Absorption of Ester Prodrugs in Caco-2 and Rat Intestine Models

Xin He, Mitsuru Sugawara, Yoh Takekuma, and Katsumi Miyazaki*

Department of Pharmacy, Hokkaido University Hospital, Kita-ku, Sapporo 060-8648, Japan

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The aim of this study was to elucidate the absorption mechanism in Caco-2 and rat intestine models in order to improve the accuracy of prediction of oral absorption of ester prodrugs. Pivampicillin and cefcapene pivoxil hydrochloride (CFPN-PI), ester-type oral antibiotics, were chosen as model ester prodrugs. The level of esterase activity in Caco-2 cells was lower than that measured in the rat jejunum when p-nitrophenyl acetate was used as a substrate. Almost complete ester hydrolysis occurred before the ester prodrugs reached the basolateral side of the monolayer, and the disappearance of prodrugs was thought to be due to metabolism or transport after addition to the apical side of the monolayer. When pivampicillin and CFPN-PI were used, the amounts of ampicillin and cefcapene (CFPN) produced by hydrolysis of prodrugs were increased because intracellular degradation of prodrugs resulted in intracellular accumulation. On the other hand, when ampicillin or CFPN was used, only a small amount of the drug reached the basolateral side of the monolayers and no intracellular accumulation was observed. The permeability of CFPN-PI, the solubility of which is dependent on the acidity of gastric juice, across a Caco-2 monolayer or rat intestine, was also investigated by using an in vitro system that mimics the physiological state of the human gastrointestinal tract. The oral absorption of CFPN-PI in humans is predicted to be good either in the Caco-2 model or in the rat intestine model. It is concluded that our system may be a valuable tool for evaluation of oral absorption of ester prodrugs metabolized during permeation through the intestinal epithelium. Broader evaluation of such a system is warranted.

A monolayer of Caco-2 cell, cells derived from human colon adenocarcinoma, is a well-established in vitro model for prediction of the absorption of orally administered compounds in humans (6, 7). We previously reported the successful application of a Caco-2 cell monolayer or rat intestine for the prediction of absorption of relatively water-soluble drugs in humans by using an in vitro system that simulates the physiological condition in the gastrointestinal (GI) tract. In our previous study, prediction of the absorption of pivampicillin, which is the pivaloyloxymethyl ester of ampicillin, in humans was evaluated, and it was possible to predict the oral absorption of pivampicillin in humans based on the cumulative permeation of pivampicillin across a Caco-2 cell monolayer or a rat intestine using the in vitro system. However, the time course of permeation into the receiver compartment using a Caco-2 cell monolayer was different from that using a rat intestine (5). This difference was thought to be due to species differences and differences between enzyme activities in homogenates from Caco-2 cells and from the rat small intestine. The aim of this study was to elucidate the absorption mechanism in order to improve the accuracy of prediction of oral absorption of ester prodrugs.

pH-related variations in absorption in humans could be accurately predicted using the above-mentioned system that takes into account drastic pH change in the GI tract (4). Cefcapene pivoxil hydrochloride (CFPN-PI), an ester-type oral cephem antibiotic, has been used in Japan since 1997. CFPN-PI, the solubility of which is dependent on the acidity of gastric juice (3), is hydrolyzed by esterase in intestinal epithelial cells to CFPN, which has broad and potent antibiotic activity against gram-positive bacteria, including Staphylococcus aureus and Streptococcus spp., as well as gram-negative bacteria, including Escherichia coli and Serratia spp. Therefore, CFPN-PI was chosen as a model compound to evaluate oral absorption in humans using this system.

MATERIALS AND METHODS

Materials. Pivampicillin and ampicillin were kindly donated by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan), and Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. CFPN-PI and CFPN sodium were gifts from Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan). p-Nitrophenyl acetate was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fluorescein isothiocyanate (FITC)-dextran (molecular weight, 4,400) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were of the highest grade available and were used without further purification.

Animals. Male Wistar rats weighing 250 to 300 g (NRC Haruna, Gunma, Japan) were fasted overnight before use either in the permeation study or for harvesting intestinal tissue for homogenate preparation.

Cell culture. Caco-2 cells were purchased from the American Type Culture Collection (Rockville, Md.). The cells were routinely maintained in plastic culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, N.J.). These stock cells were subcultivated before reaching confluence. The growth medium was Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, Ohio), 1% nonessential amino acids (Gibco), and 4 mM glutamine without antibiotics. The monolayer cultures were grown in a CO₂ incubator (5% CO₂) at 37°C. The cells were harvested with 0.25% trypsin and 0.2% EDTA (0.5 to 1 min at 37°C), resuspended, and seeded into a new flask. Cells between the 29th and 52nd passages were used.

For the transport studies, Caco-2 cells were seeded on a Snapwell or a Transwell (0.4-μm pore size, 1-cm² growth area; Corning Costar Co.) at a cell density of 8 × 10⁴ cells/filter. The cell monolayer was fed a fresh growth medium every 2 days and was then used on the 20th to 28th days (Snapwell) or on the 10th to 14th days (Transwell) for the transport experiments. To evaluate the integrity of the monolayer, transepithelial electrical resistance (TEER) was measured using a Millicell-ERS (Millipore Corp., Bedford, Mass.). TEER of the filter was subtracted from the total TEER measurements of Caco-2 cell epithelia. The Caco-2...
monolayers were used when their TEERs were >600 Ω·cm². After the transport experiment was finished, the permeation rate of 100 μM FITC-dextran was measured to check that the barrier function had been maintained during the experiment. The permeation rate of FITC-dextran to the receiver compartment over a period of 1 h was less than 0.1%.

Studies of transepithelial transport and cell accumulation (1, 12). Prior to each experiment, Caco-2 monolayers on Transwell inserts were washed with transport medium. For the determination of transepithelial flux of ester prodrugs and their active metabolites across Caco-2 monolayers, the Caco-2 monolayers were preincubated with transport medium (pH 6.0) for 5 min. After which TEER values were measured to check monolayer integrity. The medium was then replaced by 0.5 ml of transport medium with the test compound on the apical side, and the basolateral side was bathed with 1.5 ml of transport buffer (pH 7.4). Prior to use in enzyme assays, protein content of all preparations was determined according to the method of Lowry et al. (9) using bovine serum albumin as a standard. Freshly scraped Caco-2 monolayers grown in a 75-cm² (1 cm²) cell disrupter for 5 min at 14,000 g. The samples were then centrifuged for 5 min, the supernatants were adjusted to 0.176 mg/200 μl. Initial rates of degradation of 100 μM p-nitrophenyl acetate by esterase in Caco-2 cell homogenate and homogenates from segments of rat intestinal mucosa were determined by adding 200 μl of enzyme preparation to 2 ml of prewarmed substrate solution. Two milliliters of ice-cold methanol was added and vortexed to stop enzymatic activity. The absorbance of the mixture was measured at 405 nm to determine the release of p-nitrophenol using a 557 Spectrophotometer (Hitachi Co., Ltd.). Initial rates (nanomoles per second) of p-nitrophenol formation were calculated from the slope of the first linear part of the absorbance-versus-time curve. Preliminary studies had shown linearity for the degradation of 100 μM p-nitrophenyl acetate between 15 and 60 s for both enzyme preparations. All results are expressed as nanomoles per second per milligram of protein.

Degradation of p-nitrophenyl acetate by homogenates of Caco-2 cells and rat intestine (1). Freshly scraped Caco-2 monolayers grown in a 75-cm² flask were homogenized in 5 ml of transport medium (TM) (Hanks’ balanced salt solution, supplemented with 10 mM HEPES and 25 mM glucose) using a cell disruptor for 20 s at 4°C. The jejunal of the rat was rapidly excised, and a 5-cm-long segment was cut along the longitudinal axis and washed with ice-cold Hanks’ balanced salt solution to remove intestinal contents. The intestinal mucosa of segments was scraped with a glass slide. The scrapings were homogenized at 4°C in 5 ml of cold TM using a cell disrupter for 20 s. After centrifugation of the crude Caco-2 and intestinal tissue homogenates at 14,000 × g for 5 min, the supernatants were harvested and kept at 4°C. Protein content of all preparations was determined by two-tailed Student test or one-way analysis of variance and set at P < 0.05.

Degradation of pivampicillin and CFPN-PI in the presence of homogenates of Caco-2 cells or rat intestine. Two milliliters of solution containing 10 μM of the ester prodrug pivampicillin or CFPN-PI was preincubated at 37°C, and 200 μl of homogenate from Caco-2 cells or from rat intestine was added. Samples (each 200 μl) were taken at predetermined time points and added to 200 μl methanol and vortexed to stop enzymatic activity. The samples were then centrifuged for 5 min at 14,000 × g. The supernatant was injected into an high-performance liquid chromatography column for determination of the ester prodrugs as well as their active metabolites.

Drug absorption prediction system. As shown in Fig. 1, our system for predicting drug absorption takes into account drug dissolution and pH change in the GI tract. In this system, CFPN-PI (15 mg, solid form) is added to a drug-dissolving vessel (modeled stomach, pH 1.8, 10 ml) and the dissolved drug is transferred to a pH adjustment vessel (modeled intestine, pH 6.0, 10 ml). Each of these vessels is a plastic vial. The compositions of the drug-dissolving solution...
TABLE 1. Compositions (millimolar) of flowing solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Drug-dissolving solution (pH 1.8)</th>
<th>pH adjustment solution* (pH 6.8)</th>
<th>Receiver solution* (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>10.7</td>
<td>5.37</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.88</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>61.4</td>
<td>199</td>
<td>137</td>
</tr>
<tr>
<td>Na₃HPO₄</td>
<td>0.68</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.52</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.81</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>13.6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td>13.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*pH was adjusted to 6.0 with Tris before addition of NaOH. *pH was adjusted to 7.4 with Tris.

(pH 1.8), pH adjustment solution (pH 6.8), and receiver solution (pH 7.4) are shown in Table 1. In the pH adjustment vessel, a solution (pH 6.0) prepared by mixing drug-dissolving solution and pH adjustment solution (1:1) was used. The flow rate (0.5 ml/min) of each solution is controlled by a peristaltic pump. The drug solution is transferred to the donor compartment of a side-by-side diffusion chamber (Corning Costar Co.). Mounted between the donor and receptor compartments is a Caco-2 monolayer grown on a Snapwell or a jejunum removed from a rat. The drug permeates to the receptor compartment of the side-by-side diffusion chamber and is collected by a fraction collector every 5 min over a period of 200 min. In order to prevent the hydrolysis of CFPN-PI, the samples obtained were frozen immediately. A silicon tube (inner diameter, 0.5 mm) was used to connect each vessel and the compartment. All of the solutions and both vessels and both compartments were preheated to 37°C and maintained at that temperature. When the jejunum of a rat was used, the buffer was preoxygenated with O₂-CO₂ (95:5) mixture gas. Under the condition of bubbling with mixture gas, transport of drugs from the donor compartment to the receptor compartment across the rat intestine was measured.

Analysis. The concentrations of drugs were determined by high-performance liquid chromatography (L-6000; Hitachi Co., Ltd, Tokyo, Japan) using an L-4200H UV-VIS detector (Hitachi). Separation of drugs was carried out on a reversed-phase column (ODS; Hitachi catalog no. 3053) at 55°C and maintained at that temperature. The elution time (495 nm) and an emission wavelength of 514 nm. The concentrations of drugs were determined by high-performance liquid chromatography (L-6000; Hitachi Co., Ltd, Tokyo, Japan) using an L-4200H UV-VIS detector (Hitachi). Separation of drugs was carried out on a reversed-phase column (ODS; Hitachi catalog no. 3053) at 55°C and maintained at that temperature. The elution time (495 nm) and an emission wavelength of 514 nm.

RESULTS AND DISCUSSION

Studies of ester hydrolysis, transepithelial transport, and esterase activity. Since most drug absorption from the GI tract occurs via passive processes, absorption is favored when the drug is in a nonionized and more lipophilic form. Ester prodrugs are commonly used to enhance membrane permeability of drugs that have an acidic moiety. Increase in the lipophilicity of the parent hydrophilic compounds results in enhanced transepithelial transport by passive diffusion (2, 14). The influence of metabolism on transepithelial flux of the prodrugs pivampicillin and CFPN-PI as well as their active metabolites ampicillin and CFPN was studied using Caco-2 cells. The rate of transport of each drug across Caco-2 monolayers appeared to be linear with time (data not shown). The results are given in Table 2. Higher \( P_{\text{app}} \) values were obtained for prodrugs than for active acids. When CFPN-PI was added to the apical side of the monolayers, only CFPN appeared and CFPN-PI was not detected in the basolateral side. On the other hand, when pivampicillin was used, ampicillin and only small amounts of pivampicillin were detected in the basolateral side. These results suggest extensive ester hydrolysis and an increased transepithelial flux of the active metabolite during transport. At the end of flux studies, the cell accumulation of ampicillin produced from pivampicillin in the Caco-2 monolayers was greater (\( P < 0.001 \)) than that of ampicillin when ampicillin was added to the apical side. The same tendency was found in the case of CFPN (Table 2). Intracellular degradation of prodrugs resulted in intracellular accumulation. From the results of studies on transport of ampicillin and CFPN, it was concluded that no intracellular entrapment occurred.

The esterase activities of Caco-2 cells and the small intestine of the rat were studied. p-Nitrophenyl acetate was used as a model substrate at a concentration of 100 μM. The initial rates of p-nitrophenol production were linear with time (data not shown). The results showed that the enzymatic activity in homogenates from the small intestines of rats (6.04 ± 1.78 nmol/s/mg of protein) was 13.4-fold higher than that in homogenates from Caco-2 cells (0.45 ± 0.01 nmol/s/mg of protein). This is in agreement with results reported by Augustijns et al. (1).

Esterase-mediated degradation of the prodrugs was assessed by incubation of both prodrugs with homogenates obtained from Caco-2 cells and the small intestines of rats. Ester hydrolysis is indicated in Fig. 2 by a decrease in the amounts of prodrugs and the appearance of active metabolites. It was shown that the disappearance occurs in accordance with the esterase activity of homogenates, and pivampicillin appeared to be a better substrate for homogenate from the rat intestine than CFPN-PI.

Absorption prediction studies. We have reported the successful application of a Caco-2 monolayer or rat intestine for the prediction of absorption of the water-soluble prodrug pivampicillin, the pivaloyloxymethyl ester of ampicillin, in humans (5). In this work, the amount of CFPN-PI eluted into the donor compartment of the side-by-side diffusion chamber after addition of CFPN-PI to the drug-dissolving vessel was measured using the system shown in Fig. 1. Measurement showed that 97.1% of CFPN-PI was eluted into the donor compartment. A very small amount of CFPN was also detected. The total amount of CFPN-PI eluted into the donor compartment, i.e., the sum of the amount of CFPN-PI and the amount of CFPN produced from CFPN-PI in the donor compartment,
was 97.9%. The time courses of efflux of CFPN-PI to the donor compartment are shown in Fig. 3a.

Permeation of CFPN-PI across a Caco-2 monolayer and a rat intestine using the system was also studied. The time courses of the permeation of CFPN-PI into the receiver compartment are shown in Fig. 3b. When 15 mg of CFPN-PI was added, CFPN produced from CFPN-PI was detected in the receiver compartment of the side-by-side diffusion chamber, but CFPN-PI was below the detectable limit. The calculation of the amount of decomposition of CFPN-PI was based on the amount of CFPN that was produced by hydrolysis of CFPN-PI. In the case of a Caco-2 monolayer, 2.53 µg of decomposed CFPN-PI was estimated based on the amount of CFPN produced (2.22 µg). The cumulative permeation of CFPN-PI was calculated to be 0.0168%. On the other hand, in the case of rat intestine, it was estimated that 4.28 µg of CFPN-PI was decomposed into CFPN (3.76 µg), and the cumulative permeation was 0.0285%. As found in our previous studies, relatively water-soluble drugs that permeated across a Caco-2 monolayer at cumulative permeation rate of more than 0.03% or at a rate over 0.04% in the rat intestine can be almost completely absorbed in humans. When the cumulative permeation rate across a Caco-2 monolayer is lower than 0.03% or below 0.04% in the rat intestine, there is a good linear correlation between cumulative permeation across a Caco-2 monolayer and oral absorption in humans (R = 0.967) or between cumulative permeation across a rat intestine and oral absorption in humans (R = 0.959) (5). Based on these results, the oral absorption of CFPN-PI is predicted to be 66.5% in the Caco-2 model and 77.1% in the rat intestine model (Fig. 4). Laboratory prediction of oral absorption is categorized by <40% in laboratory tests as poor, 40 to 60% as moderate, and >60% as good. The results were shown in Table 3.

CFPN-PI, with pKa of 3.7, is an ester prodrug of the active...
free-acid metabolite CFPN. The site of absorption of CFPN-PI was found to be the upper and middle parts of the small intestine in preclinical studies in animal models (rats and dogs), and the absorption ratios in experimental animals were found to be 25 and 35% in fasted and fed states, respectively, using a 14C-radiolabeled compound (10). Since it is impossible in Japan to administer a radiolabeled compound in humans, intestinal absorption of CFPN-PI must be predicted from the sum of the drug excreted in the urine and bile. The absorption ratio of CFPN was estimated on the basis of the urinary recovery ratio, because it is not metabolized in the body (8, 11) and almost all CFPN absorbed is excreted into urine but not into bile (13). The urinary recovery ratio of CFPN-PI in healthy adult volunteers within 6 h was reported to be 40% (11).

Absorption from the GI tract is affected by many factors, such as gastric emptying time, variation of gastric pH, intestinal motility, surface area for absorption, blood flow to the site of absorption, physical state of the drug, and its concentration at the site of absorption. Furthermore, regardless of the site, absorption is dependent on drug solubility. Our proposed system can accommodate large amounts of solid drugs, simulating the process of drastic pH change in the GI tract, and the dissolution process can also be monitored. In this system, the drug eluted into the donor compartment of the side-by-side diffusion chamber was proposed as almost 100%. However, in the case of humans, it is possible that the absorption of CFPN-PI is reduced due to incomplete dissolution in the intestine, because the solubility of CFPN-PI dropped sharply under an alkaline condition (3). Moreover, when CFPN-PI was used, the amount of CFPN produced by hydrolysis of CFPN-PI was increased because intracellular degradation of CFPN-PI resulted in intracellular accumulation of CFPN. However, if CFPN-PI is hydrolyzed before transport, CFPN produced from

![FIG. 4. Relationship between cumulative Caco-2 (a) or rat intestine (b) permeation and oral absorption in humans (data from reference 5). Predicted oral absorptions of CFPN-PI based on this relationship are plotted (■). Each point represents the mean with standard error of the mean (error bar) of three to five experiments.](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>Drug and model</th>
<th>Amt added to dissolving vessel (mg)</th>
<th>Total amt eluted in donor side (%)</th>
<th>Amt of parent drug detected in receiver side (µg)</th>
<th>Amt of active metabolite detected in receiver side (µg)</th>
<th>Amt decomposed (µg)</th>
<th>Cumulative permeation (%)</th>
<th>Laboratory prediction of absorption</th>
<th>Absorption in humans within 6 h (excreted in urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pivampicillin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Level</td>
<td>% Level</td>
</tr>
<tr>
<td>Caco-2</td>
<td>60</td>
<td>94.7 ± 1.8</td>
<td>8.55 ± 0.99</td>
<td>16.77 ± 3.03</td>
<td>22.25 ± 4.01</td>
<td>0.051 ± 0.008</td>
<td>100 Good</td>
<td>70 Good</td>
</tr>
<tr>
<td>Rat intestine</td>
<td>60</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>23.58 ± 2.72</td>
<td>31.29 ± 3.61</td>
<td>0.052 ± 0.006</td>
<td>100 Good</td>
</tr>
<tr>
<td>CFPN-PI</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.22 ± 0.53</td>
<td>2.53 ± 0.60</td>
<td>0.0168 ± 0.004</td>
<td>66.5 Good</td>
</tr>
<tr>
<td>Rat intestine</td>
<td>15</td>
<td>97.9 ± 2.9</td>
<td>ND</td>
<td>ND</td>
<td>3.76 ± 0.93</td>
<td>4.28 ± 1.06</td>
<td>0.0285 ± 0.008</td>
<td>77.1 Good</td>
</tr>
</tbody>
</table>

* Data are means ± SEM of the results of three to five experiments. Parent drug: pivampicillin, CFPN-PI; active metabolite: ampicillin, CFPN. The data about pivampicillin are from reference 5.

* ND, not detected.
CFPN-PI outside the cells almost cannot be absorbed along the GI tract. From these factors, we conclude that a relatively higher oral absorption of CFPN-PI predicted from Caco-2 or rat intestine model than that obtained from clinical experiment in humans is acceptable. Further experiments are in progress. In conclusion, our in vitro system may be a valuable tool for evaluation of oral absorption of ester prodrugs metabolized during permeation through the intestinal epithelium.

REFERENCES