Intestinal Absorption of Acyclovir β-Glucoside: Comparative Study with Acyclovir, Guanosine, and Kinetin β-Glucoside

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Received June 1, 1998; accepted September 17, 1998

Purpose. To characterize the intestinal absorption of a β-glucose conjugate of acyclovir (9-[(2-hydroxyethoxy) methyl] guanine, ACV) and compare it to ACV and its analogues in terms of stability and transport by Na^+/glucose cotransporter (SGLT1).

Methods. ACVβ glc was enzymatically synthesized using cellulase. Intestinal absorption experiments were performed with rat everted small intestine. Conformation of the glucose moiety was analyzed by NMR spectroscopy.

Results. The ACVβglc was stable on the mucosal side, and was transported to the serosal side in all regions of the small intestine. However, significant contribution of SGLT1 to the transport of ACVβglc was not observed. NMR spectroscopic analysis indicated that the glucose conformation of ACVβglc was the $^4C_1$ chair form, identical to β-glucose or SGLT1-transportable β-glucosides reported previously. Therefore, other factors such as molecular size and charge due to aglycone may cause no transport of ACVβglc by SGLT1. On the other hand, the hydrophilicity of ACVβglc was much higher than of ACV, suggesting water solubility-derived improvement of intestinal absorption of ACV.

Conclusions. ACVβglc is stable and absorbable, but it is not transported by SGLT1. No involvement of SGLT1 in the ACVβglc transport is not due to glucose conformation.

KEY WORDS: acyclovir; β-glucoside; intestinal absorption; SGLT1; conformation; oil/water partition.

INTRODUCTION

We have reported previously that glucose or galactose conjugation of compound resulted in intestinal absorption via a novel route by Na^+/glucose cotransporter (SGLT1) (1–3). Other researchers also reported glucosides transport mediated by SGLT1 in rat intestine and in *Xenopus* oocytes expressing SGLT1 (4,5). β-Glucose conjugates are the most rapidly transported among α-, β-glucosides and α-, β-galactosides (2,3). Acyclovir (9-[(2-hydroxyethoxy) methyl] guanine, ACV), an anti-viral drug, has been reported to be poorly absorbed from the intestine (6,7). Therefore, in this study we synthesized a β-glucose conjugate of ACV (ACVβglc) (Fig. 1) and examined its intestinal absorption. Furthermore, intestinal absorption of ACVβglc analogues, guanosine (2-amino-1,7-dihydro-6H-purin-6-one β-riboside, Guo) and kinetin β-glucoside (6-furfurylaminopurine β-glucoside, KNTβglc) (Fig. 1), were examined in terms of stability and transport. Structural requirements for SGLT1-mediated transport are also discussed.

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MATERIALS AND METHODS

Materials

ACV, KNTβglc and cellulase (Penicillium funiculosum) were purchased from Sigma Chemical Co. (MO, USA). Cellobiose was from Nacalai Tesque (Kyoto, Japan). Guo was from Wako Pure Chemicals (Osaka, Japan).

Synthesis of ACVβglc

ACVβglc was enzymatically synthesized according to a method reported previously (8). Briefly, cellobiose (15 g) was dissolved in 35 ml of sodium acetate buffer (pH 5.5). ACV (225.2 mg) was dissolved in DMSO (15 ml), and mixed with the cellobiose/acetate buffer solution. Finally, cellulase (5000 units/ml final concentration) was added to the mixture to be stirred for 3 hr at 37°C. Periodically, a portion (0.1 ml) of the reaction mixture was checked for transglucosylation by HPLC assay.

Purification of ACVβglc

Perchloric acid (70%, 3.85 ml) was added to the reaction mixture (50 ml) to stop the transglucosylation reaction. One hundred and ten milliliters of purified water was added to the resultant mixture. The mixture was divided into 4 glass tubes, and centrifuged at 3000 rpm for 30 min. The supernatant was filtered with glass fiber membrane (0.4 μm pore size). Into each tube, in which precipitates of denatured cellulase remained, 40 ml of purified water and 0.5 ml of perchloric acid (70%) were added, and mixed. After centrifugation of the mixture at 2500 × g for 20 min, the same procedures were repeated. Supernatants were pooled and sodium bicarbonate was added to a pH of 5.5. The resultant solution was concentrated to around 50 ml by evaporation under reduced pressure at 40 °C, and applied to a Sephadex G-10 column (9 cm i.d., 70 cm length). ACVβglc was eluted with water at a flow rate of 4.0 ml/min). Each fraction (15 ml/tube) was checked by HPLC assay. Fractions containing ACVβglc were collected and concentrated by the evaporation described above. Three milliliters of concentrated ACVβglc was applied to a preparative HPLC using an ODS column (21.5 mm I. D., 300 mm length). ACVβglc was eluted with the mobile phase (2% methanol, 0.05% phosphoric acid in water) at a flow rate of 4.0 ml/min. Each fraction (10 ml/tube) was checked by HPLC assay. Fractions containing ACVβglc were collected and concentrated by the evaporation described above. Finally, ACVβglc was obtained as yellow powder after freeze-drying. Recovery of ACVβglc by this method was about 75%. Mass spectrum of ACVβglc by ESI spectrometry showed molecular ion peaks, $[M+H]^+$ at 388.0, $[M+Na]^+$ at 409.5 and $[M+K]^+$ at 426.4.

β-Glucosidase Treatment

Purified ACVβglc was incubated in 0.05M sodium phosphate buffer (pH 6.8) in the presence of α-glucosidase (10 units/ml) or in 0.05M sodium acetate buffer (pH 5.0) in the presence

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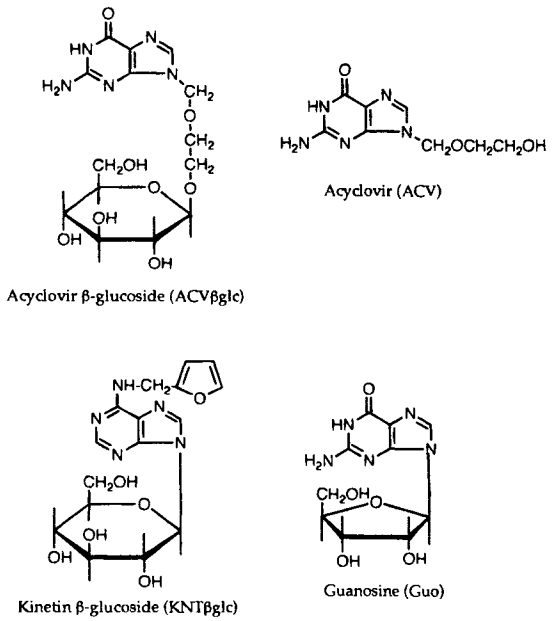

Fig. 1. Structures of acyclovir (ACV), acyclovir β-glucoside (ACVβglc), guanosine (Guo) and Kinetin β-glucoside (KNTβglc).

of β-glucosidase (10 units/ml) for 2 hr at 37°C. One hundred microliters of reaction mixture was sampled and mixed with 0.1 ml of 10% perchloric acid for the following HPLC assay.

NMR Spectrum

ACVβglc was dissolved in D_2O, and lyophilized. This procedure was performed twice. The ^1H NMR (500 MHz) spectrum of ACVβglc in D_2O was then obtained.

Oil/Water Partition

Two milliliter of 250 μM ACV or ACVβglc solution in 0.05 M sodium phosphate buffer (pH. 7.4) was added to 6 ml of *n*-butanol in a glass tube. After mixing for 30 sec, the mixture in a glass tube was kept in an incubator at 37°C for 2 hr. Every 10 min during the incubation, the mixture was mixed for 30 sec. The mixture was separated into oil and aqueous phases by centrifugation for 10 min at 3000 rpm. Two hundred microliters of the aqueous layer was mixed with 50 μl of p-aminosalicylic acid solution for the following HPLC assay. A 5 milliliter sample of the oil layer was evaporated under reduced pressure, and the resultant residue was dissolved in 200 μl of 250 μM *p*-aminosalicylic acid for the following HPLC assay.

Intestinal Absorption

Intestinal absorption of ACVβglc, Guo and KNTβglc was performed with everted small intestine (1). Briefly, male Wistar rats (180-230g, Japan Slc Inc., Japan) fasted overnight were anesthetized with ether, and intestinal blood was removed by saline perfusion. The upper, middle or lower part of the small intestine was removed and everted. The upper part of small intestine (10 cm) was defined as the region between 2 cm and 12 cm below the Treitz ligament. The middle part of small intestine (10 cm) was defined as the region between points 5 cm above and below the half-way point between the Treitz ligament and the ileocecal junction. The lower part of small intestine (10 cm) was the region between 2 and 12 cm above the ileocecal junction. Everted small intestine was placed in 30 ml of incubation medium (113.3 mM NaCl, 4.83 mM KCl, 1.214 mM KH_2PO_4, 1.205 mM $MgSO_4$, 16.96 mM $NaHCO_3$, 10.18 mM Na_2HPO_4, 0.645 mM $CaCl_2$, pH 7.4) containing peptide in a beaker through which gas (95%O_2, 5% CO_2) was bubbled at 37°C after being connected to a disposable 10 ml plastic syringe in a manner similar to the method reported by Doluisio *et al.* (9). The serosal side was filled with 5 ml of drug free incubation medium. The method of Doluisio *et al.* was performed to mix and sample the serosal solution. Incubation media (100 μl) were sampled from both the serosal and the mucosal sides up to 60 min. The samples were mixed with 100 μl of internal standard solution (250 μM *p*-aminosalicylic acid in 10% perchloric acid for ACV, ACVβglc and Guo, or 250 μM 2,4-dihydroxybenzoic acid in 10% perchloric acid for KNTβglc) for the following HPLC assay. The mixture was centrifuged at 11,000 × g for 5 min by a benchtop centrifuge (KM-15200, Kubota Ltd, Tokyo, Japan). The resultant supernatant was applied to HPLC.

HPLC Assay

The HPLC system consisted of a pump (655A-11, Hitachi Ltd., Tokyo, Japan), a UV detector (655A, Hitachi Ltd., Tokyo, Japan), and an integrator (D-2500, Hitachi Ltd., Tokyo, Japan). The UV detector and a flow rate were set at 254 nm for ACVβglc, ACV and Guo or at 260 nm for KNTβglc, and 1.5 ml/min, respectively, and an ODS column (80Tm, 6 mm i.d. × 15 cm length, Tosoh Corp., Japan) was used. For the assays of ACVβglc and ACV, the mobile phase consisted of 3% methanol and 0.05% phosphoric acid in water and the internal standard was *p*-aminosalicylic acid. For the assay of Guo, the mobile phase consisted of 5% methanol and 0.05% phosphoric acid in water and the internal standard was 250 μM *p*-aminosalicylic acid. For the assay of KNTβglc, the mobile phase consisted of 20% methanol, 0.05% phosphoric acid and 0.1% KNO_3 and the internal standard was 200 μM 2, 4-dihydroxybenzoic acid.

RESULTS

Stability and Transport of ACVβglc and ACV in Upper Part of Small Intestine

Figure 2a and b show time courses of ACVβglc and ACV concentrations on the mucosal side and the serosal side, respectively. ACVβglc and ACV were stable on the mucosal side. ACVβglc and ACV appeared on the serosal side with similar concentration-time profiles, but the transported amount of ACVβglc to the serosal side was approximately a half of that of ACV. Metabolites were not observed on either the mucosal or serosal side.

Stability and Transport of Guo and KNTβglc in Upper Part of Small Intestine

Figure 3a and b show time courses of KNTβglc and Guo concentrations on the mucosal side and the serosal side, respectively. Guo concentration on the mucosal side was decreased with time, whereas guanine formed and its concentration

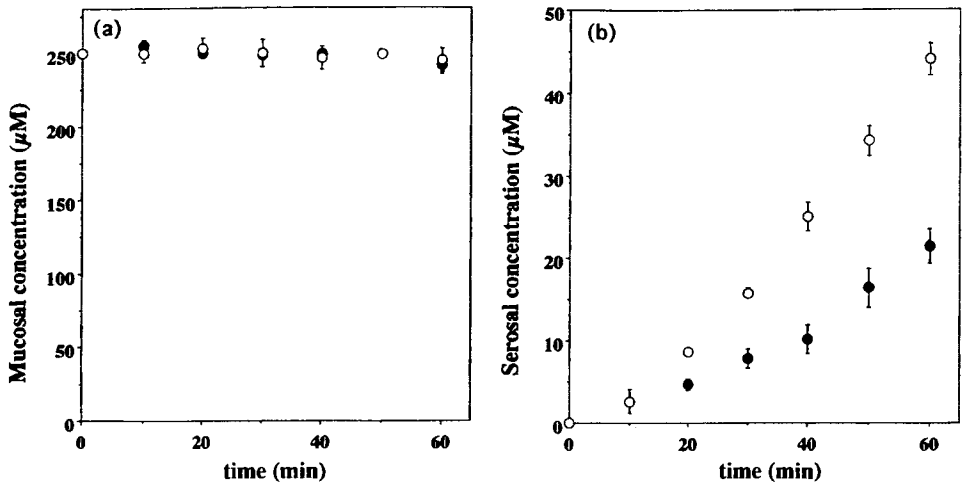

Fig. 2. Time courses of concentrations of ACV (○) and ACVβglc (●) on the mucosal (a) and serosal (b) sides. Data represent means ± S.E. (n = 3).

increased with time. Guo appeared on the serosal side, but its serosal concentration decreased after 10 min. On the other hand, KNTβglc was stable on the mucosal side and appeared on the serosal side.

Effect of Transport Inhibitors on the Transport of ACVβglc and KNTβglc in Upper Region of Small Intestine

Figure 4a and b show serosal concentrations of ACVβglc and KNTβglc in absorption experiments in the presence of phloridzin (SGLT1 inhibitor) or in the absence of Na^+ (cosubstrate of SGLT1). Transport of ACVβglc and KNTβglc from the mucosal to the serosal sides were not significantly inhibited by the presence of phloridzin or by the absence of Na^+.

Absorption of ACVβglc in the Middle and Lower Regions of Small Intestine

Figure 5 shows time courses of serosal appearance of ACVβglc in absorption experiments in the middle and lower regions of small intestine. ACVβglc was transported faster in the middle region than in the lower region, although at early stages (approximately 10 min after start), ACVβglc was transported faster in the lower region than in the upper or middle region.

NMR Spectrum of ACVβglc

Chemical shift and vicinal coupling constants of ACVβglc were tabulated (Table 1) with glucose and glucosides data. $J_{1,2}$ was 8.0, indicating that the relationship between protons at C1 and C2 positions is equatorial-axial. $J_{2,3}$, $J_{3,4}$, $J_{4,5}$, were more than 9.0, and similar to those of β-glucose, acetaminophen β-glucoside (APAPβglc), β-methylglucose (β-MG) and p-nitrophenyl β-glucoside (p-NPβglc).

Oil/Water Partition Coefficient

Oil/water partition coefficient ($PC_{O/W}$) and oil/buffer partition coefficient ($PC_{O/B}$) of ACVβglc and ACV were tabulated

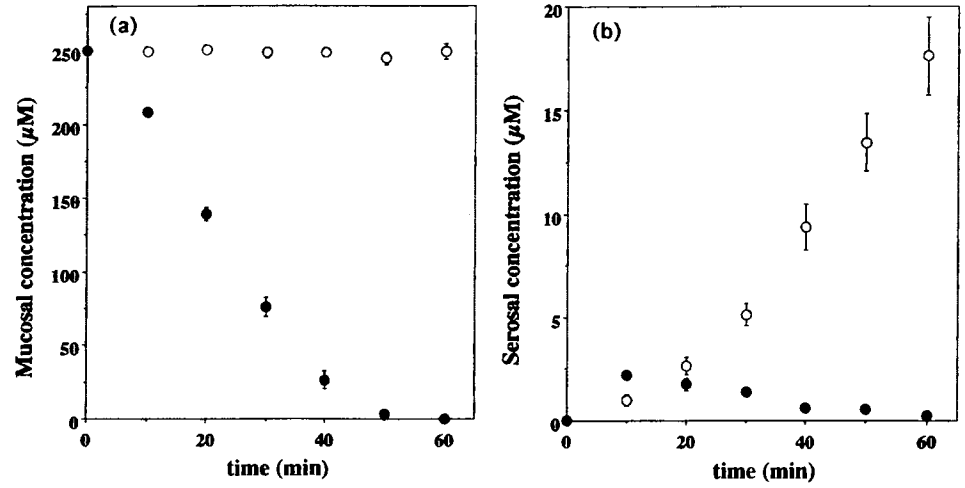

Fig. 3. Time courses of concentrations of Guo (●) and KNTβglc (○) on the mucosal (a) and serosal (b) sides. Data represent means ± S.E. (n = 3–4).

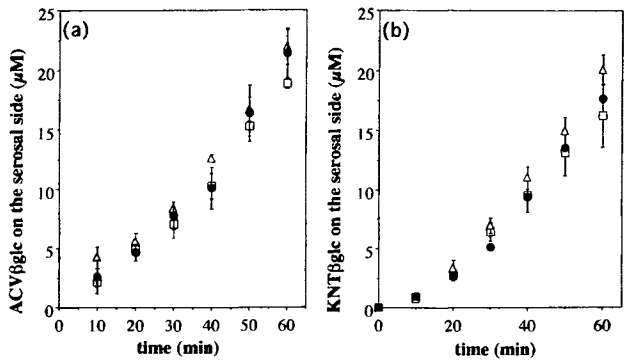

Fig. 4. Effect of transport inhibitors on the serosal appearance of ACVβglc (a) and KNTβglc (b). (●) control; (□) phloridzin; (△) Na^+-free. Data represent means ± S.E. (n = 3).

Table 1. Vicinal Coupling Constants of ^{1}H-NMR of β-Glucose Conjugates

	ACVβglc	APAPβglc[a]	β-glucose[b]	β-MG[a]	p-NPβglc[a]
$J_{1,2}$	8.0	7.6	8.0/8.1	8.0	7.5
$J_{2,3}$	9.2	9.1	9.2/8.5	9.2	—
$J_{3,4}$	9.2	9.1	9.1/8.8	9.2	9.4
$J_{4,5}$	9.7	9.8	9.8/9.0	9.7	9.4
$J_{5,6}$	2.2	2.3	2.3/2.0	2.2	2.2
$J_{5,6'}$	6.3	5.7	5.8/5.0	6.1	5.7
$J_{6,6'}$	12.3	12.5	12.3/12.2	12.4	12.3

[a] Data from a previous report (16).
[b] Data from reports of Curatolo *et al.* (15)/Koch and Perlin (14). APAPβglc, acetaminophen β-glucoside; β-MG, β-methylglucose; p-NPβglc, p-nitrophenyl β-glucoside.

in Table 2. $PC_{O/W}$ and $PC_{O/B}$ of ACVβglc were much lower than that of ACV.

DISCUSSION

ACVβglc and KNTβglc were stable on the mucosal side during transport to the serosal side (Fig. 2 and 3). On the other hand, Guo, β-ribose conjugate of guanine, was degraded to guanine easily on the mucosal side (Fig. 3), and Guo appeared on the serosal side only at early stages in absorption experiments. Guo is metabolized by purine nucleoside phosphorylase in rat small intestine (10). Therefore, it is considered that the metabolic degradation of Guo resulted in poor absorption, even though it is a substrate for nucleoside transporter in intestine (11). On the other hand, since ACVβglc and KNTβglc were stable, it was suggested that purine nucleosides in β-glucose conjugate form might be stable.

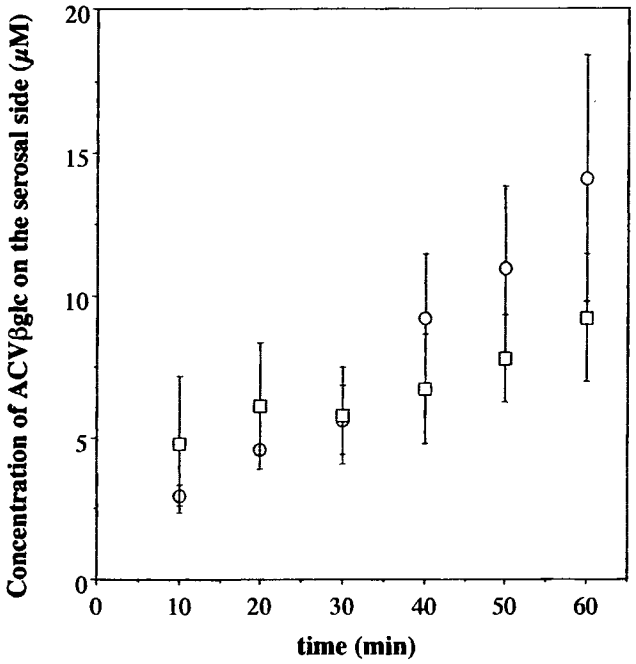

Fig. 5. Serosal appearance of ACVβglc in the middle (○) and lower (□) regions of the small intestine. Data represent means ± S.E. (n = 3).

The transport of ACVβglc and KNTβglc in the upper region of small intestine were not significantly inhibited in the presence of phloridzin or in the absence of Na^+ (Fig. 4), indicating that β-glucose conjugates of ACV and kinetin, which possess purine (guanine and adenine, respectively) moiety, were not transported by SGLT1. These results also indicate that ACVβglc was not transported by Na^+/nucleoside cotransporter, since intestinal Na^+/nucleoside cotransporter is inhibited by phloridzin (12). Vicinal coupling constants obtained from the NMR spectrum of ACVβglc (Table 1) indicate that conformation of the glucose moiety of ACVβglc is the $^4C_1$ chair form, based on the Karplus equation (13). Vicinal coupling constants ($J_{2,3}$, $J_{3,4}$, $J_{4,5}$) of ACVβglc were similar to those of β-glucose (14,15) (or β-methyl glucose (16)), p-NPβglc (16) and APAPβglc (16), which are transported by SGLT1, indicating that the glucose ring is in $^4C_1$ form (Table 1). Therefore, the lack of involvement of SGLT1 in ACVβglc absorption does not seem to be due to glucose conformation, but due to the effects of the aglycone moiety itself. For example, characteristics of aglycone due to molecular size or electric charge prevent the interaction of glucose moiety with SGLT1 and/or the transport of glucose moiety by SGLT1.

The order of the transport rate of ACVβglc in each region of small intestine was upper > middle > lower. ACVβglc appeared on the serosal side with an approximate 10 min of lag time. On the other hand, ACVβglc was transported faster in the lower region than in the upper or middle region at this early stage. The reason for the rapid absorption of ACVβglc within 10 min observed in the lower region is unclear at present.

Although β-glucose conjugation did not cause ACV to be transported by SGLT1, it did result in a 10 fold increase in hydrophilicity (Table 2). It has been suggested that poor absorption of ACV from gastrointestine might be due to limited solubility (17). Therefore, it is suggested that an increase of

Table 2. Oil/Water and Oil/Buffer Partition Coefficients of ACVβglc and ACV

Compounds	Oil/water	Oil/buffer[a]
ACVβglc	0.039 (0.042×10^{-2})	0.033 (0.027×10^{-2})
ACV	0.253 (0.543×10^{-2})	0.208 (0.117×10^{-1})

Note: Values represent means ± S.E. (n = 4). Oil phase was n-butanol.
[a] 0.05M Sodium phosphate buffer (pH 7.4).

solubility of ACV in water by glucose conjugation results in improvement of absorption from solid dosage form, because the glucose conjugation to ACV resulted in only one second decrease in intestinal transport (Fig. 2).

In summary, ACVβglc (β-glucose conjugate of ACV) was absorbed from all regions of the small intestine. The order of the transport rate of ACVβglc was upper > middle > lower, although ACVβglc transport at early phases was greatest in the lower region of small intestine. Although significant involvement of SGLT1 in ACVβglc transport was not observed, it is suggested the higher absorption rate of ACVβglc than of ACV due to their solubility. No transport of ACVβglc by SGLT1 was not due to the conformation of the glucose moiety of ACVβglc.

ACKNOWLEDGMENTS

We thank Dr Y. Shida for Mass spectrometry analysis and Ms C. Sakuma for NMR spectrometry analysis. Authors also thank Ms. K. Furumiya, Ms. N. Hisaki and Ms. K. Kanie for technical assistance.

REFERENCES

1. T. Mizuma, K. Ohta, M. Hayashi, and S. Awazu. Intestinal active absorption of sugar-conjugated compounds by glucose transport system: implication of improvement of poorly absorbable drugs. *Biochem. Pharmacol.* **43**:2037–2039 (1992).
2. T. Mizuma, K. Ohta, M. Hayashi, and S. Awazu. Comparative study of active absorption by the intestine and disposition of anomers of sugar-conjugated compounds. *Biochem. Pharmacol.* **45**:1520–1523 (1993).
3. T. Mizuma, K. Ohta, and S. Awazu. The β-anomeric and glucose preferences of glucose transport carrier for intestinal active absorption of monosaccharide conjugates. *Biochim. Biophys. Acta.* **1200**:117–122 (1994).
4. Y. Wang, R. Grigg, A. Mccormack, H. Symonds, and C. Bowmer. Absorption of N^4-D-glucopyranosylsulphamethazine by rat everted intestinal sacs. *Biochem. Pharmacol.* **46**:1864–1866 (1993).
5. M. Panayotova-Heiermann, D. D. D. Loo, and E. M. Wright. Kinetics of steady-state currents and charge movements associated with the rat Na^+/glucose cotransporter. *J. Biol. Chem.* **270**:27099–27105 (1995).
6. P. De Miranda and M. R. Blum. Pharmacokinetics of acyclovir after intravenous and oral administration. *J. Antimicrob. Chemother.* **12**:29–37 (1983).
7. S. Straus, M. Seidlin, H. Takiff, S. Bachrach, J. Digiovanna, K. Western, T. Creagh-Kirk, L. Lininger, and D. Alling. Suppression of recurrent genital herpes with oral acyclovir. *Clin. Res.* **31**:543 (1983).
8. T. Mizuma, S. Masubuchi, and S. Awazu. Cellulase-catalyzed transglucosylation of acetaminophen and acyclovir: preparative enzymatic synthesis of β-glucose conjugate. *Pharm. Res.* **14**:1647–1650 (1998).
9. J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky. Drug absorption I: an in situ rat gut technique yielding realistic absorption rates. *J. Pharm. Sci.* **58**:1196–1200 (1969).
10. R. A. Stow and J. R. Bronk. Purine nucleoside transport and metabolism in isolated rat jejunum. *J. Physiol.* **468**:311–324 (1993).
11. D. Vijayalakshmi and J. A. Belt. Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. *J. Biol. Chem.* **263**:19419–19423 (1988).
12. Q-Q Huang, C. M. Harvey, A. R. P. Paterson, C. E. Cass, and J. D. Young. Functional expression of Na^+-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. *J. Biol. Chem.* **268**:20613–20619 (1993).
13. M. Karplus. Contact electron-spin coupling of nuclear magnetic moments. *J. Chem. Phys.* **30**:11–15 (1959).
14. H. J. Koch and A. S. Perlin. Synthesis and ^{13}C NMR spectrum of D-glucose-3-d. bond-polarization differences between the anomers of D-glucose. *Carbohydr. Res.* **15**:403–410 (1970).
15. W. Curatolo, L. J. Neuringer, D. Ruben, and R. Haberkorn. Tow-dimensional J-resolved ^1H-nuclear magnetic resonance spectroscopy of α, β-D-glucose at 500 MHz. *Carbohydr. Res.* **112**:297–300 (1983).
16. T. Mizuma, Y. Nagamine, Y. Dobashi, and S. Awazu. Factors that cause β-anomeric preference of Na^+/glucose cotransporter for intestinal transport of monosaccharide conjugates. *Biochim. Biophys. Acta.*, **1381**:340–346 (1998).
17. L. D. Lewis, A. S. E. Fowle, S. B. Bittiner, A. Bye, and P. E. T. Isaacs. Human gastrointestinal absorption of acyclovir from tablet, duodenal infusion and sipped solution. *Br. J. Clin. Pharmacol.* **21**:459–462 (1986).