The Role of Inter-segmental Differences in P-glycoprotein Expression and Activity along the Rat Small Intestine in Causing the Double-peak Phenomenon of Substrate Plasma Concentration

Sho WADA1, Takashi KANO1, Suzune MITA1, Yoko IDOTA1, Kaori MORIMOTO1, Fumiyoshi YAMASHITA2 and Takuo OGIHARA1, *

1Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan
2Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

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Summary: Conflicting results have been reported on segmental differences in expression of P-glycoprotein (P-gp) along the small intestine of animals and humans. In this study, we investigated P-gp mRNA and protein levels within each of nine segments of rat small intestine. In addition, P-gp activity in each segment was evaluated in terms of permeability of rhodamine123 (Rho123), a typical P-gp substrate, using the serial intestinal non-everted sac method. The P-gp mRNA levels tended to increase from the duodenum to the ileum, with peaks in the upper and lower ileum, while P-gp protein level reached its maximum in the middle ileum. The activity of P-gp was also the highest in the middle ileum, and was highly correlated with P-gp protein level. The double-peaked plasma concentration profile that was observed following oral administration of Rho123 to rats could be well reproduced by an intestinal compartmental kinetic model incorporating inter-segmental differences of absorption and excretion rate constants. Our results suggest that the heterogeneous distribution of P-gp along the small intestine plays a key role in causing the double-peak of plasma concentration of P-gp substrates following oral administration to rats.

Keywords: permeability-glycoprotein (P-gp); rhodamine123; plasma double-peak phenomenon; small intestine; compartment model

Introduction

Permeability-glycoprotein (P-glycoprotein, P-gp, MDR1, ABCB1), which belongs to the ATP-binding cassette transporter superfamily, is extensively expressed not only in multidrug-resistant tumor cells1–3 such as carcinoma cells, lymphoma cells and leukemia cells, but also in the intestine, kidneys, brain, and adrenal glands of normal animals.4–7 P-gp plays an important role in the barrier function of these tissues by pumping xenobiotics out of cells. Many clinically used drugs such as paclitaxel,8 fexofenadine,9 and oseltamivir10,11 have been recognized as P-gp substrates, and thus, P-gp has received considerable attention as a potential determinant of the oral bioavailability of its substrates in humans and animals.12–16

Double-peaks of plasma concentration have often been observed following oral administration of P-gp substrates to humans and experimental animals. The double-peak is thought to result from various factors, among which inter-segmental variation of P-gp activity along the small intestine is considered important. It has been reported that vinblastine, which is a typical substrate of P-gp, was absorbed in the duodenum and ileum, but not in the jejunum, and a double-peak of vinblastine plasma concentration was observed in rats administered the drug orally.17 On the other hand, absorption of verapamil, another typical P-gp substrate, showed no remarkable variation among segments of the intestine and moreover, no double-peak of plasma concentration was observed in rats.17 Therefore, it has been suggested that P-gp substrates can be divided into at least two types, i.e. the vinblastine-type, for which the intestinal absorption is influenced by P-gp, and verapamil-type, for which the intestinal absorption is unaffected by P-gp. In other words, a link was suggested between the occurrence of double-peaks of plasma concentration and the inter-segmental differences of small-intestinal absorption of P-gp substrates. However, the mRNA levels and activities of P-gp were not consecutively quantified throughout the small intestine in that work, and no information was provided about P-gp protein levels. Moreover, no mathematical modeling was done to examine whether the double-peak phenomenon can be interpreted quanti-
tatively in terms of inter-segmental differences of P-gp expression and activity.

It has been reported that the mRNA level of P-gp simply increases along the small intestine, and many studies support this result. In this study, we divided the small intestine into nine segments and measured the mRNA and protein levels of P-gp in each segment. We then used the serial intestinal non-everted sac method with rhodamine123 (Rho123) as a P-gp substrate to investigate the P-gp activity in each segment, and examined the correlations of P-gp activity and mRNA and protein levels among intestinal segments. Moreover, because the double-peak phenomenon of plasma concentration was observed in rats orally administered Rho123, we performed model analysis to determine whether the double-peak of Rho123 plasma concentration could be explained by the inter-segmental differences of P-gp activity along the intestine. In addition, the influx permeability of Rho123 was low, and its efflux/influx ratio was more than 10 according to in vitro experiments using Caco-2 cells. These findings indicated that Rho123 was a typical P-gp substrate of vinblastine-type. Therefore we selected Rho123 as a substrate to observe the double peak phenomenon in the plasma concentration of rats.

Materials and Methods

Chemicals and animals: Rho123 was purchased from Sigma-Aldrich (St. Louis, MO). Verapamil hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The animal study was performed according to the Guidelines for the Care and Use of Laboratory Animals at Takasaki University of Health and Welfare and approved by the Committee of Ethics of Animal Experimentation of the university. Seven- or eight-week-old Wistar rats were purchased from SLC Japan (Hamamatsu, Japan).

mRNA analysis by real-time PCR: The 8-week-old male rats were deprived of food for 12 h before experimentation. The whole small intestine was quickly excised and washed several times with ice-cold phosphate-buffered saline (PBS). Mucosal cell layers on the small intestine were prepared as previously described. Brie

In vivo study using rats: Male rats were deprived of food overnight before experiments. Rho123 was dissolved in distilled water with or without verapamil (200 µM). The Rho123 solution was orally administered to rats in a single dose of 2 mg/10 mL/kg, and was intravenously administered to rats in a bolus dose of 0.2 mg/1 mL/kg. Blood samples were withdrawn from the jugular vein of rats with a heparinized syringe at designated times under anesthesia induced with diethyl ether. Blood samples were centrifuged (1,700 g) for 15 min at 4°C to obtain plasma. All samples were stored at 4°C after collection. The concentrations of Rho123 in plasma were determined as described below.

Assay method: The Rho123 concentrations in samples were measured immediately after the experiment. A 100 µL aliquot of each sample was transferred to a 96-well microplate, and the fluorescence of Rho123 in the sample was measured using a WALLAC Multilabel/Luminescence Counter (PerkinElmer, Waltham, MA) at wavelengths of 485 nm for excitation and 538 nm for emission.

Mathematical analyses of plasma concentration of Rho123: A model incorporating the P-gp activities and the gastrointestinal (GI) transit rate constants were evaluated (Fig. 1). Distribution volume and the elimination rate constant (ke) of Rho123 were
calculated from the results of the in vivo study described above. The gastric emptying rate constant (dX0/dt), the transit rate constant for each segment (dXn/dt) and the absorption rate constants into the central compartment (dY/dt) are represented as follows:

\[
\frac{dX_0}{dt} = -k_1 \cdot X_0
\]

\[
\frac{dX_n}{dt} = k_n \cdot X_{n-1} + \frac{1}{k_{yn}} \cdot Y - (k_{syn} + k_{n+1}) \cdot X_n
\]

\[
\frac{dY}{dt} = \sum (k_{syn} \cdot X_n) - \left(\sum k_{yn} + ke\right) \cdot Y \quad (n = 1 \sim 9)
\]

Y: central compartment, X0: gastric compartment, Xn: intestinal compartment, kn (n = 1–9): GI transit rate constant, ksyn: absorption rate constant from Xn to Y, kyn (n = 1–9): intestinal excretion rate constant from Y to Xn.

**Correlation and statistical analysis:** Correlations among all of these parameters, mRNA of P-gp, protein level of P-gp, Rho123 permeability, ksyn and kyn in intestinal segments were examined. The correlation between kn and reported GI transit rate constants was also examined. Data for the transport experiment and the plasma concentration of Rho123 were expressed as mean ± S.E.M. Statistical analysis was performed using Student’s t-test. In particular, the paired t-test for pharmacokinetics study was utilized. The differences and the correlations between means were considered to be significant when the p-value was less than 0.05.

**Results**

**Segmental differences of mRNA and protein levels of P-gp along the small intestine:** The mRNA and protein levels of P-gp in the nine segments of small intestine were examined. The mRNA levels tended to increase from the duodenum to the sixth segment (upper ileum; the first peak), followed by a higher peak at the ninth segment (lowest part of ileum; the second peak) (Fig. 2). Similarly, the protein levels increased from the duodenum to the seventh segment (middle ileum), which showed the maximum level of P-gp protein (Fig. 3).

**Serosal-to-mucosal Rho123 transport by the serial intestinal non-everted sac method:** Three sacs (the duodenum, the lowest part of the jejunum, and the lowest part of the ileum) were selected from the nine sacs of the small intestine, and the time course of Rho123 transport from the serosal to the mucosal side was studied (Fig. 4A). The transport of Rho123 reached the steady state at 30 min. Thus, the incubation time of 30 min was used in subsequent studies. Then, the serosal-to-mucosal transport of Rho123 at the nine intestinal sacs was compared (Fig. 4B). Generally, the transport of Rho123 was higher at the lower part of the small intestine. The maximum absorption was observed in the seventh segment (middle ileum). The inter-segmental difference disappeared when 200 µM verapamil was added concurrently (Fig. 4B).

**Plasma concentration profiles for Rho123 after oral and intravenous administration to rats:** Figure 5 shows the plasma concentration of Rho123 after oral administration of Rho123 (2 mg/kg) to rats. Distribution volume of Rho123 was 2.70 L/kg, and ke value was 0.39 h⁻¹, when this compound was intravenously administrated to rats. A double-peak of plasma concentration was observed when Rho123 alone was orally administered to rats. The first maximum was 18.3 ng/mL at 30 min, and this was followed by a higher peak of 19.1 ng/mL at 1 h after administration. When we normalized the higher peak in the first or second peak, the statistical difference between each peak and trough was observed (p < 0.05).
On the other hand, when 200 µM verapamil was administered concurrently, the double-peak of Rho123 disappeared, and bioavailability of Rho123 was increased from 6.0% to 7.7%.

Mathematical analysis of the double-peak phenomenon of Rho123: We attempted to model the double-peak phenomenon of Rho123 plasma concentration in rats by considering the segmental differential absorption rate, intestinal excretion rate and GI transit rate in the nine intestinal compartments (Fig. 1). Converged values of the parameters in the model are summarized in Table 1. The double-peak of Rho123 plasma concentration was well reproduced in the fitting curve in this model (Fig. 6). The proportionality constant of \( k_{yn} \) to Rho123 permeability was 0.039 loop/µL/h.

Correlation analysis: The correlation coefficients among observed parameters are summarized in Table 2. No significant correlation between mRNA and protein levels of P-gp in the segments was observed. Rho123 permeability correlated with both mRNA and protein levels of P-gp in the segments was observed.

The \( k_{yn} \) value was inversely correlated with Rho123 permeability obtained by means of the non-everted sac method. Moreover, \( k_n \) correlated well with reported GI transit rate constants in the GITA model28) (Supplemental Figs. 1 and 2).

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**Table 1. Converged parameters**

<table>
<thead>
<tr>
<th>( n )</th>
<th>( k_n(\text{h}^{-1}) )</th>
<th>( k_{yn}(\text{h}^{-1}) )</th>
<th>( k_{yn}(\text{h}^{-1}) )</th>
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<tr>
<td>1</td>
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<td>16.86</td>
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<td>0.017</td>
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<td>6</td>
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<td>0.330</td>
<td>0.733</td>
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<td>1.000</td>
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<tr>
<td>9</td>
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<td>0.097</td>
<td>0.817</td>
</tr>
<tr>
<td>10</td>
<td>0.38</td>
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**Table 2. Correlation coefficients**

<table>
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<th>mRNA</th>
<th>Protein</th>
<th>Rho123 permeability</th>
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<td>0.05</td>
<td>0.03</td>
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**Fig. 4. Serosal-to-mucosal transport of Rho123 (3 µM) by rat small-intestinal segments, determined by the non-everted sac method**

(A) Duodenum, middle jejunum, and terminal ileum were selected, and the excreted amount of Rho123 was measured at 37°C in HBSS (pH 7.4) containing 3 µM Rho123. Each point is the mean ± S.E.M. of three experiments. *p < 0.05, significant difference from duodenum.

(B) Excreted amount of Rho123 was measured in the absence (white column) and presence (black column) of 200 µM verapamil for 30 min at 37°C in HBSS (pH 7.4) containing 3 µM Rho123. Each point is the mean ± S.E.M. of three experiments. *p < 0.05, significant difference from duodenum.

**Fig. 5. Plasma concentration of Rho123 after oral administration (2 mg/kg) in the absence or presence of 200 µM verapamil**

Sampling times were 10, 20, 30, and 40 min, and 1, 2, 3, 4, and 6 h after oral administration. Rho123 dissolved in distilled water (open circle) or in 200 µM verapamil solution (closed circle) was orally administered. Each point is the mean ± S.E.M. of three experiments.

**Fig. 6. Observed plasma concentrations of Rho123 (open circle) and those calculated (solid line) by a compartment model**
of P-gp. It has been reported that protein expression levels are addition, mRNA levels of P-gp did not correlate with protein levels activity. Thus, P-gp expression does not simply increase along the Fig. 4B

The time-concentration profile after oral administration of Rhod123 to rats showed a typical double-peak of plasma concentration (Fig. 5). The double-peak disappeared when 200 μM verapamil was concurrently administered. These results suggested that P-gp activity in the small intestine might be involved in the double-peak phenomenon, in accordance with previous results. 

Previously, Kagan et al. investigated the role of P-gp in region-specific GI absorption of talinolol, a P-gp substrate, in rats. They observed the typical double peak in plasma concentration profile, with oral dosing of this compound. They also proposed a model with three divided compartments in the small intestine and wall compartments to explain the plasma concentration profile. However, the reappearance of the double peak profile was imperfect. Therefore, we developed a model incorporating the P-gp activities of the nine intestinal segments (Fig. 1). We also incorporated inter-segment GI transit rate constants (k1–k10) into our compartment model, because the GI transit rate constant was reported to be an essential parameter for predicting the absorption behavior of orally administered drugs.

The fitting curve of the model, in which compartmental excretion rate was fixed as a factor proportional to Rhod123 permeability well reproduced the double-peak phenomenon found in the in vivo study (Fig. 6). kmax values were inversely correlated with the Rhod123 permeability. It has been reported that P-gp acts not only as an absorption barrier by transporting its substrates from epithelial cells into the intestinal lumen but also as intestinal excretion from blood into the lumen. Therefore, P-gp plays important roles in both kmax and kmin, which correspond to intestinal absorption and intestinal excretion, respectively.

Considering that the amount of Rhod123 excreted in bile is not large, these results suggest that GI transit rate constant and segmental differences of P-gp activity are important for the double-peak phenomenon of P-gp substrate plasma concentrations. There are many reports about factors that influence the double-peak phenomenon, including intestinal pH, GI transit rate constant, absorption site of drug, enterohepatic circulation and gastric emptying rate constant. However, our results indicate that inter-segmental differences in the expression of P-gp along the small intestine play a key role in generating the double-peak of plasma concentration of P-gp substrates.

In conclusion, our intestinal compartment model indicated that inter-segmental differences of P-gp activity along the small intestine are a key factor in causing the double-peak phenomenon in the plasma concentration of P-gp substrates. This model should also be applicable to analyze the double-peaks of plasma concentration of other P-gp substrates, such as amiodarone, fexofenadine, vinblastine and oseltamivir, in experimental animals and humans. On the other hand, a simpler model with a number of minimum essential rate-constants and a number of compartments in which the double-peak phenomenon appears in plasma concentration could be established. In any event, further study is needed.

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