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Transport and uptake of clausenamide enantiomers in CYP3A4-transfected Caco-2 cells: An insight into the efflux-metabolism alliance

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ABSTRACT

The present study developed a CYP3A4-expressed Caco-2 monolayer model at which effects of the efflux-metabolism alliance on the transport and uptake of clausenamide (CLA) enantiomers as CYP3A4 substrates were investigated. The apparent permeability coefficients (P_{app}) of (−) and (+)CLA were higher in the absorptive direction than those in the secretory direction with efflux ratios (ER) of 0.709 ± 0.411 and 0.867 ± 0.250 ($\times 10^{-6}$ cm/s), respectively. Their bidirectional transports were significantly reduced by 75.6–87.5% after treatment with verapamil (a *P*-glycoprotein inhibitor) that increased the rate of metabolism by CYP3A4, whereas the CYP3A4 inhibitor ketoconazole treatment markedly enhanced the basolateral to apical flux of (−) and (+)CLA with ERs being 2.934 ± 1.432 and 1.877 ± 0.148 ($\times 10^{-6}$ cm/s) respectively. These changes could be blocked by the duel CYP3A4/*P*-glycoprotein inhibitor cyclosporine A, consequently, P_{app} values for CLA enantiomers in both directions were significantly greater than those obtained by using verapamil or ketoconazole, and their ERs were similar to those following (−) or (+)-isomer treatment alone. Furthermore, the uptake of (−)CLA was more than that of (+)CLA in the transfected cells. Incubation with ketoconazole decreased the intracellular concentrations of the two enantiomers. This effect disappeared in the presence of a CYP3A4 inducer dexamethasone. These results indicated that CYP3A4 could influence *P*-gp efflux, transport and uptake of CLA enantiomers as CYP3A4 substrates and that a duel inhibition to CYP3A4/*P*-glycoprotein could enhance their absorption and bioavailability, which provides new insight into the efflux-metabolism alliance and will benefit the clinical pharmacology of (−)CLA as a candidate drug for treatment of Alzheimer's disease.

1. Introduction

There are two principal proteins in the human intestine, cytochrome P450 (CYP) 3A4 and *P*-glycoprotein (*P*-gp). These proteins form a so-called drug efflux-metabolism alliance or transporter-enzyme alliance [1,2], and affect certain drug absorption and bioavailability. CYP3A4 is localized to the endoplasmic reticulum of the columnar epithelial cells lining the intestinal lumen, comprising an average of 70% of the total CYPs in the intestine [3,4] and metabolizing more than 50% of the drugs on the market [5]. It has extensive substrate overlap with *P*-gp, which is located in the apical membrane of the same intestinal epithelial cells and effluxes its substrates back into the intestinal lumen. These two proteins work to limit the permeation of drugs and xenobiotics across epithelial cells and perform physiologically protective functions. Recent studies using CYP3A4-transfected Caco-2 (human colon carcinoma) cells have shown that for dual CYP3A4 and *P*-gp substrates, selective inhibition of the transporter might yield significant effects on the extent of metabolism by CYP3A4 [1,6]. This gives valuable insight into the interactive nature of transporters and enzymes in the intestine.

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LS180/AD50 and its parent LS180/WT are also a human colon carcinoma cell lines that were the first used to observe the interaction of shared CYP3A4/*P*-gp substrates early in 1995 [3]. LS180 cells simultaneously express CYP3A4, CYP3A5, and *P*-gp when induced by several drugs. Treatment of LS180 cells with reserpine, rifampicin(Rif) and phenobarbital strongly up-regulates the co-expression of *P*-gp by 29-, 16-, 14-fold, and CYP3A4/5 by 7-, 3-, 6-fold relative to untreated controls, respectively [3].

Caco-2 cells also highly express the *P*-gp efflux transporter and are frequently used to investigate the drug transport and absorption. However, these cells lack or under express the oxidative metabolizing enzymes of the intestinal tract, including CYP3A4 [7,8]. Thus, to study drug transport and metabolism simultaneously, two Caco-2 cell models expressing high levels of CYP3A4 were developed by induction with 1α, 25-dihydroxy vitamin-D_3 [9] and by transfection of CYP3A4-bearing vectors [7,8,10]. In CYP3A4-transfected Caco-2 cells, the induction of sodium butyrate (4 mmol/L) increased the co-expression levels of *P*-gp and CYP3A4 by 1.9- and 10.5-fold over the control levels [11]. Following treatment of the cells with the *P*-gp inhibitor GG918, the absorption rate of sirolimus (an anesthetic agent and a dual CYP3A4/*P*-gp substrate) increased 58%, whereas its metabolism rate decreased 25% relative to the control, indicating the obvious effects of *P*-gp inhibition on CYP3A4 metabolism [5].

In the present study, first a eucaryotic expression vector, pcDNA3.1/myc-his(−)B-CYP3A4, was constructed and transfected into Caco-2 cells. Then the transfected cells were used to observe the transport and uptake of 3S, 4R, 5R, 6S-clausenamide [(−)CLA] and 3R, 4S, 5S, 6R-CLA [(+)CLA; Fig. 1] in the presence of Rif. It has been known that (−)CLA is a eutomer, while (+)CLA is a distomer [12–18]. (−)CLA increased the brain acetylcholine content and ameliorated memory deficits in the mouse amnesia model induced by anisodine. It also increased choline acetyltransferase activity in fetal rat frontal cortex cultures and stimulated proliferation of the neuronal cells [15,16]. Moreover, (−)CLA potentiated the basal synaptic transmission and induced a long-term potentiation (LTP) in dentate gyrus of anesthetized and freely moving rats [17,18]. Resent studies have further demonstrated that (−)CLA facilitates synaptic transmission at hippocampal Schaffer collateral-CA1 synapses [19]. These data indicates that (−)CLA has potential nootropic and antidementia effects and is currently being developed to treat Alzheimer's disease. On the other hand, pharmacokinetic studies showed that there was a significant stereoselective difference in AUC_{0-12h} values 1256 ± 308 min mg/mL ($n = 6$) for (−)CLA and 2446 ± 540 min mg/mL($n = 6$; $P < 0.001$) for (+)CLA after oral administration to rats [20]. Studies on the first-pass metabolism of CLA enantiomers following an oral dose of 100 mg/kg in rabbits also showed that (−)CLA had a smaller AUC_{0-8h} (1001 ± 487 h μg/mL, $n = 5$) than did (+)CLA(2453 ± 1101 h μg/mL, $n = 4$; $P < 0.01$) in the portal vein. Both (−) and (+)CLA were subjected to an intermediate degree of first-pass metabolism with rates of hepatic extraction 64.7% for (−)-isomer and 50.8% for (+)-isomer, and intrinsic metabolic clearances of (−) and (+)CLA being 186.3 and 107.2 (L/h), respectively [21]. Meanwhile, in the *in vitro* metabolic system with rat liver microsomes, (−)CLA was mainly metabolized to 7-hydroxy-, 5-hydroxy- and 4-hydroxy-CLA, and 7-hydroxylation was a preferential pathway with V_{max}/K_m value of 0.135 μl/min/mg, but (+)CLA was mainly metabolized to 4-hydroxy-CLA with V_{max}/K_m value of 0.547 μl/min/mg, its 7-hydroxyl and 4-hydroxyl metabolites were very small [22]. It was further found that the major metabolizing enzyme for CLA enantiomers was CYP3A 1/2 via its specific inducers and selective inhibitors [23]. Recent studies by using Caco-2, KBv and rat brain microvessel endothelial cells expressing *P*-gp at high levels have demonstrated that (−)CLA can be a weak *P*-gp inhibitor and (+)CLA can be a modulator with concentration-dependent biphasic effects on *P*-gp activity [24]. These data indicate that CYP3As and *P*-gp are associated with the transport and metabolism of CLA enantiomers in animal intestines. Therefore, this study developed a CYP3A4-expressed Caco-2 cell model at which the interplay of CLA enantiomers with the two proteins as an efflux-metabolism alliance was investigated.

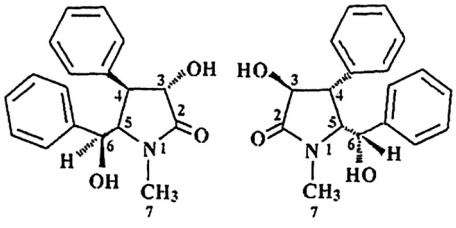

Fig. 1. Structures of (−)-3S, 4R, 5R, 6S-clausenamide and (+)-3R, 4S, 5S, 6R-clausenamide.

The results obtained from the present study suggest that more CLA enantiomers are metabolized by CYP3A4 when *P*-gp is inhibited, causing their bidirectional transport levels to be significantly reduced; whereas the inhibition of CYP3A4 leads to enhanced secretory transport and decreased intracellular uptake of the (−)- or (+)-isomer; Furthermore, the absorptive transport of the two enantiomers, in particular the (−) enantiomer, is increased after both CYP3A4 and *P*-gp are inhibited by cyclosporine A(CsA). These results provide new insight into the efflux-metabolism alliance and the clinical pharmacology of (−)CLA as an active eutomer for treatment of Alzheimer's disease.

2. Materials and methods

2.1. Chemicals, reagents, cell lines and plasmids

(−) and (+)CLA were supplied kindly by Prof. L. Huang's laboratory in the present institute (Fig. 1) [12–14,20] and Guangzhou Nuohao Medical Technology Co., Ltd. (Guangzhou, China). The optical purity of each CLA isomer was over 99%. Caco-2 cells (passage 32) were obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China). Tissue culture plates (6-well) were obtained from Corning Costar (Cambridge, MA, USA). Fetal bovine serum (FBS), minimum essential medium (MEM), non-essential amino acids (NEAA), pcDNA3.1/myc-his(−)B vector, Lipofectine2000 and Trizol were purchased from Invitrogen Corporation (Grand Island, New York, USA). SC125488(pCMV6-XL4; *Homo sapiens* CYP3A4, transcript variant 1 as transfection-ready DNA, NM_017460.3) was purchased from Origene Technologies (Beijing, China). Dimethyl sulfoxide (DMSO), G418 sulfate and sodium butyrate (NaB) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 12-*O*-tetradecanoylphorbol-13-acetate(TPA) was purchased from ALEXIS Corporation (San Diego, CA, USA). Rif, CsA, verapamil hydrochloride (Ver), ketoconazole (Ket), dexamethasone (Dex) and glipizide were purchased from the National Institutes for Food and Drug Control (Beijing, China). TIANprep Mini Plasmid Kit, 2 × Taq Platinum PCR MasterMix and DNA Gel Extraction Kits were from TransGen Biotech CO., LTD (Beijing, China). pEASY-T1Cloning Kits, T4 DNA ligase, Trans5α competent cells and Trans2K Plus DNA Markers were also from TransGen Biotech CO., Ltd. (Beijing, China). dNTP Mixture, rTaq, Kpn I and Xho I were from TAKARA Biotechnology Co., Ltd. (Dalian, China). The mouse monoclonal antibody raised against CYP3A4 and the mouse monoclonal antibody raised against amino acids of 1040–1280 of *P*-gp were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse anti-GAPDH monoclonal antibody and peroxidase-conjugated goat anti-mouse IgG secondary antibody were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Western luminescent detection kits were from Vigorous Biotechnology