Regular Article

Highlighted Paper selected by Editor-in-Chief

Transport Characteristics of 5-Aminosalicylic Acid Derivatives Conjugated with Amino Acids via Human H⁺-Coupled Oligopeptide Transporter PEPT1

Tatsushi Yuri, Yusuke Kono, Tomofumi Okada, Tomohiro Terada, Seiji Miyauchi, and Takuya Fujita

*Graduate School of Pharmaceutical Sciences, Ritsumeikan University; 1–1–1 Nohjihigashi, Kusatsu, Shiga 525–8577, Japan; †Ritsumeikan Global-Innovation Research Organization (R-GIRO), Ritsumeikan University; 1–1–1 Nohjihigashi, Kusatsu, Shiga 525–8577, Japan; ‡College of Pharmaceutical Sciences, Ritsumeikan University; 1–1–1 Nohjihigashi, Kusatsu, Shiga 525–8577, Japan; and §Faculty of Pharmaceutical Sciences, Toho University; Funabashi, Chiba 274–8510, Japan.

Received November 28, 2019; accepted January 27, 2020

5-Aminosalicylic acid (5-ASA) is used as first line therapy for symptom remission and maintenance of inflammatory bowel disease (IBD). Because 5-ASA is well absorbed from the small intestine when orally administered, several 5-ASA formulations for selective delivery to the colon have been developed and used in clinical practice. However, its delivery efficiency to local inflamed colonic sites remains low. Intestinal H⁺-coupled oligopeptide transporter 1 (PEPT1) expression in the colon is low, whereas its expression is induced in the colon under chronic inflammation conditions, such as IBD. Therefore, we considered that PEPT1 would be a target transporter to improve 5-ASA delivery efficiency to local colonic lesions. We evaluated the transport characteristics of dipeptide-like 5-ASA derivatives, which were coupling glycine (Gly), lysine, glutamic acid (Glu), valine (Val) and tyrosine to amino or carboxyl group of 5-ASA, in Caco-2 cells. [³H]Gly-cylsarcosine (Gly-Sar) uptake into Caco-2 cells was inhibited by all 5-ASA derivatives. In addition, 5-ASA derivatives (Gly-ASA, Glu-ASA and Val-ASA), which were coupled by glycine, glutamic acid and valine to amino group of 5-ASA, were taken up in a pH- and concentration-dependent manner and their uptake was inhibited by excess Gly-Sar. Two-electrode voltage-clamp experiment using human PEPT1 expressing Xenopus oocytes showed that Gly-ASA, Glu-ASA and Val-ASA induced marked currents at pH 6.0. Taken together, these results showed that these 5-ASA derivatives are transportable substrates for PEPT1.

Key words 5-aminosalicylic acid; prodrug; Caco-2 cell; oligopeptide transporter

INTRODUCTION

5-Aminosalicylic acid (5-ASA) is a conventional therapeutic for inflammatory bowel disease (IBD) and is used as a first line drug for remission induction and maintenance in IBD patients. Additionally, a very high 5-ASA dose (1.5–3.0 g/d) is usually required for IBD treatment because intestinal absorption of orally administered conventional 5-ASA is rapid and efficient in the upper small intestine. This absorption of 5-ASA might cause some adverse effects, such as nephrotic syndrome, myopericarditis and fever, and could result in a low concentration in the colonic region. Therefore, its oral delivery efficiency to the inflamed local colon is low. The ideal 5-ASA colonic delivery system should decrease the systemic absorption from the small intestine while increasing delivery efficacy of the drug to the colon. To resolve this issue, several new 5-ASA dosage forms (pH-dependent controlled release capsule and enteric coat) have been developed for the efficient targeting of the colon.

In addition to new dosage formulations, various 5-ASA prodrugs improve its delivery efficacy to the colon. 5-ASA has two interactive functional groups, carboxylate and amine, which easily react with various chemical reagents such as polysaccharides, polymers and amino acids. Many types of polysaccharides have been used as non-toxic chemical agents for the design of 5-ASA prodrugs. Polysaccharides could attach to the 5-ASA via ester bonds (such as dextran, hydroxypropyl methylcellulose or chondroitin sulfate) or an amide bond (chitosan) based on their functional group. These macromolecular 5-ASA prodrugs could not be absorbed from the small intestine. Furthermore, they might be water soluble and release 5-ASA in media containing rat cecal and colonic content.

Amino acids are among the moieties that have been used to prepare 5-ASA prodrugs. Based on these functional groups, amino acids could attach to the 5-ASA via ester, amide or azo bonds. In particular, amide bond conjugates of 5-ASA with amino acids are structurally similar to dipeptide. Therefore, these 5-ASA conjugates might be recognized as dipeptides and might be transported into the cells via H⁺-coupled oligopeptide transporter PEPT1.

PEPT1 expressed in the brush border membranes of small intestinal epithelial cells accepts small peptide, which comprises two or three amino acids as substrates. In addition to di/tripeptides, PEPT1 is responsible for the intestinal absorption of peptide-like drugs such as oral β-lactam antibiotics. Therefore, PEPT1 has been used as a target transporter to improve the intestinal absorption of poorly absorbed compounds with amino acid modification. In clinical practice, the oral bioavailability of valacyclovir and valganciclovir,
amino acid prodrugs of acyclovir and ganciclovir, respectively, has increased because of PEPT1-mediated transport compared with parent drugs.\textsuperscript{18,19} Interestingly, while PEPT1 is not detectable or poorly expressed under normal conditions,\textsuperscript{14,20} colonic PEPT1 is up-regulated in IBD.\textsuperscript{18,19}

Here, we report the transport characteristics of dipeptide-like 5-ASA derivatives conjugated with several amino acids (glycine (Gly), lysine (Lys), glutamic acid (Glu), valine (Val) and tyrosine (Tyr)) via PEPT1 in Caco-2 cells, their electrophysiological properties utilizing heterologous expression of human PEPT1 (hPEPT1) in Xenopus laevis (X. laevis) oocytes. We expect that targeting PEPT1 contribute to the enhancement of the therapeutic effect of 5-ASA and the reduction of the non-specific distribution, and consequently reduce the adverse effects.

**MATERIALS AND METHODS**

**Materials** 5-ASA derivatives conjugated with amino acid such as glycine, lysine, glutamic acid, valine and tyrosine into NH$_2$ or COOH-group of 5-ASA (Fig. 1) were purchased from the Chemical Soft Co., Ltd. development laboratory (Mie, Japan). $[^3]$H-Glycylsarcosine (Gly-Sar; specific radioactivity, 29.4 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). Gly-Sar was purchased from Sigma-Aldrich Japan (Tokyo, Japan). 5-ASA was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 2-(N-Morpholino)ethanesulfonate (Mes), 2-[4-(2-hydroxyethyl)-a-piperazinyl]ethanesulfonic acid (Hepes) and tris(hydroxyymethyl)aminomethane (Tris) were obtained from Nacalai Tesque (Kyoto, Japan). Culture reagents were obtained from Gibco Life Technologies (Waltham, MA, U.S.A.) or Nacalai Tesque. Other chemicals used were of the highest purity available.

**Cell Culture and Uptake Studies** The human colon adenocarcinoma cell line Caco-2 was obtained from DS Pharma Biomedical (Osaka, Japan). Caco-2 cells were routinely cultured with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100U/mL penicillin G and 100 µg/mL.\textsuperscript{23,24} Caco-2 cells were subcultured in 24-well plate at a seeding density of 3 × 10$^5$ cells/cm$^2$. Uptake of $[^3]$H-Gly-Sar was measured 19-23 d after seeding at 37°C as described previously.\textsuperscript{23} In most experiments, the uptake buffer comprised 25mM Mes/Tris (pH 6.0), 140mM NaCl, 5.4mM KCl, 1.8mM CaCl$_2$, 0.8mM MgSO$_4$ and 5mM d-glucose. Uptake was initiated by removing the culture medium from the 24-well plate, washing the cells with 1.0mL uptake buffer and adding 250µL of the fresh uptake buffer containing $[^3]$H-Gly-Sar ($2µCi; 60nM$) and unlabeled Gly-Sar. After incubation for 15 min, the buffer was removed by aspiration and the cells were washed twice with 2.0mL ice-cold uptake buffer. The cells were solubilized with 500µL 0.2M NaOH/1% sodium dodecyl sulfate (SDS), aliquots (400µL) of samples were transferred to a counting vial and the radioactivity associated with the cells was measured using liquid scintillation spectrometry (Model LS6000; Beckmann, Fullerton, CA, U.S.A.).

In experiments using 5-ASA and 5-ASA derivatives, extraction solution (water/acetonitrile = 10/90) was added to the cells after the uptake period. After standing for 1 h at room temperature, the solutions were centrifuged (13500 ×g for 10 min at 4°C) and the supernatants were evaporated at 60°C. The pellets were resolved with mobile phase for HPLC analysis and were subjected to HPLC determination.

**Preparation of Caco-2 Cell Homogenate and Degradation of 5-ASA Derivatives** Caco-2 cells grown for 15–18 d were washed twice with ice-cold Ca$^{2+}$/Mg$^{2+}$-free phosphate buffered saline (PBS(-)), detached by a scraper and homogenized using a Teflon protein assay kit (Thermo Fisher Scientific) using bovine serum albumin as a standard. The reaction was terminated by adding 10-excess volume of acetonitrile at appropriate time. The reaction mixtures were centrifuged at 13500 ×g for 10 min at 4°C and the resultant supernatants were evaporated at 60°C. The pellets were resolved with mobile phase for HPLC analysis and subjected to HPLC determination.

**X. laevis Oocytes Expressing hPEPT1 and Electrophysiology** The procedures for preparation of oocytes from X. laevis, synthesis of human PEPT1 cRNA and microinjection of cRNA into the oocytes have been described previously.\textsuperscript{25,26} Surgically, mature oocytes (stage IV or V) from Xenopus laevis were injected with 50nL RNA solution containing 50ng human PEPT1 cRNA. All electrophysiological measurements were performed after 3–6 d by incubating oocytes in buffer comprising 88 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 0.33 mM Ca(NO$_3$)$_2$, 2.4 mM NaHCO$_3$ and 10 mM Mes/Tris at pH 6.5 (modified Barth’s solution). The membrane potential was clamped at −50mV. The oocyte was superfused with the perfusion buffer (96mM NaCl, 2mM KCl, 1mM MgCl$_2$, 1.8mM CaCl$_2$, 5mM Hepes/Tris (pH 7.5)). After the current stabilized, the oocyte was superfused with NaCl buffer (100mM NaCl, 2mM KCl, 1mM MgCl$_2$, 1mM CaCl$_2$, 10mM Mes/Tris (pH 6.0)), followed by the same buffer containing Gly-Sar (0.25mM) and/or 5-ASA derivatives (1mM). After application of the test compound, the currents were recorded; once the currents reached the maximum and steady.
where the test compound was washed out with the perfusion buffer (pH 7.5). The substrate-induced current at a given membrane potential was calculated as the difference between the steady-state currents recorded in the absence and presence of substrate.

**HPLC Measurement of 5-ASA Derivatives**

5-ASA derivatives were quantified according to a reversed-phase HPLC (Shimadzu, Kyoto, Japan) with a fluorescence detector (RF-10XL) and COOSMOSIL 5C18-AR-II column (3.0 mm i.d. × 150 mm; Nacalai Tesque). The eluent was 20 mM phosphate buffer (pH 2.2): methanol = 80:20 for ASA-Val, ASA-Tyr, Val-ASA and Tyr-ASA. Fluorescence detection was done at excitation 311 nm and emission 449 nm. The injection volume was 5 μL and total flow rate was 0.4 mL/min.

**Data Analysis**

For saturation kinetics of Gly-Sar and 5-ASA derivatives transport, the data were fitted to the Michaelis–Menten equation:

\[
V = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

where \( V_{\text{max}} \) is the maximal velocity of test compound transport, \( K_m \) is the Michaelis constant, \( S \) is the test compound concentration. To confirm the single transport system of test compound uptake in Caco-2 cells, Eadie-Hofstee transformation was performed:

\[
V' = V_{\text{max}} - K_m \frac{V'}{[S]}
\]

where \( V' \) is the saturable component of test compound transport.

To calculate the half maximal inhibitory concentration (IC50), the data were fitted to the sigmoidal-logistic 3 parameter equation:

\[
V = \frac{A}{1 + \left(\frac{[S]}{IC_{50}}\right)^n}
\]

Where \( A \) is the transport velocity in the absence of the tested inhibitory compound and \( n \) is Hill coefficient.

Linear or non-linear regression analyses were performed using SigmaPlot (version 13.0; Jandel Scientific, San Rafael, CA, U.S.A.). The fitting quality was determined by evaluating the correlation coefficient \( r \) and standard error of parameter estimations. Results are expressed as mean ± standard deviation (S.D.). Statistical comparisons were performed with unpaired or paired t test and ANOVA, as appropriate.

**RESULTS**

### Degradation Properties of 5-ASA Derivatives in Caco-2 Cell Homogenate

To evaluate the stability of 5-ASA derivatives, we initially assessed the degradation properties of 5-ASA derivatives in Caco-2 cells homogenate. All 5-ASA derivatives were stable in PBS(−) at 37°C. Moreover, 5-ASA derivatives conjugated with amino acid into the carboxyl group of 5-ASA (ASA-amino acid derivatives) were not degraded in Caco-2 cell homogenate, whereas the derivatives conjugated with amino acid into the amino group of 5-ASA (amino acid-ASA derivatives: Gly-ASA, Lys-ASA, Glu-ASA, Val-ASA and Tyr-ASA) were converted to parent drug (5-ASA) in Caco-2 cell homogenate (Supplementary Fig. 1). Their half-lives \( (t_{1/2}) \) were approximately 199, 3.2, 136, 30, and 53 min, respectively (Table 1). Therefore, protease inhibitors cocktail was added to the uptake buffer.

### Effects of 5-ASA Derivatives on Gly-Sar Uptake in Caco-2 Cells

Uptake of Gly-Sar into Caco-2 cells is driven like 5-ASA derivatives impact on PEPT1-mediated uptake PEPT1 system. 27,28)

**Table 1. Degradation Rate Constants and Half-Lifves of 5-ASA Derivatives in Caco-2 Cell Homogenate**

| Derivative | Half-life (min) |
|------------|----------------|
| ASA-Gly    | No degradation |
| ASA-Lys    | No degradation |
| ASA-Glu    | No degradation |
| ASA-Val    | No degradation |
| ASA-Tyr    | No degradation |

The Caco-2 homogenate (1 mg protein/mL) was mixed with an equal volume of 10 μM 5-ASA derivatives, and incubated at 37°C for an appropriate time. The apparent first-order degradation rate constants \( k_{\text{app}} \) of 5-ASA derivatives were determined by plotting the logarithm of 5-ASA derivatives remaining as a function time. The degradation half-life was calculated by the following equation: \( t_{1/2} = \ln 2/k \). Results are expressed as mean ± S.D. (n = 4).

### Table 2. Inhibitory Effects of 5-ASA Derivatives on Gly-Sar Uptake in Caco-2 Cells

| Unlabeled compound | [3H]Gly-Sar uptake pmol/mg protein/15 min (%) |
|--------------------|---------------------------------------------|
| None               | 166 ± 5.62 | 100 |
| Gly-Sar            | 15.0 ± 0.50 | 9 |
| 5-ASA              | 79.1 ± 9.93 | 48 |
| ASA-Gly            | 56.3 ± 8.75 | 34 |
| ASA-Lys            | 39.1 ± 0.45 | 23 |
| ASA-Glu            | 2.85 ± 0.72 | 2 |
| ASA-Val            | 21.6 ± 1.60 | 13 |
| ASA-Tyr            | 14.2 ± 4.59 | 9 |
| Gly-ASA            | 11.5 ± 3.39 | 7 |
| Lys-ASA            | 24.1 ± 10.6 | 14 |
| Glu-ASA            | 5.01 ± 1.00 | 3 |
| Val-ASA            | 3.95 ± 0.27 | 2 |
| Tyr-ASA            | 12.1 ± 1.28 | 7 |

[3H]Gly-Sar (1 μM) uptake was measured in Caco-2 cells at an extracellular pH of 6.0. Unlabeled compounds were used at a concentration of 10 mM. Results are expressed as mean ± S.D. (n = 3).
Fig. 2. Influence of pH on 5-ASA Amino Acid Derivative Uptake in Caco-2 Cells

Uptake of (A) ASA-amino acid derivatives (1 mM) and (B) amino acid-ASA derivatives (1 mM) was measured in Caco-2 cells. The uptake was conducted for 15 min in the uptake buffers of varying pH. Values are mean ± S.D. (n = 4).
We compared the influence of extracellular pH on amino acid-ASA and ASA-amino acid derivative uptake (Fig. 2). Because 5-ASA derivatives were partially metabolized to 5-ASA and each amino acid with in Caco-2 cells (Table 1), uptake amounts of 5-ASA derivatives were calculated as the sum of the amounts of the unchanged and degraded form. ASA-amino acid derivative uptake decreased when the pH changed from 5.0 to 8.5 (Fig. 2A). The influence of pH on amino acid-ASA derivative uptake differed greatly from that on ASA-amino acid derivative uptake (Fig. 2B). The process of amino acid-ASA derivative uptake showed a distinct pH optimum around pH 6.0. Increasing the pH from 5.0 to 6.0 stimulated amino acid-ASA derivative uptake, but a further increase above pH 6.5 reduced this uptake.

We next assessed the concentration-dependent inhibitory effect of Gly-Sar on 5-ASA derivative uptake in Caco-2 cells (Fig. 3A). 5-ASA derivative uptake experiments were performed with a 15-min incubation period because we confirmed that the cellular uptake of 5-ASA was linear for up to 15 min.

Fig. 3. Inhibitory Effect of Gly-Sar on the Uptake of 5-ASA and 5-ASA Amino Acid Derivatives in Caco-2 Cells

Uptake of 1 mM ASA-amino acid derivatives (A) and amino acid-ASA derivatives (B) was measured with a 15-min incubation in the uptake buffer at pH 6.0 over a Gly-Sar concentration range of 0–10 mM. Each point represents mean ± S.D. (n = 4).
30 min (data not shown). Gly-Sar did not change cellular uptake of ASA-amino acid derivatives. However, cellular uptake of Gly-ASA, Glu-ASA and Val-ASA were decreased by Gly-Sar addition in a concentration dependent manner; their IC_{50} values were 3.4 ± 0.6 mM, 4.6 ± 1.5 mM and 10.2 ± 2.1 mM, respectively (r^2 = 0.91, 0.70 and 0.78, respectively; Fig. 3B). Lys-ASA and Tyr-ASA uptake was not affected by Gly-Sar addition even at a concentration of 10 mM. These results suggest that three 5-ASA derivatives, Gly-ASA, Glu-ASA and Val-ASA, are preferentially recognized and transported by PEPT1 as substrates.

Figure 4 shows the uptake of amino acid-ASA derivatives into Caco-2 cells that occurred via a saturable process. The cellular uptake of three compounds, Gly-ASA, Glu-ASA and Val-ASA was saturable with K_m values of 1.3 ± 0.1 mM, 1.0 ± 0.1 mM and 0.59 ± 0.08 mM (r^2 = 0.99, 0.95 and 0.95), respectively. In addition, the V_{max} value was 487 ± 13.1 nmol/mg protein/15 min for Gly-ASA, 362 ± 17.5 nmol/mg protein/15 min for Glu-ASA, and 591 ± 21.2 nmol/mg protein/15 min for Val-ASA. However, Lys-ASA and Tyr-ASA uptake into Caco-2 cells showed linear profiles (Fig. 4). Cellular uptake of 5-ASA was also linear (data not shown). When the cellular uptake amount at 0.5 mM was compared, Gly-ASA, Glu-ASA and Val-ASA showed higher uptake than 5-ASA (Table 3). On the other hand, the uptake of Lys-ASA and Tyr-ASA in Caco-2 cells was lower than that of 5-ASA because of their poor affinity for PEPT1.

### Identification of 5-ASA Derivatives Transport in Caco-2 Cells
Figures 3B and 4 indicate that cellular uptake of Gly-ASA, Glu-ASA and Val-ASA might be mediated by PEPT1. We therefore selected Val-ASA as a representative derivative and investigated the kinetic nature of inhibition of PEPT1-mediated Gly-Sar transport caused by Val-ASA (Fig. 5). In the absence of Val-ASA, Gly-Sar transport was saturable with a K_m value of 2.1 ± 0.1 mM and a V_{max} value of 315 ± 2.7 nmol/mg protein/15 min (r^2 = 0.99). The presence of 0.5 mM Val-ASA further increased the K_m value to 10.5 ± 0.7 mM, but V_{max} value did not change significantly (404 ± 13.27 nmol/mg protein/15 min; r^2 = 0.99). Moreover, as shown in Fig. 5B, the Gly-Sar uptake was inhibited by Val-ASA in a concentration-dependent manner, and the IC_{50} value was 0.14 ± 0.026 mM (r^2 = 0.99). These data show that Val-ASA is a competitive inhibitor of PEPT1-mediated Gly-Sar transport and the apparent K_i value of Val-ASA for PEPT1-mediated Gly-Sar transport is 0.14 mM. This value is relatively comparable with the K_m value (0.59 mM) for Val-ASA transport in Caco-2 cells (Fig. 4). These results indicate that the transport system of Val-ASA is identical to that of Gly-Sar in Caco-2 cells which is mediated by PEPT1.

### Electrophysiological Characterization of hPEPT1-Mediated Transport of 5-ASA Derivatives
hPEPT1 transports di/tripeptide and orally active β-lactam antibiotics in an electrogenic manner; the transport process is coupled to the transport of H^+ with a 1:1 stoichiometry for H^+ and substrate. Therefore, the transport activity of hPEPT1 for any compound as a potential substrate can be monitored by determining if the compound could induce inward currents in hPEPT1-

---

Table 3. Uptake Amounts of 5-ASA and 5-ASA Derivatives in Caco-2 Cells

| Uptake (nmol/mg protein/15 min) | 5-ASA | Gly-ASA | Lys-ASA | Glu-ASA | Val-ASA | Tyr-ASA |
|---------------------------------|-------|---------|---------|---------|---------|---------|
| 5-ASA                           | 110 ± 3.76 | 126 ± 2.24 | 6.69 ± 0.36 | 122 ± 9.01 | 271 ± 32.2 | 34.2 ± 5.28 |

Uptake of 5-ASA (0.5 mM) or 5-ASA derivatives (0.5 mM) was measured in Caco-2 cells with a 15-min incubation in the uptake buffer at pH 6.0. Results are expressed as mean ± S.D. (n = 4).
expressing oocytes. Three compounds of 5-ASA derivatives, Gly-ASA, Glu-ASA and Val-ASA, induced significant inward currents (44 ± 6.2, 56 ± 11 and 59 ± 8.6% compared with normalized Gly-Sar current; Fig. 6A). All examined 5-ASA derivatives inhibited Gly-Sar uptake when uptake was measured using radiolabeled tracer (Table 2). This shows that all 5-ASA derivatives compete with [3H]Gly-Sar in the uptake process, but only Gly-ASA, Glu-ASA and Val-ASA are actually transported across the oocyte membrane and thus could induce inward current. We then characterized the transport of these compounds further using the same electrophysiological approach. As shown in Fig. 6B, the I–V relationship for the induced currents exhibited a slightly positive slope for the above three compounds, which indicates that hyperpolarization enhanced the substrate-induced currents. Conversely, the I–V relationship for the current induced by Lys-ASA exhibited a small but detectable negative slope, which suggests that this particular derivative might inhibit the constitutive inward current of H+ through hPEPT1, which might be driven by the intracellular negative membrane potential even in the absence of a transportable substrate.

DISCUSSION

In this study, we investigated the PEPT1-mediated transport of dipeptide-like 5-ASA derivatives in Caco-2 cells. Although the mechanism of 5-ASA efficacy has not been clarified enough, it is considered to inhibit cyclooxygenases and lipooxygenases, scavenge effective radicals, and inhibit nuclear factor-kappaB (NF-κB) activation in the large intestinal inflamed epithelium.29,30 Therefore, PEPT1-targeted delivery of 5-ASA could not only decrease its non-specific distribution but also improve the therapeutic effect.

The derivatives modified with amino acids in the carboxyl group of 5-ASA were stable in Caco-2 homogenate (Table 1). Conversely, the derivatives modified with amino acids in the amino group of 5-ASA were hydrolyzed in Caco-2 homogenate (Table 1, Supplementary Fig. 1). These results suggest that the type of amino acid and the introduced position of 5-ASA might be determining factors for recognition by hydrolytic enzymes. The degradation of the compounds with dipeptide-like structures seems to be mediated by metabolic enzymes such as aminopeptidases and carboxylpeptidases. Various aminopeptidases are functionally expressed in Caco-2 cells and each aminopeptidase has a different affinity to small peptide structure, whereas the enzymatic activity of carboxylpeptidases in Caco-2 cells is very low or none.31,32 In our previous study, chemical modification of phenylalanylglycine (Phe-Gly) with fatty acids to the N-terminal portion was more stable than the native Phe-Gly.33,34 However, Phe-Gly chemically modified with fatty acid to the C-terminal portion exhibited no significant increase in stability compared with the
native Phe-Gly. These indicated that the introduction of fatty acid to the N-terminal portion might reduce Phe-Gly degradation against aminopeptidases, which are abundantly expressed in the brush border of the intestinal epithelium. Therefore, it is reasonable that amino acid-ASA derivatives are more rapidly degraded than ASA-amino acid derivatives and their degradation rates differ depending on the amino acid. In fact, aminopeptidase activities in human large intestine have been reported to be 10-fold higher than those in Caco-2 cells. Therefore, 5-ASA derivatives would be more rapidly degraded in human large intestine tract.22,23)

Uptake of Gly-Sar into Caco-2 cells is driven by an inwardly directed H+ gradient and mediated by PEPT1. Gly-Sar was used as a model dipeptide because it is highly resistant to both plasma membrane and intracellular dipeptidases. We therefore investigated the effect of a high concentration (10 mM) of 5-ASA and various 5-ASA derivatives on [3H]Gly-Sar uptake in Caco-2 cells (Table 2). All compounds inhibited Gly-Sar uptake. However, uptake of 5-ASA and ASA-amino acid derivatives into Caco-2 cells was not affected by increasing Gly-Sar concentration (Fig. 3A), which indicates that these compounds were not accepted by PEPT1. We assume that these compounds may bind to the substrate-binding site of PEPT1 and subsequently inhibit Gly-Sar uptake, but they would not be transported via PEPT1. However, amino acid-ASA derivatives, especially Gly-ASA, Glu-ASA and Val-ASA, inhibited Gly-Sar uptake in a concentration-dependent manner (Fig. 3B) and their cellular uptake was saturable with K_m values of 1.3 ± 0.1, 1.0 ± 0.1 and 0.59 ± 0.08 mM, respectively (Fig. 4). These results indicate that Gly-ASA, Glu-ASA and Val-ASA might be substrates for PEPT1. In addition, comparing the cellular uptake amount at 0.5 mM, Gly-ASA, Glu-ASA and Val-ASA showed higher uptake than 5-ASA (Table 3). Since these 5-ASA derivatives are predominantly transported into Caco-2 cells by PEPT1, these derivatives would be transported into the inflamed epithelium more efficiently and selectively than 5-ASA.

In addition to the cellular uptake study, we directly investigated the charge transfer with PEPT1-mediated transport of amino acid-ASAs in Xenopus oocytes expressing PEPT1. At pH 6.0, Gly-ASA, Glu-ASA and Val-ASA evoked marked inward currents (Fig. 6). Conversely, Lys-ASA and Tyr-ASA elicited a weak signal. These results agree very well with data obtained in Caco-2 cells. Interestingly, in Caco-2 cells, the uptake of Gly-Sar (1 mM) is lower than those of 5-ASA derivatives (1 mM) (Figs. 4, 5A), whereas in Xenopus oocytes, the induced current by Gly-Sar (0.25 mM) was higher than those of 5-ASA derivatives (1 mM). At present, the reason for this discrepancy is not known. We hypothesize that posttranslational modifications may play a role. When cloned PEPT1 was expressed heterologously in Xenopus oocytes, transport activity was measured as inwardly current. Posttranslational modifications of PEPT1 such as glycosylation in Xenopus oocytes might be different from those in well-differentiated Caco-2 cells, and therefore posttranslational modifications of the transporter protein are likely to occur these conditions.

PEPT1 has broad recognition of substrates and can transport the compounds with not only di/tripeptide structure29,30 but also amino acid esters.18,37,38 Furthermore, Börner et al.29 screened transport of alanine (Ala) amide derivatives in Caco-2 cells and Xenopus oocytes expressing PEPT1 and demonstrated that PEPT1 accepts alanine aryl amides as substrates. Taken together with the above reports and our present results, the conjugation position of amino acid might play a key role in the interaction of 5-ASA derivatives with the substrate-binding site of PEPT1 and transport in intact form across the plasma membrane. PEPT1 is reported to be most abundantly expressed in the small intestine, with the highest expression in the duodenum and lower levels toward the ileum, and failed to detect its expression in colon.42,43 However, controversial reports suggested the presence of PEPT1 in healthy colons of mice, rabbits and humans44–47 and expression in the colonic cells that is upregulated under intestinal inflammatory conditions. Furthermore, the mucosal pH in colon tends to decrease in inflammatory conditions such as IBD45 because of the reduced mucosal bicarbonate secretion and increased mucosal and bacterial lactate and short-chain fatty acid (acetate, propionate and butyrate) production. Nugent et al. reported that the pH in the large intestine is 5.3–6.8 at the onset of IBD.45 Therefore, upregulated PEPT1 in the IBD colon would properly function and transport amino acid 5-ASA derivatives to the inflamed lesion. Furthermore, increased expression of PEPT1 is reported to be involved in the induction and progression of IBD by transporting the inflammatory peptide, such as N-formylmethionyl-leucyl-phenylalanine (fMLP), muramyl dipeptide (MDP) and L-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP), produced from microbiota in colon.46) Therefore, we assume that 5-ASA derivatives could inhibit the transport of inflammatory small peptides into the large intestinal epithelium via PEPT1 and this may enhance the therapeutic effect of the derivatives.

Because PEPT1 is highly expressed in the small intestine compared with the large intestine, Gly-ASA, Glu-ASA and Val-ASA are more rapidly absorbed than a parent drug (5-ASA) in the small intestine. Therefore, colon selective delivery systems should be used to avoid absorption from the small intestine. We have already reported 5-ASA colon-specific delivery system using chitosan capsules and azopolymer.47–49 We are preparing chitosan-microcapsules incorporating 5-ASA derivatives and evaluating their therapeutic effect in rat colitis. Using this formulation, 5-ASA derivatives might reach the large intestine efficiently and might be selective accumulated in inflamed regions.

Acknowledgments We would like to thank Ms. Hitomi Yamashima and Mr. Takahiro Nishi (Ritsumeikan University) for technical assistance. This study was supported in part by a Grant from the Strategic Research Foundation at Private Universities and a Grant-in-Aid for Scientific Research (C) (Grant Number: 17K08430) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ritsumeikan Global Innovation Research Organization (R-GIRO) Project at Ritsumeikan University and the Sasagawa Scientific Research Grant from The Japan Science Society.

Author Contributions Participated in research design: Yuri, Kono, Terada, Fujita. Conducted experiments: Yuri, Okada, Miyauchi. Contributed reagents or analytic tools: Yuri, Kono, Miyauchi, Fujita. Performed data analysis: Yuri, Kono, Miyauchi, Fujita. Wrote or contributed to the writing of the manuscript: Yuri, Kono, Fujita.
Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Ham M, Moss AC. Mesalamine in the treatment and maintenance of remission of ulcerative colitis. Expert. Rev. Clin. Pharmacol., 5, 113–121 (2012).
2) Abinusawa A, Tenjara S. Release of 5-aminosalicylic acid (5-ASA) from mesalamine formulations at various pH levels. Adv. Ther., 32, 477–484 (2015).
3) Novis BH, Koznets Z, Chen P, Bernheim J. Nephrotic syndrome after treatment with 5-aminosalicylic acid. BMJ, 296, 1442 (1988).
4) Nair AG, Cross RR. Mesalamine-induced myopericarditis in a pediatric patient with Crohn’s disease. Cardiol. Young, 25, 383–386 (2015).
5) Slim R, Amara J, Nasras R, Honein K, Jaoude JB, Yaghi C, Dinali F, Sayegh R. Isolated fever induced by mesalamine treatment. World J. Gastroenterol., 19, 1147–1149 (2013).
6) Goyanes A, Hatton GB, Merchant HA, Basit AW. Gastrointestinal release behaviour of modified-release drug products: dynamic dissolution testing of mesalazine formulations. Int. J. Pharm., 484, 103–108 (2015).
7) Chourasia MK, Jain SK. Pharmaceutical approaches to colon targeted drug delivery systems. J. Pharm. Pharm. Sci., 6, 33–66 (2003).
8) Shahdadi Sardo H, Saremnejad F, Bagheri S, Akhgari A, Afrasiabi, Vahid. The online version of this article contains supplementary materials.
9) Clerici C, Gentili G, Boschetti E, Santucci C, Aburbeh AG, Nanni M, Iseki S, Tsuji A. Immunolocalization and pharmacological relevance of oligopeptide transporter PepT1 in intestinal absorption of β-lactam antibiotics. FEBS Lett., 392, 25–29 (1996).
10) Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng T, Heder MA, Madara JL. Colon epithelial hPepT1 expression occurs in inflammatory bowel disease: transport of bacterial peptides influences expression of MHC class I molecules. Gastroenterology, 120, 1666–16/9 (2001).
11) Wojtal KA, Eloranta JJ, Hruez P, Gutmann H, Drewe J, Staumann B, Gutmann H, Drewe J, Staumann B. Expression cloning of a cDNA from rabbit small intestine related to protein-coupled oligopeptide transporter over-expressed in Hela cells: electrogenic efflux and existence of a newly observed channel-like state. Arch. Biochem. Biophys., 445, 165–176 (2003).
12) Fujita T, Majikawa Y, Umehisa S, Okada N, Yamamoto A, Ganapathy V, Leibach FH. S. Enhancement of the small intestinal uptake of phenylalanylglycine via a H+/oligopeptide transporter over-expressed in Hela cells: electrogenic efflux and existence of a newly observed channel-like state. Arch. Biochem. Biophys., 445, 165–176 (2003).
13) Mackenzie B, Liu DW, Fei Y, Liu WJ, Ganapathy V, Leibach FH, Wright EM. Mechanisms of the human intestinal H+ -coupled oligopeptide transporter hPepT1. J. Biol. Chem., 271, 5430–5437 (1996).
14) Fujisawa Y, Kitagawa T, Miyake S, Nara T, Kamo N, Miyachi S. Measurement of electric current evoked by substrate transport via bi-directional H+ /oligopeptide transporter over-expressed in Hela cells: electrogenic efflux and existence of a newly observed channel-like state. Arch. Biochem. Biophys., 445, 165–176 (2003).
15) Saito H, Inui K. Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line CaCo-2. Am. J. Physiol., 265, G289–G294 (1993).
16) Brandsch M, Miyamoto Y, Ganapathy V, Leibach FH, Expression and protein kinase C-dependent regulation of peptide/H+ co-transport system in the CaCo-2 human colon carcinoma cell line. Biochem. J., 299, 253–260 (1994).
17) Peskar BM, Dreyling KW, May B, Schaarschmidt K, Goebell H. Possible mode of action of 6-aminosalicylic acid. Dig. Dis. Sci., 52 (Suppl. 1), 515–568 (1997).
18) Irlan J, Jawele DP. Mechanism of action of 5-aminosalicylic acid and its derivatives. Clin. Sci., 78, 119–125 (1990).
19) Howell S, Kenny AJ, Turner AJ. A survey of membrane peptides in two human colonic cell lines, CaCo-2 and HT-29. Biochem. J., 284, 505–601 (1992).
20) Quan YS, Fujita T, Tohara D, Tsuji M, Kohyama Y, Yamamoto A, Muranishi S. Enhancement of the small intestinal uptake of phenylalanine glycine via a H+/oligopeptide transport system by chemical modification with fatty acids. Life Sci., 64, 1243–1252 (1999).
21) Fujita T, Morishita Y, Ito H, Kuribayashi D, Yamamoto A, Muranishi S. Enhancement of the small intestinal uptake of phenylalanine glycine (Phe-Gly) across the intestinal membrane by chemical modification with various fatty acids. Drug Metab. Pharmacokinetic., 18, 33–32 (2003).

Exp. Ther., 275, 1631–1637 (1995).

18) Ganapathy ME, Huang W, Wang H, Ganapathy V, Leibach FH, Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. Biochem. Biophys. Res. Commun., 246, 470–475 (1998).
19) Balimane PV, Tamai I, Guo A, Nakanishi T, Kitada H, Leibach FH, Tsuji A, Sinko PJ. Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. Biochem. Biophys. Res. Commun., 250, 246–251 (1998).
20) Sai Y, Tamai I, Sunikawa H, Hayashi K, Nakanishi T, Amano O, Numata M, Iseki S, Tsuji A. Immunolocalization and pharmacological relevance of oligopeptide transporter PepT1 in intestinal absorption of β-lactam antibiotics. FEBS Lett., 392, 25–29 (1996).
21) Merlin D, Si-Tahar M, Sitaraman SV, Eastburn K, Williams I, Liu X, Heder MA, Madara JL. Colon specific delivery systems for 5-aminosalicylic acid and cystine. Drug Synthesis and properties of μ-N,N'-bis(5-aminosalicyl)-L-cystine as a colon-specific deliverer of 5-aminosalicylic acid and cystine. Drug, 299, 253–260 (1994).
22) Kono Y, Iwasaki A, Matsuoka K, Fujita T. Effect of mechanical agitation on cationic liposome transport across an unstirred water layer in CaCo-2 Cells. Biol. Pharm. Bull., 39, 1293–1299 (2016).
23) Novis BH, Korzets Z, Chen P, Bernheim J. Nephrotic syndrome after treatment with 5-aminosalicylic acid. BMJ, 296, 1442 (1988).
24) Abinusawa A, Tenjarla S. Release of 5-aminosalicylic acid (5-ASA) from mesalamine formulations at various pH levels. Adv. Ther., 32, 477–484 (2015).
25) Goyanes A, Hatton GB, Merchant HA, Basit AW. Gastrointestinal release behaviour of modified-release drug products: dynamic dissolution testing of mesalazine formulations. Int. J. Pharm., 484, 103–108 (2015).
26) Fujita T, Majikawa Y, Umehisa S, Okada N, Yamamoto A, Ganapathy V, Leibach FH. Receptor ligand-induced up-regulation of the H+ /peptide transporter PEPT1 in the human intestinal cell line CaCo-2. Biochem. Biophys. Res. Commun., 261, 242–246 (1999).
27) Chourasia MK, Jain SK. Pharmaceutical approaches to colon targeted drug delivery systems. J. Pharm. Pharm. Sci., 6, 33–66 (2003).
35) Acartürk F, Parlutan ZI. A comparative study on the rectal amino-peptidase enzymatic activities of different species. Pharmazie, 58, 211–213 (2003).

36) Tamai I, Nakahashi T, Nakahara H, Sai Y, Ganapathy V, Leibach FH, Tsuji A. Improvement of L-dopa absorption by dipeptidyl derivation, utilizing peptide transporter PepT1. J. Pharm. Sci., 87, 1542–1546 (1998).

37) Sugawara M, Huang W, Fei YJ, Leibach FH, Ganapathy V, Ganapathy ME. Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. J. Pharm. Sci., 89, 781–789 (2000).

38) Tao W, Zhao D, Sun M, Wang Z, Lin B, Bao Y, Li Y, He Z, Sun Y, Sun J. Intestinal absorption and activation of decitabine amino acid ester prodrugs mediated by peptide transporter PEPT1 and enterocyte enzymes. Int. J. Pharm., 541, 64–71 (2018).

39) Börner V, Fei YJ, Hartrodt B, Ganapathy V, Leibach FH, Neubert K, Brandsch M. Transport of amino acid aryl amides by the intestinal H+/peptide cotransport system, PEPT1. Eur. J. Biochem., 255, 698–702 (1998).

40) Jappar D, Wu SP, Hu Y, Smith DE. Significance and regional dependency of peptide transporter (PEPT) 1 in the intestinal permeability of glycylsarcosine: in situ single-pass perfusion studies in wild-type and Pept1 knockout mice. Drug Metab. Dispos., 38, 1740–1746 (2010).

41) Ford D, Howard A, Hirst BH. Expression of the peptide transporter hPepT1 in human colon: a potential route for colonic protein nitrogen and drug absorption. Histochem. Cell Biol., 119, 37–43 (2003).

42) Freeman TC, Bentsen BS, Thwaites DT, Simmons NL. H/dipeptide transporter (PepT1) expression in the rabbit intestine. Pflugers Arch., 430, 394–400 (1995).

43) Ziegler TR, Fernández-Estívariz C, Gu LH, Bazargan N, Umekunne K, Wallace TM, Diaz EE, Rosado KE, Pascal RR, Galloway JR, Wilcox JN, Leader LM. Distribution of the H+/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. Am. J. Clin. Nutr., 75, 922–930 (2002).

44) Wuensch T, Schulz S, Ulrich S, Lill N, Stezl T, Rubio-Alia I, Loh G, Chamaillard M, Haller D, Daniel H. The peptide transporter PEPT1 is expressed in distal colon in rodents and humans and contributes to water absorption. Am. J. Physiol. Gastrointest. Liver Physiol., 305, G66–G73 (2013).

45) Nugent SG, Kumar D, Rampton DS, Evans DF. Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosalicylates and other drugs. Gut, 48, 571–577 (2001).

46) Ingersoll SA, Ayadurai S, Charania MA, Laroui H, Yan Y, Merlin D. The role and pathophysiological relevance of membrane transport PepT1 in intestinal inflammation and inflammatory bowel disease. Am. J. Physiol. Gastrointest. Liver Physiol., 302, G484–G492 (2012).

47) Tozaki H, Fujita T, Odoriba T, Terabe A, Okabe S, Muranishi S, Yamamoto A. Validation of a pharmacokinetic model of colon-specific drug delivery and the therapeutic effects of chitosan capsules containing 5-aminosalicylic acid on 2,4,6-trinitrobenzenesulphonic acid-induced colitis in rats. J. Pharm. Pharmacol., 51, 1107–1112 (1999).

48) Tozaki H, Fujita T, Komokie J, Kim SI, Terasihma H, Muranishi S, Okabe S, Yamamoto A. Colon-specific delivery of budesonide with azopolymer-coated pellets: therapeutic effects of budesonide with a novel dosage form against 2,4,6-trinitrobenzenesulfonic acid-induced colitis in rats. J. Pharm. Pharmacol., 51, 257–261 (1999).

49) Tozaki H, Odoriba T, Okada N, Fujita T, Terabe A, Suzuki T, Okabe S, Muranishi S, Yamamoto A. Chitosan capsules for colon-specific drug delivery: enhanced localization of 5-aminosalicylic acid in the large intestine accelerates healing of TNBS-induced colitis in rats. J. Control. Release, 82, 51–61 (2002).