabolic activity, MDCK cells can form polarized monolayers with tight junctions when grown on semi-permeable supports and possess BBB-like passive permeability, being able to accurately distinguish passive from effluxed compounds (Hellinger et al., 2012). The stability of the DBH inhibitors was determined beforehand and the quantification of these compounds was achieved using validated high-performance liquid chromatography (HPLC) techniques with diode-array detection (DAD).

2. Methods

2.1. Chemicals and reagents

Etamicastat, nepicastat and zamicastat were kindly supplied by BIAL-Portela & C[a]., S.A. (S. Mamede do Coronado, Portugal). Acetonitrile (HPLC gradient grade) and dimethyl sulfoxide (DMSO) were acquired from Fisher Scientific (Leicestershire, UK). All the remaining chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Cell culture

MDCK II parent cells and human *MDR1*- and *ABCG2*-transfected cells were purchased from the Netherlands Cancer Institute (NKI-AVL, Amsterdam, Netherlands). The cells were cultured in Dulbecco's modified Eagle's medium with 0.04 M sodium bicarbonate and supplemented with 10% of heat-inactivated fetal bovine serum (Gibco Life Technologies, ThermoFisher Scientific, Waltham, MA, USA), 100 μg/mL streptomycin and 100 I·U/mL penicillin. Cells were grown in T-75 flasks (Orange-Scientific, Braine-l'Alleud, Belgium), passaged twice a week using a 0.25% Trypsin-EDTA solution and cultured at 37 °C in 5% CO_2 and 95% relative humidity.

2.2.1. Cell viability studies

The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay was selected to estimate the influence of DBH inhibitors on cell viability. Briefly, MDCK II, MDCK-MDR1 and MDCK-BCRP cells were seeded in 96-well plates at a density of 1×10^4 cells per well (Orange Scientific, Braine-l'Alleud, Belgium). After 24 h, the culture medium was removed, 200 μL of medium (control cells) or with each DBH inhibitor at different concentrations was added to the wells, and the cells were incubated for 4 h (5, 10, 20, 50, 80, 100 μM) or 30 min (only 100 μM). Lastly, the MTT solution (0.5 mg/mL) was added to each well and the plates were incubated for 2 h at 37 °C in 5% CO_2. At the end of this period, the MTT solution was replaced with 100 μL of DMSO. Absorbance was measured at 570 nm with a reference wavelength of 620 nm on a Biotek Synergy HT microplate reader (Biotek Instruments®, Winooski, VT, USA).

2.2.2. Stability studies

The stability of DBH inhibitors was evaluated in order to assess degradation levels during assays. The solutions were prepared in concentrations corresponding to low (QC1) and high (QC3) quality control samples of the respective analytical calibration curve and data were compared before (reference sample) and after (stability sample) exposure to assay conditions [120 min maximum at 37 °C in Hanks balanced salt solution (HBSS) with 10 mM HEPES pH 7.4]. Compounds were considered stable if the percentage of the ratio between stability and reference samples was kept between 85 and 115%, according to the limit of ± 15% established in the EMA guideline for the stability of analytical samples (European Medicines Agency, 2011).

2.2.3. Intracellular accumulation studies

P-gp and BCRP inhibitors were identified by performing intracellular accumulation studies. Initially, MDCK II, MDCK-MDR1 and MDCK-BCRP were seeded in 12-well plates (Corning Costar, NY, USA) at 3.0×10^5 cells per well for 48 h. Verapamil and Ko143 were used as positive controls of P-gp or BCRP inhibition, respectively. The cells were incubated for 30 min in the absence (negative control) or presence of verapamil (100 μM), Ko143 (0.5 μM) or DBH inhibitor (100 μM in 0.1% DMSO). Then, for 1 h at 37 °C, 10 μM rhodamine-123 or Hoechst 33342 were added to the cells as P-gp or BCRP substrates, respectively. Intracellular accumulation was stopped by washing the cells thrice with ice-cold phosphate buffer. Cell lysis was performed with Triton X-100 (0.1%, v/v) at room temperature during 30 min and an aliquot of cell lysate was used to measure the amount of accumulated substrate utilizing the Biotek Synergy HT microplate reader (Biotek Instruments, Winooski, VT, USA) in fluorescence mode (excitation and emission wavelengths of 485/528 nm for rhodamine-123; and 360/460 nm for Hoechst 33342). The concentrations were normalized according to the protein content of cell lysates, determined with the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, CA, USA). Additional concentrations of etamicastat, nepicastat or zamicastat were tested when a significant P-gp or BCRP inhibition was observed ($p < 0.05$) in order to determine the concentration of DBH inhibitor that inhibits substrate efflux by 50% (IC_{50}).