Drug Metab. Pharmacokinet. 28 (2): 98–103 (2013).

Regular Article

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 WADA[1], Takashi KANO[1], Suzune MITA[1], Yoko IDOTA[1], Kaori MORIMOTO[1], Fumiyoshi YAMASHITA[2] and Takuo OGIHARA[1,*]

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

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

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 cells[1–3] 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 oseltamivir[10,11] 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-

Received January 13, 2012; Accepted July 19, 2012
J-STAGE Advance Published Date: July 31, 2012, doi:10.2133/dmpk.DMPK-12-RG-005
**To whom correspondence should be addressed:* Takuo OGIHARA, Ph.D., *Laboratory of Biopharmaceutics, Department of Pharmacology, Faculty of Pharmacy, Takasaki University of Health and Welfare, 60, Nakaorui-machi, Takasaki 370-0033, Japan. Tel. +81-27-352-1180, Fax. +81-27-352-1118, E-mail: togihara@takasaki-u.ac.jp*

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.[18–24] 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.[25] These findings indicated that Rho123 was a typical P-gp substrate of vinblastine-type.[17] 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 ice were scraped off and immediately added to RNAlater™ RNA Stabilization Reagent (QIAGEN, Hilden, Germany). Total RNA was isolated using an RNeasy Mini Kit (QIAGEN), and 5 μg RNA from each sample was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The resulting cDNA was amplified by THUNDERBIRD™ qPCR Mix (TOYOBO, Osaka, Japan). The primer pairs used were as follows: P-gp (forward, 5′-agtggtgggcagaagcagagga-3′; reverse, 5′-tcaacttcatttcctgctgtctgc-3′), and hGAPDH (forward, 5′-agatcatcagcaatgcctcc-3′; reverse, 5′-tgtggtcatgagtccttcca-3′). The specificity was checked by analyzing the melting curves, and results were calculated using the comparative cycle threshold method, in which the amount of target mRNA was normalized to the internal control hGAPDH.

Western blot analysis: The sample proteins of the small intestine were prepared as previously described.[26] Briefly, the 8-week-old male rats were deprived of food for 12 h before experimentation, and the small intestine was quickly removed and washed with ice-cold saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Mucosa was scraped off with a slide glass and homogenized in buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4) and 1 mM PMSF. The homogenate was centrifuged at 3,000 × *g* for 10 min to get rid of the nuclear and mitochondrial fractions, and the supernatant was again centrifuged at 15,000 × *g* for 30 min. The pellet was resuspended in 0.5 mL of a buffer containing 50 mM mannitol, 50 mM Tris-HCl (pH 7.4), and 1 mM PMSF, and stored at −30°C until use. Cellular protein was determined using a protein assay kit with bovine serum albumin as a standard.

Sample protein (50 μg) was loaded onto e-PAGEL (5–12%) (ATTO Corporation, Tokyo, Japan), electrophoresed for 2 h, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 5% skim milk and incubated for 2 h at ambient temperature with a primary antibody, mouse anti-P-gp C219 (Signet, Dedham, MA) or mouse anti-Na^+/K^+ ATPase (Sigma-Aldrich),[27] and for 1 h with secondary antibody, anti-mouse Ig HRP-linked antibody. Bands were visualized using an enhanced chemiluminescence detection method with the ECL Plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK). Band intensity of the Western blot was analyzed using NIH Image (http://rsb.info.nih.gov/nih-image/). For relative quantification, the optical density value was determined for equal-size pixels drawn around bands, with background values taken below each band of interest to account for non-specific antibody staining in the lane. Each density value of target protein was normalized to the membrane marker Na^+/K^+ ATPase.

Serosal-to-mucosal transport experiment using the serial intestinal non-everted sac method: Male rats were fasted overnight and anesthetized with diethyl ether. The whole small intestine was quickly excised after ligation of the bile duct, and washed several times in ice-cold saline (0.9% NaCl). The inner side of the small intestine (mucosal side) was filled with HBSS (pH 6.0) solution, and tied at both ends to form a non-everted sac. Nine sacs (each 10 cm in length) of segments (*i.e.*, duodenum, upper jejunum, middle jejunum, lower jejunum, distal jejunum, upper ileum, middle ileum, lower ileum, and terminal ileum) were prepared. These sacs were then immersed in HBSS (pH 7.4) containing 3 μM Rho123 with or without 200 μM verapamil. After a designated time, the inner solution was collected with a syringe for quantitation of the transported Rho123. Rho123 permeability was obtained by dividing the transported amounts by the concentration of Rho123 in the medium.

***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[28] were evaluated (**Fig. 1**). Distribution volume and the elimination rate constant (ke) of Rho123 were