Determination of the enantioselectivity of six chiral aryloxy aminopropanol drugs transport across Caco-2 cell monolayers

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Abstract This study aimed to determine the transepithelial transport characteristics of chiral drug enantiomers across Caco-2 cell monolayers, a model of human intestinal epithelial membrane. Six chiral aryloxy enantiomers (atenolol, sotalol, celiprolol, carvedilol, metoprolol and propafenone) were tested in bi-directional transport studies. The separation and quantitation of these enantiomers were performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using 2, 3, 4, 6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a pre-column derivatizing agent. Bi-directional transport studies demonstrated that celiprolol and carvedilol exhibited significant enantioselectivity in polarized transport at the concentration range tested. The efflux ratio (ER) for (R)-(+)–celiprolol was 8.96, but it was much lower for (S)-(−)-celiprolol which is 3.42 at the concentration of 96.0 μM; carvedilol had the same transport behavior as celiprolol while the difference between the ER values of two enantiomers was not as significant as celiprolol at the concentration of 5.0 μM. They are 2.41 for (R)-(+)–carvedilol and 1.98 for (S)-(−)-carvedilol. But in the transport studies of racemic atenolol, sotalol, metoprolol and propafenone, no enantioselective transport were observed over the concentration range tested. Because P-glycoprotein (P-gp) is highly expressed in Caco-2 cells, we inferred that P-gp might participate in the transport processes of celiprolol and carvedilol in chirally discriminative ways.

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KEY WORDS
Chiral aryloxy aminopropanol drugs; Transport; Enantioselectivity; P-gp; Caco-2 cell

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1. Introduction

Chiral drugs, which took more than 50% of all current drugs, have been paid more and more attention for their clinical applications. A pair of enantiomers would be discriminated as different molecules by chiral environment such as enzymes, receptors, carries, etc. Biomembrane permeability and cellular uptake of chiral drugs may be also stereoselective if intracellular macromolecules recognize the enantiomers in chirally discriminative ways. Therefore, the pharmacodynamics, pharmacokinetics and toxicology between the enantiomers could be stereoselective.

Aryloxy aminopropanol drugs, such as atenolol, sotalol, celiprolol, carvedilol, metoprolol and propafenone, were widely used to treat cardiovascular diseases and administrated mainly by racemes. Studies proved that the pharmacokinetics of some aryloxy aminopropanol compounds, for example, plasma protein binding, liver metabolism, bile excretion, were stereoselective1–3, and the enantiomers’ pharmacological activities are different from each other4. For example, (S)(+)-sotalol hydrochloride and (R)(−)-isomer have similar anti-arrhythmia activity but the comprehensive effect of β-adrenergic blockers is actually attributed by (R)(−)-isomer5. Due to these stereo-different pharmacological profiles, the uncovering of the transport stereoselectivity of aryloxy aminopropanol drugs was important in clarifying the pharmacokinetics of these drugs; furthermore, the uncovering of pharmacokinetic behaviors of these drugs will facilitate the understanding of the pharmacological differences.

To evaluate the transportation mechanisms and absorption of aryloxy aminopropanol drugs and to further explain the pharmacological differences of these drugs, we investigated the transepithelial transport of six chiral aryloxy aminopropanol drugs (atenolol, sotalol, celiprolol, carvedilol, metoprolol and propafenone) in Caco-2 cell model, which has been widely accepted as an in vitro model for intestinal drug absorption (Fig. 1). The chiral RP-HPLC method using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a pre-column derivatizing agent was used to separate and assay these enantiomers, which was previously described by our group6. In this study, we will concentrate on the transport characteristics of these chiral drug enantiomers across Caco-2 cells.

2. Materials and methods

2.1. Materials

Racemic atenolol and racemic metoprolol tartrate were purchased from National Institutes for Food and Drug Control (Beijing, China). Racemic sotalol hydrochloride was provided by Dong Dong Chemical Pharmaceutical Company (Taizhou, Zhejiang, China). Racemic celiprolol hydrochloride was provided by Hai Zheng Pharmaceutical Company (Zhejiang, China). Racemic propafenone hydrochloride was purchased from Sigma. Racemic carvedilol was provided by Tian Heng Pharmaceutical Company (Ningbo, Zhejiang, China). Lucifer yellow was purchased from Biochemika. Methanol and acetonitrile were of HPLC grade. Other solvents used were of analytical grade.

2.2. Cell culture

Caco-2 cells (35 passage) obtained from Chinese Academy of Medical Sciences (CAMS, Beijing, China) were grown in a humidified atmosphere of 5% CO2 at 37°C. Cells were cultured in high-glucose Dulbecco’s modified eagle’s medium.

Figure 1 The structures of six aryloxy aminopropanol drugs (atenolol, sotalol, celiprolol, carvedilol, metoprolol and propafenone), the chiral carbon atom was labeled by “*”. 
with initial TEER values higher than 450 Ω/cm² and the \( P_{\text{app}} \) value lower than 0.2 × 10⁻⁶ cm/s were used.

2.3. Transport experiments

The stock solutions of racemic drugs were made in Hank’s Balanced Salt Solution (HBSS), containing 25 mM HEPES, pH 7.4, and were diluted to a series of concentration (Table 1). The solutions were filtered through 0.22 μm filter membrane for sterilizing. To ensure the transport experiments were conducted in the sink conditions, the incubation time in Caco-2 cell monolayers of each drug was determined by preliminary experiments. The transport studies were completed before the receiver concentration exceeded 10% of the concentration in the donor compartment.

A solution containing individual concentration of these drugs. The transport rate of six enantiomers of drug was calculated using the following equation:

\[
V = \frac{dQ}{dt} \cdot A
\]

(1)

where \( dQ/dt \) is the rate of appearance of drugs on the receiver side, \( A \) is the surface area of the monolayer and \( C_0 \) is the initial concentration in the donor compartment.

Data was expressed as the mean ± SD (\( n = 5 \)). Differences in \( P_{\text{app}} \) of enantiomers were evaluated using paired t-test. A \( P \) value < 0.05 was considered to be statistically significant.

3. Results

We have investigated the transport across Caco-2 cell monolayers of six chiral aryloxy aminopropanol compounds. The experimental results indicated that the transports of racemic atenolol, sotalol, metoprolol and propafenone were not enantioselective in the concentration ranges tested. The transport rates of (S)-enantiomers were similar to (R)-enantiomers both in the absorptive direction and the secretory direction (\( P > 0.05 \)). During the transport process of enantiomers, neither concentration-dependence nor directionality (\( P_{\text{app}} \) (AP→BL))

![Table 1](image1.png)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Racemic concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>75.0 187.5 375.0 750.0 1500</td>
</tr>
<tr>
<td>Sotalol</td>
<td>130.0 325.0 650.0 1300 3250</td>
</tr>
<tr>
<td>Celiprolol</td>
<td>96.0 240.0 480.0 960.0 2400</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>5.0 12.5 25.0 50.0 100.0</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>6.0 15.0 30.0 75.0 150.0</td>
</tr>
<tr>
<td>Propafenone</td>
<td>2.0 5.0 10.0 25.0 50.0</td>
</tr>
</tbody>
</table>

![Table 2](image2.png)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Incubation time (h)</th>
<th>Racemic conc. (μM)</th>
<th>( P_{\text{app}} ) (AP→BL) (× 10⁻⁶ cm/s)</th>
<th>( P_{\text{app}} ) (BL→AP) (× 10⁻⁶ cm/s)</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>4.0</td>
<td>75.0</td>
<td>0.162 ± 0.139</td>
<td>0.150 ± 0.101</td>
<td></td>
</tr>
<tr>
<td>Sotalol</td>
<td>4.0</td>
<td>130.0</td>
<td>0.358 ± 0.006</td>
<td>0.385 ± 0.049</td>
<td></td>
</tr>
<tr>
<td>Celiprolol</td>
<td>3.0</td>
<td>96.0</td>
<td>0.785 ± 0.309</td>
<td>2.218 ± 0.349</td>
<td></td>
</tr>
<tr>
<td>Carvedilol</td>
<td>2.0</td>
<td>5.0</td>
<td>9.935 ± 2.631</td>
<td>11.27 ± 1.849</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>3.0</td>
<td>6.0</td>
<td>4.319 ± 0.556</td>
<td>4.487 ± 0.673</td>
<td></td>
</tr>
<tr>
<td>Propafenone</td>
<td>1.0</td>
<td>2.0</td>
<td>12.90 ± 1.171</td>
<td>13.06 ± 1.106</td>
<td></td>
</tr>
</tbody>
</table>
and \( P_{\text{app}} \) (BL→AP) did not have significant differences) were observed in the concentration ranges studied, suggesting that the transmembrane transports of these drugs were mainly by passive diffusion pathway (Table 2). A series of concentrations of atenolol, sotalol, metoprolol and propafenone have been tested, but the \( P_{\text{app}} \) values in both two directions and efflux ratios (ER) in higher concentrations were similar to the lowest concentrations used in these experiments, so only these parameters determined at the lowest concentration were shown in Table 2.

These studies also demonstrated that transports of celiprolol and carvedilol were significantly stereoselective. In the absorptive direction, there was significant difference between the \( P_{\text{app}} \) values of (S)-(−)- and (R)-(+) -celiprolol \((P < 0.01)\). The values for (R)-(+) -isomer were almost 3-fold smaller than (S)-(−)-isomer which indicated (S)-(−)-isomer was easier to penetrate through biomembrane. The transports of celiprolol enantiomers were concentration-dependent, with the increasing of the drug’s concentration, the transport rates raised, though it dropped a little at the highest concentration of (S)-(−)-isomer. But the distinction between (S)-(−)- and (R)-(+) -celiprolol was decreased while drugs’ concentration increased. In secretory direction, the \( P_{\text{app}} \) values of (S)-(−)-celiprolol were slightly smaller than (R)-(+) -celiprolol at low concentrations \((96.0 – 480.0 \, \mu M)\) \((P < 0.05)\), but as the concentration increased to 960.0, 2400.0 \( \mu M \) the differences between celiprolol enantiomers diminished \((P > 0.05)\). Compared the bidirectional (AP→BL and BL→AP) transport, we conclude that celiprolol enantiomers have conspicuous excretion transport, and the ER value were between 9.0–2.2 in the concentration range studied (Fig. 2).

As shown in Fig. 3, the transport of carvedilol was enantioselective and excretive, but no concentration-dependence was observed. In the absorptive direction, the \( P_{\text{app}} \) values of (S)-(−)-carvedilol were greater than (R)-(+) -carvedilol, while in the secretory direction, \( P_{\text{app}} \) values of (S)-(−)-carvedilol were smaller than (R)-(+) -carvedilol \((P < 0.05)\). So the ER values of (S)-(−)-carvedilol were conspicuously smaller than (R)-(+) -carvedilol. It is likely that an excretive transporter participated in the transport process across Caco-2 cell monolayers, and the transporter favors (R)-(+) -enantiomer of the molecule. The ER values were between 2.4–1.9 in the concentrations studied (Fig. 3).

4. Discussion

In Caco-2 cell monolayers, the transports of atenolol, sotalol, metoprolol and propafenone were not stereoselective, suggesting that the differences of pharmacological effects of sotalol isomers were not caused by the differences of cell permeability between two isomers. But the enantiomers of celiprolol and carvedilol displayed different transport behaviors.
Our data are in accordance with previous studies roughly. Bachmakov et al.13 have observed that the BL→AP transport of carvedilol was greater than the AP→BL transport, and the polarized transport of cefepim has been reported by Kuo et al.14. Karlsson et al.15 reported that the basal-to-apical transport (secretion) of [14C]-cefepim (50 μM) was 5 times higher than apical-to-basal transport (absorption). And as reported, atenolol with low permeability was used as a reference compound for low intestinal absorption and was mainly transported paracellularly16–19. These results are in agreement with our observation because small P_app values for atenolol were obtained in our experiments (Table 2). Yang et al.20 have determined the permeability of seven β-blockers using Caco-2 cell line, and the P_app values of metoprolol, atenolol and sotalol were close to our results calculated as racemate. They also believed that the transmembrane transport of the three drugs were mainly by passive diffusion pathway. But some investigations indicated atenolol and sotalol had slight polarized transport13,21. These minor inconsistencies may be caused by the varied cultural conditions of Caco-2 cells and different experimental circumstances from lab to lab. The study of Bachmakov et al.22 revealed that although propafenone was not the substrate of P-gp, the inhibitory activity in P-gp-mediated digoxin transport of (S)(+)- and (R)(−)-propafenone were different. (R)(−)-propafenone reduced the digoxin transport more significantly. Comparing with our result, (R)-propafenone has a little higher ER value than (S)(+)-isomer, indicating (R)(−)-propafenone may have higher affinity to P-gp which makes (R)(−)-isomer interact with digoxin more effectively. However, they all did not investigate whether the transport characteristics of the enantiomers were different. Because the enantiomers may differ in terms of pharmacological properties and disposition, stereoselective disposition of the enantiomers can arise from absorption of the enantiomers via intestine; therefore, uncovering the transport characteristics of enantiomers, which have been evaluated in this study, has significant meaning in predicting the pharmacological effect and pharmacokinetic behaviors of chiral drugs’ enantiomers.

The transport of cefepim and carvedilol were stereoselective and excretive, which were significantly different from the other four chiral drugs, and the similar transport behavior has been observed in esmolol23 and propranolol24 enantiomers in our previous studies. It has been proved that both cefepim and carvedilol are the substrates of P-gp.25,26 There are several articles investigated the interaction between cefepim and P-gp. Karlsson et al.15 reported that the secretion of cefepim could be inhibited by typical substrates of P-gp (such as vinblastine, verapamil and nifedipine), furthermore, cefepim inhibited the basal-to-apical transport of vinblastine. These results indicated that cefepim was transported by P-gp. In our observation, the P_app values for (S)(−)-cefepim were almost 3-fold larger than (R)-(−)-isomer in the absorptive direction, and in secretory direction, they were slightly smaller than (R)(−)-cefepim, which indicated that (S)(−)-isomer was easier to penetrate through biomembrane. So, the recognition of P-gp for (R)(−)-cefepim was much stronger than (S)(−)-isomer, and (R)(−)-isomer may be a more potent substrate for P-gp. The transports of cefepim enantiomers were concentration-dependent in our experiment. With the drug’s concentration increased, the transport rates raised. For (R)(−)-isomer, it raised all the way within the concentration range tested, while it dropped a little at the highest concentration for (S)(−)-isomer (Fig. 2). The reason for this phenomenon may because (S)(−)-isomer penetrated more than (R)(−)-isomer, so it reached the steady-stage of penetration more rapidly. The penetration at 960 μM has reached the maximum and the decreasing was caused by the experimental error. On the other hand, another reason could be undefined transport mechanisms existed to make (S)(−)-isomer easier to penetrate through biomembrane, and at high concentration these transport systems were inhibited which contributed to the less permeability of (S)(−)-isomer.

The transport behaviors of carvedilol enantiomers were different but no concentration-dependence was observed. In the concentration range studied, the P_app values of (S)(−)-isomer were higher than (R)(−)-isomer in absorptive direction, while in secretory direction, it was contrary (P<0.05). It is likely that an excretive transporter participated in the transport process across Caco-2 cell monolayers, and the transporter favors (R)(−)-enantionomer (Fig. 3). Several articles have reported carvedilol-digoxin interaction in children and adults27–29. Carvedilol could increase serum concentrations and the area under the plasma concentration time-curve of orally administered digoxin. As digoxin is a typical substrate of P-gp, so carvedilol is likely to have the ability to inhibit P-gp. This result has been proved on MDR1-transfected Madin-Darby canine kidney (MDR1-MDCK) cells; these MDR1-mediated reversing effects of carvedilol on vinblastine, paclitaxel, doxorubicin and daunorubicin were similar to those of verapamil29. Taken together, P-gp is likely to be one determinant of carvedilol disposition in humans.

Combined the results above and our pre-studies of esmolol and propranolol, we presume that the chiral recognition of P-gp may lead to the stereoselective transport. But more investigations are still needed to confirm that if P-gp indeed play a role in the enantioselective recognition. Because the interactions are restricted by the shape of the binding pocket, the conformation differences between enantiomers may lead to the chiral recognition and stereoselective transport of P-gp. Further studies in molecular model are needed to help us learn more about structure activity relationship between P-gp and these drugs.

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References