Characterization of Secretory Intestinal Transport of Phenolsulfonphthalein

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Summary: It is known that secretory transport limits the oral bioavailability of certain drugs. However, there is little information on the secretion of anionic compounds in the intestine. Phenolsulfonphthalein (PSP) and p-aminohippuric acid (PAH) have been used widely as substrates for organic anion transport systems. PAH is transported in the secretory direction in the intestine. It is possible that PSP and PAH share the same transport system at the mucosal membrane. The purpose of this study was to characterize the transport system for PSP in the intestine. In the jejunum, the serosal-to-mucosal permeation rate of PSP was significantly reduced in an ATP-depleted condition, whereas a significant difference was not observed in the ileum. Some multidrug resistance-associated protein 2 (Mrp2) inhibitors inhibited PSP permeation in the jejunum. However, pravastatin, a substrate of Mrp2, did not inhibit the PSP permeation. The jejunal secretory transport of pravastatin was significantly reduced in an ATP-depleted condition and by addition of probenecid, but PSP did not affect the jejunal permeation of pravastatin. These results suggest that PSP is secreted into the intestinal lumen by Mrp2-like transporter and that two Mrp2 substrates, PSP and pravastatin, are likely to be transported by different transport systems at the mucosal membrane.

Key words: PSP; organic anion; intestinal secretion; Mrp2; pravastatin

Introduction

Various mechanisms can influence the intestinal absorption and oral bioavailability of drugs. Passive diffusion is commonly the most important mechanism of absorption. Permeation by diffusion is often predictable from a drug's physicochemical properties. A number of foreign weak electrolytes are thought to penetrate the intestinal mucosal barrier by passive diffusion of the nonionized drug species across a lipoidal membrane according to the pH-partition theory.\textsuperscript{1)} However, there have been numerous drugs exhibiting higher absorption rates after oral administration than expected from their physicochemical properties. Studies on the mechanisms of intestinal absorption of various ionic drugs have revealed that drug transporters can be classified into three systems: organic anion transport systems, organic cation transport systems, and peptide transport systems.\textsuperscript{2)} It has been reported that some drugs administered intravenously are secreted into the intestinal lumen in considerable amounts and that intestinal absorption of some compounds is limited partly because they are preferentially transported in the secretory direction.\textsuperscript{3–5)}

Among the transporters involved in secretion, P-glycoprotein (P-gp/Abcb1) has been the most extensively investigated. Although many investigations have demonstrated that various organic cationic compounds are excreted almost exclusively via P-gp, there have been few studies on intestinal secretion of other organic anions, and the precise mechanisms remain unclear.\textsuperscript{6,7)} Therefore it is important to clarify the transport mechanisms operating in the intestine. Recently, Naruhashi et al. reported that p-aminohippurate (PAH), an organic anion compound, is preferentially transported in the secretory direction in the intestine and that this process is mediated by multiple transporters.\textsuperscript{8)} It is possible that some anionic compounds are...
transported in the secretory direction via multiple transporters, resulting in poor bioavailability.

In addition to the transporters identified in the intestine, many transporters contributing to efflux/secretory transport exist in other tissues. More than 80 years ago, the results of landmark experiments using phenolsulfonphthalein (PSP, phenol red) carried out by Marshall and colleagues established the foundation for future developments in the realm of tubule secretory function. Results of subsequent studies initially using PSP and later PAH led to the development of several other important concepts. However, there is a curious difference between the behavior of PSP and that of PAH in vivo and in vitro. In addition to renal secretion, it has been reported that PSP is transported from the serosal to mucosal side of the rat intestine and that a P-gp-like transporter is involved in this permeation. However, there have only been a few studies on intestinal secretion of PSP. In this study, we used PSP as a model substrate to clarify the transport characteristics of organic anions in intestinal epithelial cells.

**Materials and Methods**

**Chemicals:** PSP, indomethacin, sulfobromophthalein (BSP), PAH, quinidine, sodium azide and sodium fluoride were purchased from Wako Pure Chemical (Osaka, Japan). Probenecid, fluorescein isothiocyanate (FITC)-dextran (molecular weight, 4,400) and 4,4'-diiodothiocyanostilbene (DIDS) were purchased from SIGMA (St Louis, MO). Pravastatin was kindly donated by Sankyo (Tokyo, Japan). All other reagents used in these experiments were of the highest grade available and used without further purification.

**Animals:** Male Wistar rats, aged 7 to 8 weeks (300–350 g in weight), were obtained from NRC Haruna (Gunma, Japan). The rats were housed at least for 1 week at 23 ± 3°C and 50 ± 10% relative humidity and were maintained on a 12-h light/dark cycle. During the acclimatization the rats were allowed free access to food and water. Animals were used without fasting before all experiments. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”.

**Everted sac studies:** Transport studies were carried out as described in a previous report with minor modification. The medium used for all experiments was Tyrode’s buffer (137 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄ and 6 mM D-glucose). The final pH of the buffer was adjusted with 1N HCl or NaOH. For everted sac studies, the jejunal and ileal were excised from the rat under anesthesia and rinsed in ice-cold saline. The intestinal segments were slid onto a glass rod and the epithelial surface was exposed. After washing the epithelial surface with ice-cold saline, 5-cm-long everted segments of intestine were isolated. These everted segments were each ligated at one end. Then 100 μL of an experimental solution containing a substrate (170 μM PSP or 100 μM pravastatin) and 100 μM FITC-dextran was injected into each segment, and each segment was ligated at the other end. The sac was immersed into 10 mL of the drug-free buffer. The buffer was prewarmed at 37°C and preoxygenated with O₂/CO₂ (95:5) mixture gas. Under bubbling with mixture gas, the amount of the substrate transported from the serosal to mucosal surfaces across the intestine was measured by sampling the mucosal buffer periodically for 60 min. All samples were analyzed by HPLC or fluorescent measurement as described below. The permeation rate of FITC-dextran was subtracted from that of PSP or pravastatin to obtain the intracellular permeation rate. In the energy-dependency studies, the buffer was preincubated at 37°C for 30 min in the presence of 10 mM sodium fluoride and 10 mM sodium azide.

**Analysis:** Substrates were determined using an HPLC system equipped with a Hitachi L-6000 pump and L-4200H UV/VIS detector described previously. The column was a Hitachi ODS Gel #3053 (4 mm i.d. × 250 mm, Hitachi, Tokyo, Japan). In the assay for PSP, a mobile phase containing 20% acetonitrile/50 mM H₃PO₄ in water, with pH adjusted to 3.0 by NaOH, was used. Each sample was eluted at a flow rate of 0.7 mL/min. In the assay for pravastatin, a mobile phase containing 30% acetonitrile/30 mM H₃PO₄ in water at a flow rate of 0.7 mL/min was used. The wavelengths of the detectors for PSP and pravastatin were 432 nm and 230 nm, respectively. The measurement of FITC-dextrane was carried out in a spectrophuorometer (F1000; Hitachi) with an excitation and emission wavelengths of 495 nm and 514 nm, respectively. Statistical significance was evaluated using ANOVA followed by a post hoc test or Student’s t-test. A value of P < 0.05 was considered significant.

**Results**

**Site specificity of the serosal-to-mucosal permeability of PSP:** The characteristics of the serosal-to-mucosal permeability of PSP were investigated in everted sac studies. It has been reported that the secretory intestinal transport of PAH required metabolic energy, but protons or hydroxyl ions were not involved as a driving force. To determine if the PSP transport requires metabolic energy, the effect of metabolic inhibitors on the serosal-to-mucosal permeability of PSP was investigated (Fig. 1). In the jejenum, the serosal-to-mucosal permeation of PSP was significantly decreased in the presence of metabolic inhibitors, whereas a significant difference was not observed in the ileum. Therefore, the...
Table 1. Effect of pH on the permeation of PSP from serosal to mucosal surfaces across the everted jejunum from Wistar rats

<table>
<thead>
<tr>
<th>Extracellular pH (serosal/mucosal)</th>
<th>Permeation (% of Control)</th>
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<tbody>
<tr>
<td>7.4/7.4</td>
<td>100</td>
</tr>
<tr>
<td>7.4/6.0</td>
<td>104.1 ± 18.4</td>
</tr>
<tr>
<td>6.0/7.4</td>
<td>94.2 ± 14.7</td>
</tr>
</tbody>
</table>

The concentration of PSP was 170 μM. Results were obtained at the end of a 60-min experiment. Each value represents the mean with S.D. of three determinations. The control value for the permeation of PSP was 0.98 ± 0.10 nmol/5-cm sac.

jejenum was used in the following experiments.

Effect of pH on the serosal-to-mucosal permeation of PSP across the everted jejunum: The effect of protons on the serosal-to-mucosal permeation of PSP was examined. As shown in Table 1, the effect of pH was negligible. Therefore, the following experiments were performed at pH 7.4.

Effect of probenecid on the serosal-to-mucosal permeation of PSP across the everted jejunum: Saitoh et al. reported that mucosal-to-serosal permeability of PSP was increased by the presence of probenecid on the serosal side and that serosal-to-mucosal permeation of PSP was not decreased by the presence of probenecid on the serosal side.12) We compared the inhibitory effect of probenecid present both on the serosal and mucosal sides and the inhibitory effect of probenecid present on the serosal side alone (Fig. 2). Serosal-to-mucosal permeation of PSP was significantly decreased by the presence of probenecid both on the serosal and mucosal sides. However, there was no significant inhibition by probenecid present on the serosal side alone. In subsequent inhibition experiments, an inhibitor was added to both mucosal and serosal sides to give a designated final concentration.

Effects of various compounds on the permeation of PSP across the everted jejunum: It has been reported that multidrug resistance-associated protein 2 (Mrp2/Abcc2) is expressed in the apical membrane of the epithelium of the small intestine and secretes various drugs into the jejunum lumen.15,16) Recently, we reported that PSP is a substrate of Mrp2.17) Thus, the effects of various compounds on serosal-to-mucosal permeability of phenolsulfonphthalein were investigated. As shown in Table 2, two known Mrp2 inhibitors, indomethacin and BSP, significantly inhibited PSP permeation in the jejunum. However, PAH and 1-naphtol, which inhibit organic anion secretion in the intestine,8,18) quinidine, a substrate of P-gp,19) and DIDS, an inhibitor of organic anion transporters,20) did not affect PSP
permeation in the jejunum. Moreover, pravastatin, a substrate of Mrp2 did not inhibit the PSP permeation.21)

**Effects of various compounds on the permeation of pravastatin across the everted jejunal**
It is possible that a transporter(s) other than Mrp2 plays a major role in the excretion of organic anions in the small intestine.8,18,22 In the second part of this study, transport properties of the serosal-to-mucosal permeation of pravastatin were investigated. Pravastatin permeation in the jejunum was reduced in an ATP-depleted condition (Table 3). The effects of probenecid and PSP on the permeation of pravastatin in the jejunum are shown in Table 3. Probenecid significantly inhibited pravastatin permeation in the jejunum, but PSP did not affect pravastatin permeation in the jejunum.

**Discussion**

Oral drug delivery is generally the most desirable means of administration, mainly because of patient acceptance, convenience in administration and cost-effective manufacturing. Drug absorption from the intestine or oral bioavailability is determined by various mechanisms. The high oral bioavailability of drugs has been explained, in part, by the existence of absorptive transporters in addition to passive diffusion mechanisms.23 On the other hand, it has been reported that secretory transport limits the oral bioavailability of certain drugs.24 Among these secretory transport systems, P-gp has been the most extensively investigated. It has been reported that P-gp mainly transports neutral or cationic drugs.23 However, there is little information on the secretion of anionic compounds in the intestine. Therefore, it is important to investigate the transport system(s) that contributes to the secretory transport of anionic compounds in the intestine.

Recently, Naruhashi et al. reported that PAH, an organic anion compound, is preferentially transported in the secretory direction in the intestine and that this process is mediated by multiple transporters.8 PAH is exclusively eliminated through the kidneys. The renal epithelial membrane transport of PAH involves exchange transport with dicarboxilic acid at the basolateral membrane via the human organic anion transporter.20 PSP is widely used clinically as a drug for testing renal function because of its high renal clearance.20 Recently, we reported that PSP as well as PAH is recognized by a rat organic anion transporter.26 Moreover, Saitoh et al. reported that PSP is transported from the serosal to mucosal sides of the rat intestine and that a P-gp like transporter is involved in this permeation.22 Thus, it is possible that PSP and PAH share the same transport system at the mucosal membrane. The purpose of this study was to characterize the intestinal efflux transport of PSP.

In the first part of this study, we investigated the serosal-to-mucosal permeability of PSP by everted sac studies. It has been reported that intestinal secretory transport of PAH requires metabolic energy.8 Therefore, to clarify the possible contribution of energy-dependent transporters to PSP transport in the intestine, the effect of metabolic inhibitors on the serosal-to-mucosal permeability of PSP was investigated. In the jejunum, metabolic inhibitors decreased PSP permeation, whereas a significant difference was not observed in the ileum. Furthermore, the effect of pH was negligible. These results suggest that jejunal secretory transport of PSP is likely to require metabolic energy, but protons or hydroxyl ions are unlikely to be the driving force for PSP transport.

Next, we compared the inhibitory effect of probenecid present both on the serosal and mucosal sides and the inhibitory effect of probenecid present on the serosal side. Serosal-to-mucosal permeation of PSP was sig-

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**Table 2. Effects of various compounds on the permeation of PSP from serosal to mucosal surfaces across the everted jejunum from Wistar rats**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Permeation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1 mM</td>
<td>46.4±8.52**</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>1 mM</td>
<td>58.4±13.4**</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>10 mM</td>
<td>107±21.9</td>
</tr>
<tr>
<td>PAH</td>
<td>1 mM</td>
<td>113±9.61</td>
</tr>
<tr>
<td>PAH</td>
<td>20 mM</td>
<td>98.1±18.1</td>
</tr>
<tr>
<td>1-Naphtol</td>
<td>1 mM</td>
<td>96.1±15.2</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1 mM</td>
<td>99.7±9.83</td>
</tr>
<tr>
<td>DIDS</td>
<td>500 μM</td>
<td>91.9±23.1</td>
</tr>
</tbody>
</table>

The concentration of PSP was 170 μM. Results were obtained at the end of a 60-min experiment. Each value represents the mean with S.D. of three to five determinations. The control value for the permeation of PSP was 1.19±0.27 nmol/5-cm sac. **P<.01, significantly different from that in the absence of compounds.

**Table 3. Effects of probenecid and PSP on the permeation of pravastatin from serosal to mucosal surfaces across the everted jejunum from Wistar rats**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Permeation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>ATP depletion</td>
<td>45.3±12.9**</td>
</tr>
<tr>
<td>1 mM Probenecid</td>
<td>68.4±4.98**</td>
</tr>
<tr>
<td>1 mM PSP</td>
<td>93.1±18.4</td>
</tr>
</tbody>
</table>

The concentration of pravastatin was 100 μM. Results were obtained at the end of a 60-min experiment. Each value represents the mean with S.D. of three to five determinations. The control value for the permeation of pravastatin was 1.34±0.14 nmol/5-cm sac. **P<.01, significantly different from the control.
significantly decreased by the presence of probenecid both on the serosal and mucosal sides, but there was no significant inhibition by probenecid present on the serosal side alone. These findings suggest that secretory transport of PSP occurs at the mucosal epithelium.

It has been reported that Mrp2 is present mainly in the mucosal membrane of the intestine and that the amount of Mrp2 gradually decreases from the jejunum to the distal ileum.\textsuperscript{15} Mrp2 is involved in the secretion of organic anions in the small intestine. Moreover, we have shown that PSP is a substrate of Mrp2.\textsuperscript{17} To characterize the secretory process for PSP by the intestine, the inhibitory effects of various compounds were determined. Three Mrp2 inhibitors, probenecid, indomethacin and BSP, significantly inhibited PSP permeation in the jejunum, whereas a significant difference was not observed in the presence of PAH. Since the secretory transport activity level of PAH is high in the lower region of the small intestine,\textsuperscript{8} it is possible that PAH did not affect the PSP permeation in the jejunum. Uwai et al. demonstrated that hydrophilic dicarboxylates with a backbone of five or more carbons, but not those with a backbone of only three or four carbons, are able to inhibit Oat1 (Slc22a6)-mediated PAH transport.\textsuperscript{27} In contrast, the preferred substrates for Oat3 (Slc22a8) are larger and more hydrophobic compounds such as estrone sulfate.\textsuperscript{28} We reported that PSP is a high-affinity substrate for rOat3 but is a relatively low-affinity substrate for rOat1.\textsuperscript{30} It is possible that substrates for the efflux transporter in the ileum include hydrophilic and small molecules such as PAH and that the preferred substrates for the efflux transporter in the jejunum are larger and more hydrophobic compounds such as PSP.

In this study, pravastatin, an Mrp2 substrate, had no effect on PSP permeation in the jejunum. In addition to pravastatin, PAH has been reported to be a substrate of Mrp2.\textsuperscript{29,30} The Km values of pravastatin and PAH for Mrp2 were 220 μM and 880 μM, respectively. The concentrations of pravastatin and PAH were 20-fold higher than the Km values for Mrp2. Vries et al. suggested that an intestinal organic anion transporter(s) that preferentially accepts Mrp2 substrates rather than Mrp2 is expressed on the mucosal membrane of both Wistar and TR\textsuperscript{-} rats.\textsuperscript{22} It is possible that total intestinal secretion of Mrp2 substrates is accounted for the contribution of Mrp2 and other transporter(s) to the intestinal secretion of Mrp2 substrates. It is possible that the contribution of Mrp2 to the intestinal secretion of PSP is markedly different from that of pravastatin and that PSP and pravastatin are mainly transported by different transport systems at the mucosal membrane. To confirm this hypothesis, we carried out a study on inhibition of secretion of pravastatin. Pravastatin permeation in the jejunum was reduced in an ATP-depleted condition. This result suggests that metabolic energy is likely to be involved as the driving force of pravastatin excretion in the jejunum. To characterize the secretory process for pravastatin by the intestine, the inhibitory effects of probenecid and PSP were determined. Probenecid significantly inhibited the pravastatin permeation in the jejunum, but PSP did not affect pravastatin permeation in the jejunum. These results further confirmed the hypothesis that PSP and pravastatin are mainly transported by different transport systems at the mucosal membrane. Moreover, it is possible that the contribution of Mrp2 to the intestinal secretion of PSP is markedly different from that of pravastatin. In addition to Mrp2, several other transporters are involved in intestinal secretion. It is essential to evaluate the contribution of Mrp2 and other transporters to the intestinal secretion of organic anions.

There are several problems for the development of oral delivery systems for drugs. One of the major problems is poor permeability through the intestinal mucosa. When the absorption of a drug candidate is poor, various approaches to improve absorption, such as prodrugs, analogs or coadministration of absorption enhancers are often undertaken. However, it is also important to understand whether poor absorption is due to the involvement of a secretory transport system and to develop an understanding of the substrate specificity of such a transport system.

In this study, we used PSP as a model substrate to clarify the transport characteristics of organic anions in intestinal epithelial cells. Although secretory transport of PSP occurs at the mucosal epithelium in vitro everted rat jejunum, PSP is exclusively eliminated through the kidneys in vivo and the contribution of intestinal secretion is minor. Secretion of organic anions involved the uptake of organic anions across basolateral membranes into cells and exit into the lumen across brush-border membranes. It is possible that the contribution of basolateral transporters to the uptake of PSP is accounted for the disposition of PSP.

In summary, PSP is actively secreted into the intestinal lumen by Mrp2 or Mrp2-like transporter. However, two Mrp2 substrates, PSP and pravastatin, are likely to be transported by different transport systems at the mucosal membrane. It is possible that the contribution of Mrp2 to the intestinal secretion of PSP is markedly different from that of pravastatin. The barrier effects of efflux transporters may be one of the critical factors limiting the bioavailability of certain drugs.

References


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