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Transport Characteristics of Candesartan in Human Intestinal Caco-2 Cell Line

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ABSTRACT: The intestinal absorptive characteristics and the efflux mechanisms of candesartan (CDS), a novel angiotensin II type 1 receptor blocker, were investigated. The Caco-2 cells were used as models of the intestinal mucosa to assess uptake and transport of CDS. The determination of CDS was performed by HPLC-Flu. In the Caco-2 cells, the uptake and absorptive transport of CDS were pH-independent (in the pH range 6.0–8.0). Passive membrane diffusion dominates the absorptive transport behavior of CDS across Caco-2 cells, while secretory transport was a concentration-dependent and saturable process. In the presence of cyclosporin A and verapamil, potent inhibitors of P-glycoprotein (P-gp), the P_{ratio} decreased from 3.8 to 2.3 and 1.8, respectively, and permeation of apical to basolateral was enhanced. Overall, the current study suggests that efflux transporters are capable of mediating the absorption and secretion of CDS, and they may play significant roles in limiting the oral absorption of CDS. Copyright © 2009 John Wiley & Sons, Ltd.

Key words: candesartan; Caco-2 cells; P-glycoprotein; intestinal absorption

Introduction

Candesartan (CDS) is a potent and selective angiotensin II type 1 (AT_1) receptor blocker, which has widely been used orally in patients with hypertension, kidney disease and heart failure [1,2]. CDS lowers blood pressure through blockade of the renin–angiotensin–aldosterone system [3]. The AT_1 binding affinity of CDS is 80 times greater than that of losartan and 10 times greater than that of EXP 3174, an active metabolite of losartan [1]. The efficacy of CDS has been shown to be much higher or at least equivalent to that of many other commonly prescribed antihypertensive agents [4,5].

Candesartan is also a long-acting angiotensin II receptor antagonist. To overcome a poor oral absorption, a series of ester prodrugs was synthesized, and candesartan cilexetil (CC) was identified as the compound that provided the best angiotensin II antagonistic activity profile after oral administration. CC is rapidly and completely converted to its active compound, CDS, during gastrointestinal absorption [6]. The extent of absolute oral bioavailability (*F*) of CC is 15%–40% [6–8], and varied in 20 Chinese healthy male volunteers (our unpublished data). It is generally believed that the low and variable *F* of CC may be explained by physicochemical factors (i.e. solubility, permeability and dissolution) as well as physiological factors (i.e. intestinal absorption, efflux and the first-pass metabolism). However, little is known about the transport characteristics of CDS in the human intestine. In recent years, the Caco-2 cell model has become a popular model of the intestinal mucosa. Because these cells have a colonic origin,

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they also express transporters normally found in the intestinal mucosa, including P-glycoprotein (P-gp), multiple drug resistance associated protein (MRP) and so on [9,10].

The present study was carried out to elucidate the characters of the uptake and transport of CDS across intestinal epithelial cells using Caco-2 cells.

Materials

CDS was sponsored by Takeda Pharmaceutical (Tianjin, China) Co. Ltd. [^{3}H] Mannitol (1 μCi/μl) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Heated inactivated fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2-(*N*-morpholino)-ethanesulfonic acid (MES), trypsin–ethylenediamine tetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acid solution, L-glutamine and penicillin–streptomycin were products from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA). Cyclosporin A (CsA) was obtained from Taishan Medical Drug Co. (Guangdong, China). Verapamil was supplied by Shanghai Xinyi Pharmaceutical Co. (Shanghai, China). Other chemicals were of reagent grade.

Methods

Cell culture

The Caco-2 cell line was obtained from American Type Culture Collection, (Rockville, MD, USA). The cells were grown routinely in culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, NJ, USA) in DMEM containing 25 mM D-glucose, 25 mM HEPES, 44 mM $NaHCO_3$, supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid solution, 1% (v/v) L-glutamine, 1% (v/v) penicillin–streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% O_2 and 90% relative humidity. The medium was replaced every 2–3 days after incubation. When the cells had reached 70–80% confluence (approximately every 5 days), they were harvested with 0.25% trypsin and 0.02% EDTA. The cells were passaged, 1:5 (one harvested suspension divided into five parts), into a new flask. For the transport experiments, the cells were seeded at a density of 5×10^4 cells/cm^2 onto a permeable polycarbonate insert (0.6 cm^2, 0.40 μm pore size, Millipore, Bedford, MA, USA) in 24-well plastic plates. Media in the culture plates was changed every 2 days for the first week following seeding then replaced every day. The quality of the monolayers grown on the permeable membrane in the transport studies was assessed by the transepithelial electrical resistance (TEER) of the monolayers at 37°C using a Millicell-ERS apparatus (Millipore) and [^{3}H]mannitol transport. Only monolayers displaying TEER values above 400 Ωcm^2 were used in the transport experiments. The permeability of mannitol was determined to be $<1\%$ per hour. These results indicated that the cell monolayers were not leaky. For the uptake study, Caco-2 cells were seeded on 24-well plastic plates (Corning Costar Corp., Cambridge, MA, USA). The cell monolayers were fed a fresh growth medium every 2 days.

Uptake studies

CDS uptake was studied using Caco-2 cell monolayer 14–16 days post-seeding. Before the experiments, the monolayers were washed twice with HBSS (pH 7.4). Then, the monolayers were preincubated at 37°C for 15 min with 0.5 ml of HBSS. After removal of HBSS, 0.5 ml of fresh HBSS containing CDS was added. The monolayers were incubated for a designated time at 37°C. The cell monolayers were washed rapidly twice with an ice-cold HBSS at the end of the incubation period and lysed with HBSS containing 1% Triton-X. Then, a part of the suspension (100 μl) was transferred to determine the content of protein using a reported colorimetric assay [11]. The remainder was centrifuged ($4000 \times g$, 15 min), and the concentration of CDS in the supernatant was determined. The CDS stock solution was freshly prepared by dissolving in dimethyl sulfoxide (DMSO) first, then diluted with HBSS-HEPES or HBSS-MES to obtain working solutions with a specified pH. The final concentration of DMSO in the HBSS was below 1% (v/v). The effects of time, concentration of CDS, pH and CsA on the uptake of CDS in Caco-2 cells were investigated.

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Transport studies

Transport of CDS across the Caco-2 cell monolayers was studied using monolayers 21–24 days post-seeding. Before the experiments, the monolayers were washed twice with HBSS (pH 7.4). After being washed, the monolayers were preincubated (37°C, 20 min) and TEER was measured. The HBSS solution on both sides of the cell monolayers was then removed by aspiration. For the measurement of the apical (AP) to basolateral (BL) transport (A→B), 0.4 ml of HBSS containing CDS (5–100 mg/l) was added on the AP side, and 0.6 ml of HBSS without the drug was added to the BL side. For the measurement of the BL to AP transport (B→A), 0.6 ml of HBSS containing CDS (5–100 mg/l) was added on the BL side, and 0.4 ml of HBSS without the drug was added to the AP side. The monolayers were incubated at 37°C, placed in a shaker (50 rpm) during the transport process to minimize the influence of aqueous boundary layer. Samples were taken from the receptor chamber at 15, 30, 45, 60, 90, 120 min followed by an immediate replacement of the same volume of prewarmed fresh HBSS. The effect of pH in AP (6.0, 6.4, 7.4 and 8.0) on the transport of 50 mg/l CDS from the AP to the BL side was examined under constant pH of the BL side (pH 7.4). The inhibition of efflux of CDS (50 mg/l) across Caco-2 cells was investigated in the presence of two common P-gp inhibitors, CsA (10 mg/l) and verapamil (10 mg/l).

HPLC analysis of CDS

The analysis was performed on an Agilent 1100 series HPLC system (Agilent Corp. Ltd, Palo Alto, CA, USA) equipped with quat-pump, a column compartment and a fluorescence detector. CDS was separated using an YMC® ODS column (250 mm, l; 4.6 mm, i.d.; 5 μm, particle size).

The mobile phase, 10 mmol/l potassium dihydrogen phosphate (adjusted to a pH 3.0 with phosphoric acid):acetonitrile (40:60, v/v) was run at a flow-rate of 1.0 ml/min at 30°C. The column eluent was monitored using a fluorescence detector at an excitation wavelength of 270 nm and an emission wavelength of 390 nm. A 20 μl aliquot of the samples obtained from the uptake or transport studies was directly injected into the HPLC column. The standard curve of CDS was linear within the range 0.01–50 mg/l ($r^2 = 0.99$).

Calculations

Apparent permeability coefficients (Papp) of CDS were calculated in both A→B and B→A according to the following equation:

$$\text{Papp} = (dQ/dt)/(A \times C_0) \quad (1)$$

in which the dQ/dt (μg/min) is the drug permeation rate, A is the cross sectional area (0.6 cm^2), and C_0 (mg/L) is the initial CDS concentration in the donor compartment at $t = 0$.

The net efflux of a test compound was assessed by calculating the ratio of Papp in the B→A versus Papp in the A→B:

$$P_{ratio} = (Papp_{B\to A})/(Papp_{A\to B}) \quad (2)$$

A ratio of substantially greater than 2.0 indicates a net efflux of the drug.

Statistical analysis

The results are expressed as mean ± SD ($n \geqslant 3$, each). A value of $p < 0.05$ was deemed to be statistically significant using the two-tailed Student's *t*-test or one-way ANOVA.

Results

Uptake of CDS in Caco-2 cells

Effects of time. Figure 1 shows the time course of the uptake of CDS (50 mg/l, pH 7.4) up to 30 min. The uptake of CDS increased with time, but the slope decreased from 15 min. Thus, a 15 min incubation was chosen arbitrarily for the uptake studies of CDS in Caco-2 cells.

Effects of pH. Figure 2 shows the effect of extracellular pH on the uptake of CDS (50 mg/l). The incubation medium used for this study was HBSS-MES (pH 6.0, 6.4) or HBSS-HEPES (pH 7.4, 8.0). In these four different pH environments, the highest uptake occurred at a pH of 7.4. There was no significant difference ($p > 0.05$) for these four different pHs.

Effects of CDS concentration. The concentration-dependence uptake of CDS was investigated. The uptake of CDS showed linearity within the range 5–250 mg/l concentration, and uptake was not saturated up to 250 mg/l (Figure 3).

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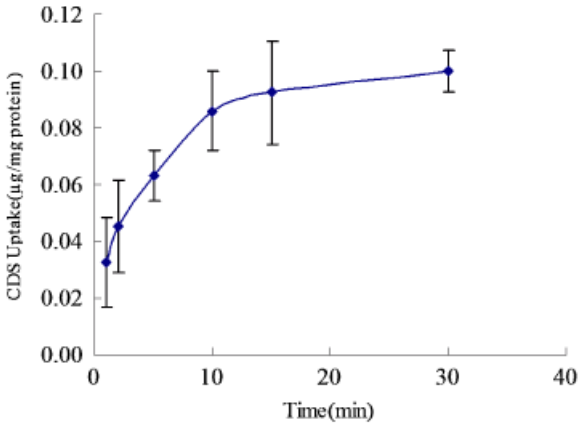

Figure 1. Time course of the uptake of CDS (50 mg/l, pH 7.4) in 37°C from 0 to 30 min

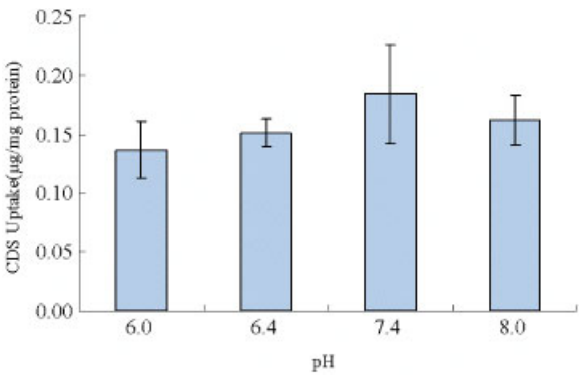

Figure 2. Influence of pH on CDS uptake in Caco-2 cells

Effects of cyclosporin A. CDS solution (0.5 ml) alone or with CsA was added to each well to determine the influence of P-gp inhibitor on the cellular drug uptake of CDS. All treatments were adjusted to 50 mg/l CDS with HBSS. Figure 4 represents the time course of the cellular uptake of CDS by Caco-2 cells with the CDS solution alone or with CsA. The absorbed amount in Caco-2 cells was significantly increased after 30 min in the presence of 10 mg/l of CsA.

Transport of CDS in Caco-2 cells

Effects of pH. The transepithelial transport of CDS by Caco-2 cells monolayers was examined at pH 6.0, 6.4, 7.4 and 8.0 on the AP side, and at pH 7.4 on the BL side. The study was performed at a 50 mg/l concentration of CDS. The effects of pH

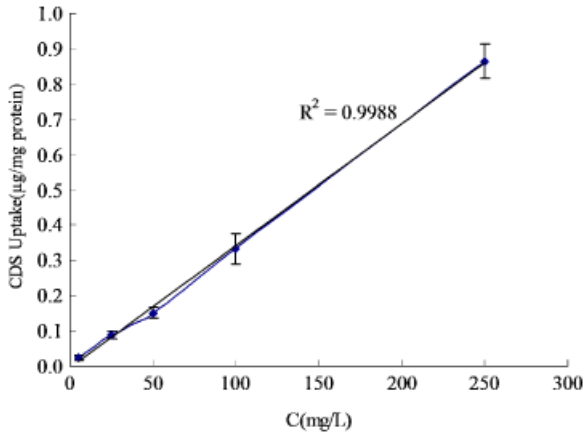

Figure 3. Effect of CDS concentration on Caco-2 cell uptake

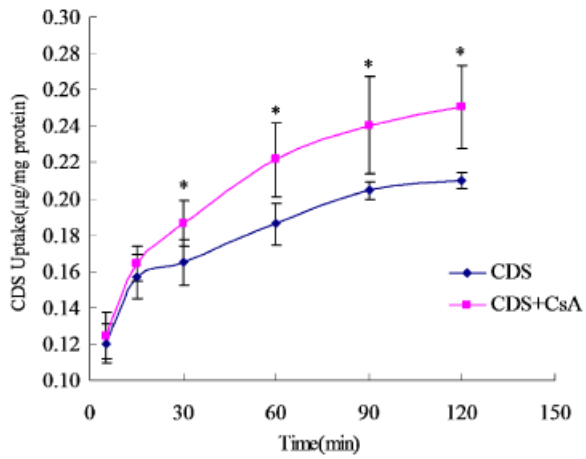

Figure 4. Effect of CsA on uptake of CDS by Caco-2 cells in 120 min

on the transepithelial transport are shown in Figure 5. The transepithelial transport of CDS was pH-independent ($p > 0.05$). Thus, the AP side medium of pH 7.4 was chosen arbitrarily for the following experiments.

Transcellular transport of CDS across Caco-2 cells. The influence of CDS concentration on both the A→B and B→A transport of CDS across the Caco-2 cell monolayers was measured. The concentration-independence absorptive transport of CDS in the concentration range 5–100 mg/L was observed. The absorption Papp (A→B) of CDS was significantly lower than the secretory Papp (B→A) over all concentration ranges ($p < 0.05$; Figure 6). The absorptive Papp was

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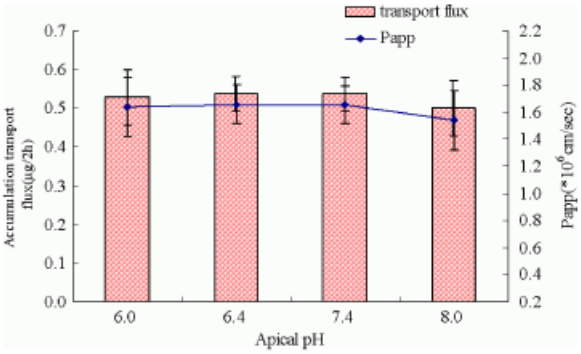

Figure 5. Effects of pH on the transepithelial transport flux and Papp of CDS by Caco-2 cell monolayers

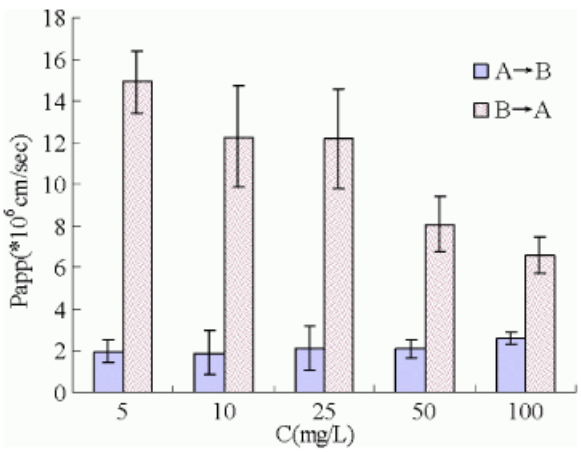

Figure 6. Concentration in directionality of the AB and BA apparent permeability (Papp) of CDS across Caco-2 cell monolayers

estimated to be $(2.2 \pm 0.9) \times 10^{-6}$ cm/s. The absorptive flux was nearly increased linearly with increasing CDS concentrations (5–100 mg/l). However, the secretory transport was seemed to be a saturable process (Figure 6).

Effects of the inhibitors on A→B and B→A permeation of CDS across Caco-2 cells. The efflux transport of CDS was investigated in the presence of CsA and verapamil. The Papp in both directions is listed in Table 1. The P_{ratio} were significantly lower than that without inhibitors. Furthermore, the permeation of A→B was enhanced and that of B→A was decreased. These results imply that the P-gp might be involved in the efflux of CDS, because the Papp and permeation of CDS across Caco-2 cell monolayers in the absorptive and secretory directions were not equal to that in the presence of the P-gp inhibitors.

Table 1. Effects of P-gp inhibitors on transport of CDS across Caco-2 cells

	Papp($*10^6$ cm/s)		P_{ratio}
	A→B	B→A	
CDS (control)	2.11±0.44	8.08±1.34	3.8
CDS+CsA	2.83±1.03	6.51±4.88	2.3[a]
CDS+verapamil	3.20±0.37	5.78±1.77	1.8[a]

[a] $p < 0.05$ versus control.

Discussion

It has been reported previously that the *F* of CC was low and variable [6–8]. Furthermore, one of the AT_1 receptor antagonists, losartan, was proved to be a substrate for P-gp [12]. This indicates that intestinal absorption may play a crucial role in the clinical use of CDS. Since CDS is the most recently marketed AT_1 receptor antagonist, the transport mechanisms of CDS in intestinal absorption has not yet been elucidated. This study investigated the uptake and the transport characteristics of CDS in Caco-2 cells.

In the present study, the uptake and the transepithelial transport of CDS across Caco-2 cell monolayers were pH-independent in the pH range 6.0–8.0. One interpretation for the results is that the pK_a of CDS is 2.48, thus, at pH 6.0 or above (the common physiological environment in the human intestine), CDS presents similar ionic forms, which restrict its absorption.

It has been observed that the uptake and absorptive transport of CDS were not concentration-independent (Figures 3, 6), suggesting the involvement of passive membrane diffusion as the dominating process. The absorptive Papp was estimated to be $(2.2 \pm 0.9) \times 10^6$ cm/s, which is within the suggested threshold value $(1–10 \times 10^{-6}$ cm/s) for medium absorbed compounds [13] and consistent with the *F* (15%–40%) of CC in the pharmacokinetic study.

In contrast to absorptive transport, the secretory transport appeared to tend to be concentration-dependent and saturable (Figure 6), suggesting the involvement of a transporter or multiple transporters. The transcellular transport data (Table 1) for

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CDS showed the secretory Papp of CDS was much higher than its absorptive Papp, suggesting that the efflux of CDS may limit the oral absorption of CDS. In the uptake study a significantly higher accumulation of CDS from 30 min to 120 min in Caco-2 cells was found in the presence of CsA compared with the CDS solution alone (Figure 4). This indicates that CDS may be a substrate for P-gp. In the transport study, the efflux of CDS was competitively inhibited by CsA and verapamil, which are potent inhibitors of P-gp [14,15], suggesting the involvement of multiple efflux transporters including P-gp in the efflux of CDS. CsA (10 mg/l) and verapamil (10 mg/l) significantly inhibited this polarized efflux, i.e. the P_{ratio} decreased from 3.8 to 2.3 and 1.8, respectively. These data indicate that the polarized efflux of CDS in Caco-2 cells was possibly due mainly to the presence of P-gp. Since the function and expression levels of intestinal P-gp are not unlimited, the saturation of secretory transport may have a relationship with the saturation of P-gp. The expression and function of P-gp are modified by genetic polymorphisms of the MDR_1 gene. MDR_1 polymorphisms have a great impact on pharmacokinetics and pharmacodynamics of P-gp substrates [16,17]. This may be one of the explanations of why the *F* of CC is low and variable.

In summary, the results of this study show that passive membrane diffusion dominates the absorptive transport behavior of CDS across Caco-2 cells. The absorption of CDS could be enhanced by P-gp inhibitors, CsA and verapamil. These results provide a rationale for the poor absorption of CDS. This study is the initial step in identifying transport mechanisms of CDS in intestinal absorption. A more precise mechanism for the efflux of CDS is needed in future studies.

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