European Journal of Pharmaceutics and Biopharmaceutics 75 (2010) 366–374

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Carboxyl group-terminated polyamidoamine dendrimers bearing glucosides inhibit intestinal hexose transporter-mediated D-glucose uptake

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ARTICLE INFO

Article history:
Received 29 December 2009
Accepted in revised form 11 April 2010
Available online 24 April 2010

Keywords:
Dendrimer
Glucoside
Hexose transporter
Conjugation
Oral anti-diabetic drug

ABSTRACT

We are investigating non-absorbable polymeric conjugates bearing glucosides via a ω-amino triethylene glycol linker as oral anti-diabetic drugs that suppress an increase in the blood glucose level after meals through inhibition of Na^+/glucose cotransporter (SGLT1). When the linker was bound to phloridzin, which is a SGLT1 inhibitor, to yield a precursor of the conjugate, the in vitro inhibitory effect on SGLT1-mediated D-glucose uptake was reduced to about one-tenth that of phloridzin. The inhibitory effect was recovered completely when the precursor was immobilized on the surface of poly(amidoamine) (PAMAM) dendrimers (generation: 3.0) by coupling with one-eighth or less of the terminal carboxyl groups. We considered that the phloridzin-derived glucose moiety on the dendrimer surface was prerequisite for SGLT1 inhibition but that the aglycon part was not always required for the inhibition. Commercially used arbutin, a SGLT1 substrate, was substituted for phloridzin whose aglycon is composed of toxic phloretin. The in vitro inhibitory effect of arbutin was about one-thirtieth that of intact phloridzin; however, the inhibitory effect of the PAMAM dendrimer–arbutin conjugates was as strong as that of the PAMAM dendrimer–phloridzin conjugates. Rat experiments further showed that the PAMAM dendrimer–arbutin conjugates significantly suppressed D-glucose-induced hyperglycemic effects. The dendritic conjugate bearing arbutin appears to be a good candidate as an oral anti-diabetic drug.

1. Introduction

Pharmacokinetics and physicochemical properties of drugs are significantly altered when drugs are conjugated with polymers [1]. Many researchers have investigated the potential of conjugation as a method that eliminates the disadvantages of the original drugs and often confers unique properties that the original drugs did not possess [1–5]. We have been investigating conjugation as a method to alter the behavior of orally administered drugs in the gastrointestinal tract [6–9]. Orally administered polymer–drug conjugates with high molecular weights are generally not absorbed through the intestinal membranes [7–12]. This characteristic limits their application as oral medicines because they are not delivered to the systemic circulation. However, the non-absorption of conjugates may render the drug safer and also result in a low incidence of drug–drug interactions in comparison with conventional low-molecular weight drugs that are absorbed systemically. Polymer–drug conjugates appear suitable for oral medication to patients with life style-related diseases who are required to take several drugs concomitantly for a long period.

Carbohydrates, which are one of the three major nutrients in the human diet, are hydrolyzed thoroughly by digestive enzymes in the gastrointestinal tract. The resulting monosaccharides are absorbed from the small intestine via influx hexose transporters [13,14]. There are two types of hexose transporters in human and rat small intestines: sodium-dependent Na^+/glucose cotransporters (SGLT) and sodium-independent glucose transporters (GLUT) [15,16]. Of these, SGLT1 is predominantly expressed in the apical membranes of the intestinal epithelial cells, and it is understood to contribute mainly to the apical membrane permeation of D-glucose [13]. Diabetes is a typical life style-related disease, and hyperglycemia results from the deficient insulin

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doi:10.1016/j.ejpb.2010.04.003

secretion or insulin resistance. For the treatment of diabetes, it is indispensable to suppress the rapid increase in the blood glucose level after a meal [17]. As mentioned before, the first step of glucose absorption is the recognition of D-glucose by SGLT1 [13,18]. Therefore, we considered that SGLT1 inhibition could be one of the most effective approaches to suppress an increase in the blood glucose level after a meal.

We have previously prepared poly(γ-glutamic acid)s (γ-PGA) bearing phloridzin via a non-biodegradable ω-amino triethylene glycol linker (PGA–PRZ) [8,9]. Phloridzin is a glucoside found in the bark and stem of apple trees and has been used experimentally as a SGLT1 inhibitor. However, it has not been examined as an oral anti-diabetic drug because phloretin is produced through the hydrolysis of the glucoside bond of phloridzin by β-glucosidase located on the apical membranes of the intestine, and it may inhibit GLUT1, which is responsible for D-glucose uptake in various tissues [18,19]. The hydrolysis also severely reduces in vivo activities of phloridzin because it results in the depletion of intramolecular glucose moieties, which are essential for the SGLT1 inhibition. Steric hindrance of the polymer chain often improves the stability of enzyme-susceptible chemical bonds [1,6]. PGA–PRZ was designed with the expectation that it remains unchanged in the gastrointestinal tract and suppresses the increase in the blood glucose level by inhibiting SGLT1 without the systemic exposure of the conjugate. The β-glucosidase-susceptible glucoside bond of phloridzin was stabilized through conjugation with γ-PGA. D-Glucose was not released from PGA–PRZ with a phloridzin content of greater than 15% incubated with rat small intestinal brush-border membrane vesicles (BBMVs), despite the immediate release of D-glucose from intact phloridzin. Data also indicated that toxic phloretin was retained in the polymer backbone of the conjugate. The conjugate with a phloridzin content of 15% (PGA–PRZ-15%) significantly suppressed the increase in the blood glucose level after oral administration of D-glucose in rats, while intact phloridzin scarcely affected the in vivo D-glucose-induced hyperglycemic effect.

Our previous studies suggest that PGA–PRZ-15% has potential as an oral anti-diabetic drug that inhibits SGLT1. However, 2 concerns still remain. We found that the chemical modification of phloridzin resulted in a ca. 90% reduction in its in vitro activities. The latent toxicity of phloretin in PGA–PRZ may not be acceptable even though phloretin remains in the non-absorbable polymer backbone: patients will take PGA–PRZ daily for an extended period of time. In this study, we succeeded to solve these problems by substituting γ-PGA and phloridzin with carboxyl group-terminated poly(amidoamine) dendrimers and arbutin, respectively.

2. Materials and methods

2.1. Materials

Carboxyl group-terminated poly(amidoamine) (PAMAM) dendrimer (1,6-diaminohexane core; generation: 3.0; terminals: 32; molecular weight: 10167.4), phloridzin, and arbutin were purchased from Sigma–Aldrich (St. Louis, MO, USA). γ-PGA (weight-average molecular weight: 382 kDa) was gifted by Meiji Seika Co., Ltd. (Tokyo, Japan). D-Glucose was obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). All other chemicals were commercial products of analytical or reagent grade and were used without further purification. Dialysis membranes with a nominal molecular weight limit of 10 kDa (Amicon Ultra-15) and membrane filters (HAWP02500, pore size: 0.45 μm) were purchased from Millipore Co. (Billerica, MA, USA). Tritiated D-glucose ([2-^{3}H], 370-740 GBq/mmol, 37 MBq/mL, in sterilized water) was furnished by Moravek Biochemicals and Radiochemicals (Brea, CA, USA). F-kit D-glucose was purchased from J.K. International Co., Ltd. (Tokyo, Japan). Glucose pilot was obtained from Aventir Biotech LLC (Carlsbad, CA, USA).

2.2. Synthesis

Phloridzin and arbutin derivatives were synthesized as shown in Schemes 1 and 2, respectively.

2.2.1. ω-Amino triethylene glycol-bound phloridzin (Am-PRZ)

Synthetic procedures of Am-PRZ, which is a precursor of the conjugate bearing phloridzin, were described previously [8]. Briefly, *N*-benzyloxycarbonylated ω-amino triethylene glycol was prepared in three reaction steps using 2-[2-(2-chloroethoxy)ethoxy]ethanol as a starting material. It was then reacted with methanesulfonyl chloride to yield its mesylate. The mesylate was reacted with 4′-phenolic hydroxyl groups of phloridzin to yield *N*-benzyloxycarbonyl-ω-amino triethylene glycol-bound phloridzin. Finally, the protecting groups were removed by catalytic reduction to yield Am-PRZ.

2.2.2. Carboxyl group-terminated PAMAM dendrimer–phloridzin conjugate (PAMAM–PRZ)

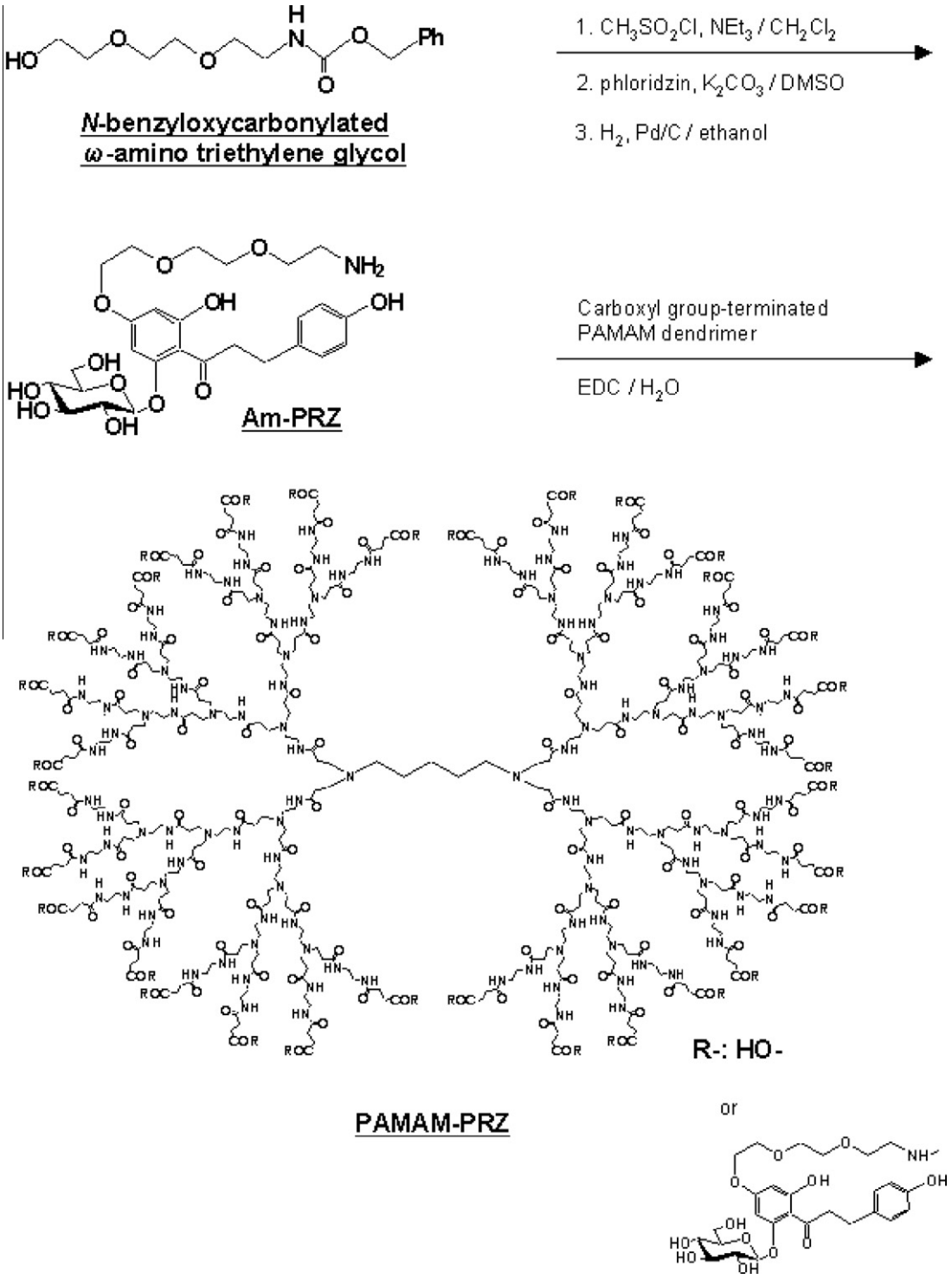
Carboxyl group-terminated PAMAM dendrimers (19.5 mg, 6.2×10^{-2} mmol as a carboxyl group equivalent) were dissolved in pure water. To this solution, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 11.8 mg, 6.2×10^{-2} mmol) was added at 0 °C. After stirring at 0 °C for 30 min, Am-PRZ (18.7–56.1 mg, 3.1×10^{-2}–9.3×10^{-2} mmol) was added. The mixture was stirred at room temperature overnight. After unreacted substances were removed by ultrafiltration (Amicon Ultra-15), the resulting solution was lyophilized for 2 days to give PAMAM–PRZ. IR: 3277, 3078, 2936, 1696, 1637, 1545, 1178, and 1074 cm^{-1}.

The content of phloridzin in PAMAM–PRZ was determined by the integral ratio of the phenyl proton signals in the phloridzin unit to the methylene proton signals in the 1,6-diaminohexane core of PAMAM dendrimer on the ^{1}H NMR spectrum of PAMAM–PRZ. PAMAM–PRZ with a X/32 phloridzin content (the ratio of the number of terminal carboxyl groups modified with phloridzin to the total number of terminal carboxyl groups) was abbreviated as PAMAM–PRZ-X/32 (Runs 1–3, Table 1).

2.2.3. ω-Amino triethylene glycol-bound arbutin (Am-ARB)

The mesylate of *N*-benzyloxycarbonylated ω-amino triethylene glycol was prepared according to the same procedure as described in the previous article [8]. The mesylate (640 mg, 1.77 mmol), arbutin (1440 mg, 5.31 mmol), and potassium carbonate (490 mg, 3.54 mmol) were dissolved in dry dimethylformamide (50 mL) under a nitrogen atmosphere. After stirring at 60 °C for 2 days, the mixture was extracted with ethyl acetate (70 mL) and saturated aqueous solution of sodium chloride (60 mL × 2). The organic layer was dried over anhydrous magnesium sulfate and then concentrated under reduced pressure. The residue was purified by silica-gel column chromatography with acetone–hexane (1:1 → 2:1 → 10:0) as an eluent, yielding *N*-benzyloxycarbonylated ω-amino triethylene glycol-bound arbutin as a yellow oil (109 mg, 13%). ^{1}H NMR (DMSO-d_6): δ 3.09–3.27 (m, 4H), 3.36–3.60 (m, 6H), 3.62–3.72 (m, 3H), 4.00 (t, 2H, J = 4.0 Hz), 4.53 (t, 1H, J = 5.2 Hz), 4.69 (d, 1H, J = 7.6 Hz), 4.95–5.04 (m, 3H), 5.25 (dd, 1H, J = 5.2, 1.2 Hz), 6.80–6.86 (m, 2H), 6.92–6.98 (m, 2H), and 7.23–7.38 (m, 5H). FAB-MS: 538.3 $(M+1)^+$. IR: 3368, 2870, 1727, 1508 cm^{-1}.

N-Benzyloxycarbonylated ω-amino triethylene glycol-bound arbutin (168 mg, 0.31 mmol) was dissolved in ethanol (5 mL). To

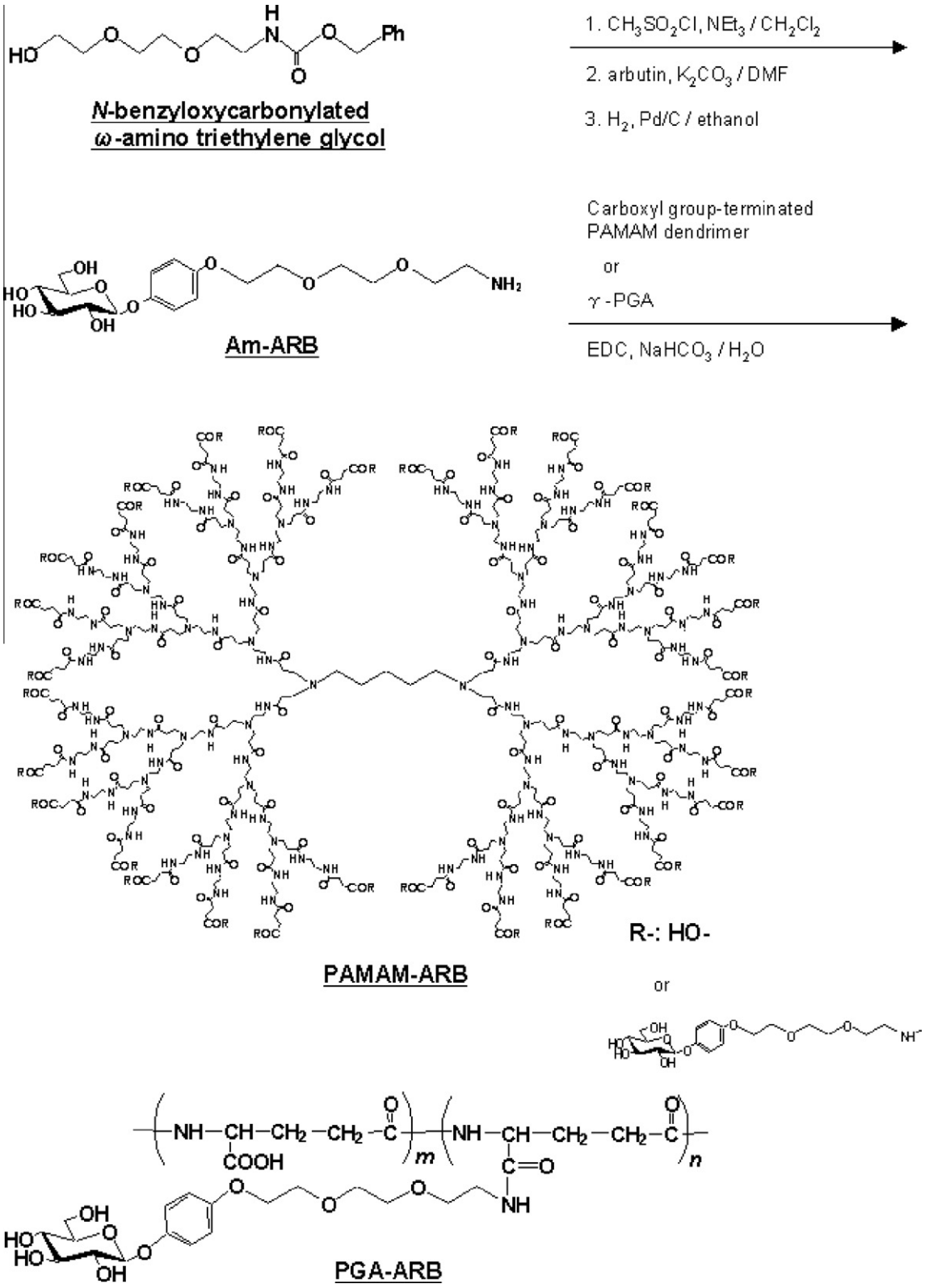


Scheme 1. Synthesis of carboxyl group-terminated PAMAM dendrimer–phloridzin conjugate (PAMAM–PRZ).

this solution, 20% palladium on carbon (165 mg) was added. The mixture was placed in an autoclave and stirred overnight under hydrogen at 7 atm. The reaction mixture was filtered to remove the palladium on carbon, and the filtrate was concentrated under reduced pressure to give Am-ARB as a pale yellow viscous liquid (109 mg, 87%). ^{1}H NMR (DMSO-d_6): δ 2.70 (t, 2H, J = 5.6 Hz), 3.07-3.29 (m, 4H), 3.37–3.59 (m, 7H), 3.62–3.71 (m, 3H), 3.99 (t, 2H, J = 4.0 Hz), 4.67 (d, 1H, J = 7.6 Hz), 6.81–6.87 (m, 2H), 6.92-6.98 (m, 2H). FAB-MS: 404.3 $(M+1)^+$. IR: 3360, 2870, 1592, 1505, and 1067 cm^{-1}.

2.2.4. Carboxyl group-terminated PAMAM dendrimer–arbutin conjugate (PAMAM–ARB)

Carboxyl group-terminated PAMAM dendrimers (33.8 mg, 1.06×10^{-1} mmol as a carboxyl group equivalent) were dissolved in pure water. To this solution, EDC (20.2 mg, 1.06×10^{-1} mmol)



Scheme 2. Synthesis of carboxyl group-terminated PAMAM dendrimer–arbutin conjugate (PAMAM–ARB) and poly(γ-glutamic acid)–arbutin conjugate (PGA–ARB).

was added at 0 °C. After stirring at 0 °C for 30 min, Am-ARB (42.2 mg, 1.06×10^{-1} mmol) was added. The mixture was then stirred at room temperature overnight. Lyophilized PAMAM–ARB was collected from the reaction mixture in the same manner as that described for the purification of PAMAM–PRZ. IR: 3278, 3074, 2927, 1638, 1560, 1214, and 1074 cm^{-1}.

The content of arbutin in PAMAM–ARB was determined by the integral ratio of the phenyl proton signals in the arbutin unit to the methylene proton signals in the 1,6-diaminohexane core of PAMAM dendrimer on the ^1H NMR spectrum of PAMAM–ARB. PAMAM–ARB with a X/32 arbutin content (the ratio of the number of terminal carboxyl groups modified with arbutin to the total

Table 1
Preparation of polymer–glucoside conjugates.

Run	Conjugates	In feed					Content of glucoside in the conjugate
		Polymers		EDC (mmol)	Glucoside derivatives		
		PAMAM (mmol)[a]	γ-PGA (mmol)[b]		Am-PRZ (mmol)	Am-ARB (mmol)	
1	PAMAM–PRZ-2/32	6.2×10^{-2}		6.2×10^{-2}	3.1×10^{-2}		2/32[c]
2	PAMAM–PRZ-4/32	6.2×10^{-2}		6.2×10^{-2}	6.2×10^{-2}		4/32[c]
3	PAMAM–PRZ-6/32	6.2×10^{-2}		6.2×10^{-2}	9.3×10^{-2}		6/32[c]
4	PAMAM-ARB-4/32	1.06×10^{-1}		1.06×10^{-1}		1.06×10^{-1}	4/32[c]
5	PGA–ARB-15%		0.81	0.81		0.27	15%[d]

[a] Molar amount as a carboxyl group equivalent of PAMAM dendrimers.
[b] Molar amount as a γ-PGA monomer equivalent.
[c] The ratio of the number of terminal carboxyl groups modified with glucosides to the total number of terminal carboxyl groups.
[d] The percentage of the number of γ-PGA monomer units modified with glucosides to the total number of γ-PGA monomer units.

number of terminal carboxyl groups) was abbreviated as PAMAM–ARB-X/32 (Run 4, Table 1).

2.2.5. γ-PGA–arbutin conjugate (PGA–ARB)

The supplied γ-PGA was hydrolyzed in an alkaline solution to reduce molecular weights as described previously [8]. Hydrolyzed γ-PGA (Mw: 57,800, as determined by gel permeation chromatography) was collected by precipitation in an acidic solution followed by filtration.

The γ-PGA (105 mg, 0.81 mmol as a γ-PGA monomer equivalent) was dissolved in aqueous solution of sodium hydrocarbonate (67.6 mM, pH: 8.0). To this solution, EDC (156 mg, 0.81 mmol) was added at 0 °C. After stirring at 0 °C for 30 min, Am-ARB (109 mg, 0.27 mmol) was added. The mixture was then stirred at room temperature overnight. Lyophilized PGA–ARB was collected from the reaction mixture as described before. IR: 3266, 2934, 1732, 1647, 1546, 1165, and 1072 cm^{-1}.

The content of arbutin in PGA–ARB was determined by the integral ratio of the phenyl proton signals in the arbutin unit to the methylene proton signals in the γ-PGA chain on the ^1H NMR spectrum of PGA–ARB. PGA–ARB with a X% arbutin content (percentage of the number of γ-PGA monomer units modified with arbutin to the total number of γ-PGA monomer units) was abbreviated as PGA–ARB-X% (Run 5, Table 1).

2.3. In vitro D-glucose uptake by rat small intestinal BBMVs

All rat experiments were approved by the Ethical Review Committee of Setsunan University. The experimental conditions of D-glucose uptake by rat small intestinal BBMVs were described previously [9].

BBMVs were isolated by the conventional divalent ion-precipitation method. Briefly, the mucosa was scraped off the luminal surface of the rat small intestine. The scrapings were homogenized, and calcium chloride was added to the homogenate. Precipitated BBMVs were collected and purified through centrifugation. All processes occurred under cool conditions. The BBMVs were finally dispersed in cold suspension buffer (HEPES: 10 mM, D-mannitol: 100 mM, KCl: 100 mM, pH 7.4) at a concentration of 0.1–0.2 mg of protein/10 μL.

BBMVs (10 μL) were incubated at 30 °C for 3 min and then mixed with 90 μL of uptake buffer (NaCl: 100 mM, D-mannitol: 100 mM, HEPES: 10 mM, pH 7.4) containing D-glucose, tritiated D-glucose, and an inhibitor (glucosides and their derivatives). The concentrations of D-glucose (including the tritiated one) and the inhibitor were adjusted to 0.01 mM and 0.01–2 mM as a glucoside equivalent, respectively. The radioactivity of the solution was adjusted to ca. 100 kBq/mL. To create sodium ion-free conditions, NaCl in the uptake buffer was replaced with an equivalent concentration of KCl. After incubation of the mixture at 30 °C for 1 min, the uptake reaction was terminated by addition of 1 mL of ice-cold stop buffer (KCl: 100 mM, HEPES: 10 mM, pH 7.4). The solution was immediately filtered through the membrane filter (HAWP02500) under reduced pressure. The filter, on which BBMVs were collected, was subsequently washed with the ice-cold stop buffer (5 mL × 2) and dried for 30 s under reduced pressure. After the filter was dissolved in 10 mL of scintillation fluid at room temperature, the radioactivity was measured with a liquid scintillation counter (LSC 3500, Aloka Co., Tokyo, Japan).

In this uptake experiment, the difference between D-glucose uptake under inhibitor-free conditions with and without sodium ions (difference A) is regarded as the uptake via SGLT1, because SGLT1 cannot transport D-glucose in the absence of sodium ions. The difference between D-glucose uptake under inhibitor-free conditions without sodium ions and that in the presence of inhibitors at various concentrations with sodium ions (difference B) is regarded as residual SGLT1-mediated uptake in the presence of inhibitors. By subtracting the percentage of difference *B* to difference *A* from 100%, the inhibitor-suppressed percentage of SGLT1-mediated D-glucose uptake is obtained (0%: no inhibition, 100%: complete inhibition). The obtained values were plotted as a function of inhibitor concentration, and the inhibitor concentration giving half-maximum inhibition (IC_{50}) of the uptake was calculated by fitting the data into the Michaelis–Menten-patterned curve.

2.4. In vitro digestive enzyme-induced degradation of phloridzin and its derivatives

Phloridzin or its derivatives (Am-PRZ or PAMAM–PRZ) was dissolved in PBS at a concentration of 1.1 mmol as a phloridzin equivalent. After the solution (0.72 mL) was mixed with BBMVs prepared in the previous section (0.08 mL), the mixture was incubated at 37 °C. Aliquots of the mixture (⩾0.1 mL) were taken at predetermined time points and cooled in an ice bath. The concentration of D-glucose released from phloridzin and its derivatives was measured using the NADP spectrometric method (F-kit D-glucose). When this kit was used under our experimental conditions, the lower limit of quantification corresponded to 0.2% of D-glucose released from phloridzin.

2.5. In vivo inhibitory effect of arbutin and its derivatives on D-glucose absorption

Seven-week-old male Wistar rats were fasted for 24 h. Oral administration of PAMAM–ARB-4/32 (dose: 0.125 mmol as an arbutin equivalent/2.5 mL of water/kg of rat body weight) was followed by oral administration of D-glucose (500 mg/2.5 mL/kg) with a 10-min interval. Blood samples were taken from the tail vein without anesthesia at predetermined time points after the D-glucose administration. The blood glucose concentration was

measured by an enzyme electrode method (Glucose Pilot). PAMAM–ARB-4/32 was substituted with arbutin (0.125 mmol/2.5 mL/kg) to compare the in vivo inhibitory effects on D-glucose absorption between the conjugate and intact arbutin.

The change in blood glucose concentration before and after D-glucose administration was calculated (the initial glucose concentration was measured before the oral administration of PAMAM–ARB-4/32 or arbutin), and the means and standard errors were determined. Each value was plotted as a function of time. The area between the curve representing the change in the glucose concentration versus time and a horizontal line representing zero change during the 120-min experiment was calculated by the trapezoidal method [8]. The value obtained (area of glucose elevation) was used as an index of the biological effect of D-glucose (this value decreases when D-glucose absorption is inhibited). Statistical significance was assessed with the unpaired Student's *t*-test, and *p* values of 0.05 or less were considered to be statistically significant.

3. Results and discussion

3.1. Potential of PAMAM–PRZ as a SGLT1 inhibitor

It is known that the substrate specificity of SGLT1 is high. Nutritional D-glucose and D-galactose are absorbed from the small intestine via SGLT1, but disaccharides are not substrates for this transporter. The Michaelis–Menten constant (*Km*) values of D-glucose and D-galactose for human SGLT1 are 0.5 mM and 0.6 mM, respectively [20].

In previous studies [8,9], we first used γ-PGA, which is a linear macromolecule, as the backbone of the conjugate bearing phloridzin. Intact phloridzin strongly inhibited D-glucose uptake by rat small intestinal BBMVs, and its glucoside-equivalent IC_{50} value was estimated to be 0.035 mM. The inhibitory effect was reduced considerably when ω-amino triethylene glycol was introduced into the 4′-hydroxyl group on the aromatic ring of phloridzin to yield the precursor of the conjugate (Am-PRZ). The glucoside-equivalent IC_{50} value of Am-PRZ was estimated to be 0.44 mM. However, no further reduction was observed in the inhibition of D-glucose uptake when Am-PRZ was conjugated with γ-PGA, irrespective of the phloridzin content.

In this study, γ-PGA was substituted with a carboxyl group-terminated PAMAM dendrimer, which is a nanometer-sized starburst macromolecule with generation-dependent numbers of terminal groups, with the aim of recovering the activity of chemically modified phloridzin. Large amounts of ligands can be immobilized on the surface of dendrimers through chemical reaction with terminal groups. It is reported that the affinity of ligands for target molecules is often improved by multivalent interactions between them [21–24]. Conjugation of Am-PRZ with PAMAM dendrimers may result in an increase in the phloridzin activity.

Carboxyl group-terminated PAMAM dendrimer–phloridzin conjugates (PAMAM–PRZ) were synthesized according to Scheme 1. We developed procedures to synthesize Am-PRZ when we previously examined γ-PGA–phloridzin conjugates (PGA–PRZ) [8]. The phloridzin unit was successfully immobilized on the dendrimer surface by coupling the amino groups of Am-PRZ with the carboxyl groups of the dendrimers in the presence of EDC. As shown in Runs 1–3 of Table 1, the number of phloridzin unit bound to PAMAM dendrimer was controlled by changing the feed ratio of Am-PRZ to the dendrimer. PAMAM–PRZ with a phloridzin content greater than 6/32 on an average was not prepared even when an excess amount of Am-PRZ was used in the reaction. It appears that Am-PRZ is so bulky that only a limited amount of Am-PRZ is introduced into the surface carboxyl groups of the dendrimer, which has an average diameter of about 5 nm.

The IC_{50} values of phloridzin and its derivatives against SGLT1-mediated D-glucose uptake are summarized in Table 2. As shown in glucoside-equivalent IC_{50} values, PAMAM-PRZ with a phloridzin content of 4/32 or less on an average strongly inhibited SGLT1-mediated D-glucose uptake. On the other hand, there was no difference in the inhibitory effect between PAMAM–PRZ with a phloridzin content of 6/32 and Am-PRZ. If the affinity of Am-PRZ on the dendrimer surface for SGLT1 was improved by the multivalent interactions, the glucoside-equivalent IC_{50} values of PAMAM–PRZ would be reduced with an increase in the phloridzin content. Contradictory data indicated that the multivalent interactions and the affinity were not linked in our case; however, we did obtain a couple of dendritic conjugates whose inhibitory effects on SGLT1-mediated D-glucose uptake were almost identical to those of intact phloridzin.

We next examined the stability of the glucoside bond of Am-PRZ immobilized on the dendrimer surface. Fig. 1 shows the release profiles of D-glucose from phloridzin and its derivatives incubated with BBMVs. D-Glucose was immediately released from intact phloridzin, but this instability was improved to some extent by chemically modifying the 4′-hydroxyl group on the aromatic ring of phloridzin, as seen in our previous study [9]. Conjugation with carboxyl group-terminated PAMAM dendrimers did not affect the stability of the glucoside bond of Am-PRZ, irrespective of the phloridzin content. When Am-PRZ was conjugated with γ-PGA, the conjugates self-assembled in water into spherical particles whose diameters were of the magnitude of 10^2 nm. The

Table 2
IC_{50} of phloridzin and its derivatives against SGLT1-mediated D-glucose uptake.

Inhibitors	IC_{50} (mM)	
	Glucoside equivalent	Dendrimer equivalent
Phloridzin	0.035[d]	–
Am-PRZ[a]	0.44[d]	–
PAMAM–PRZ-2/32[b]	0.030	0.015
PAMAM–PRZ-4/32[b]	0.057	0.014
PAMAM–PRZ-6/32[b]	0.37	0.062
PGA–PRZ-15%[c]	0.44[d]	–

(Mean of 4–5 experiments).
[a] ω-Amino triethylene glycol-bound phloridzin.
[b] Carboxyl group-terminated PAMAM dendrimer–phloridzin conjugate.
[c] Poly(γ-glutamic acid)–phloridzin conjugate.
[d] Data cited from our previous article [9].

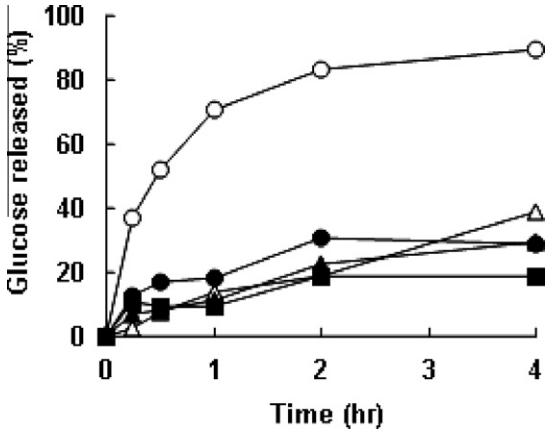


Fig. 1. Release profiles of D-glucose from phloridzin (○), Am-PRZ (Δ), PAMAM–PRZ-2/32 (●), PAMAM–PRZ-4/32 (▲), and PAMAM–PRZ-6/32 (■) incubated with rat small intestinal BBMVs at 37 °C. Each value represents the mean of two experiments.

self-assembly-induced rigid aggregation possibly prevented the glucoside bond from being hydrolyzed by β-glucosidase [9]. The glucoside bond of Am-PRZ immobilized on the dendrimer surface was probably exposed to β-glucosidase without any protection. Data showed that the difference in the affinity for SGLT1 between the respective dendritic conjugates was independent of the stability of the glucoside bond.

The glucoside-equivalent IC_{50} values of PAMAM–PRZ-2/32 and PAMAM–PRZ-4/32 correspond to a dendrimer-equivalent IC_{50} value of 0.015 mM and 0.014 mM, respectively (Table 2). When the carboxyl group-terminated PAMAM dendrimers were applied to the uptake experiment at this concentration, no SGLT1 inhibition was observed. Tyagi et al. reported that there were a couple of phloridzin-binding sites on SGLT1: one for the glucose part of phloridzin and another one for the aglycon part of phloridzin [25]. Our previous research indicated that the affinity of the glucose part remained unchanged, but that of the aglycon part disappeared after the chemical modification of the 4′-hydroxyl group on the aromatic ring of phloridzin [9].

We considered that the aglycon part was not involved in recovering the inhibitory effect of Am-PRZ through conjugation with PAMAM dendrimers at its content of 4/32 or less. Instead of the aglycon part, functional groups such as residual carboxyl groups on the dendrimer surface may cooperate with phloridzin-derived glucose moieties in inhibiting SGLT1 through some interaction with the transporter, although there is no direct evidence for our speculation. It came to our notice that there was a discrete change in the glucoside-equivalent IC_{50} values between PAMAM–PRZ-4/32 and PAMAM–PRZ-6/32. It appears that the inhibitory effect of PAMAM–PRZ-6/32 was attributed to the glucoside-equivalent IC_{50} value of Am-PRZ because the excess Am-PRZ immobilized on the dendrimer surface interfered with the above-mentioned cooperative interactions. If this speculation is correct, phloridzin could be substituted with other glucosides. The phloretin-derived latent toxicity of PAMAM–PRZ may be solved.

3.2. Potential of PAMAM–ARB as a SGLT1 inhibitor

The bearberry leaves are used traditionally as herbal medicine. Arbutin, which is the main active ingredient in bearberry leaves, is categorized as a phenyl group-bound glucoside as is phloridzin. Among these glucosides, arbutin has the simplest chemical structure with hydroquinone as an aglycon part. Arbutin is a substrate for human SGLT1, with a *Km* value of 1.3 ± 0.2 mM [26]. Orally absorbed arbutin sterilizes the urinary tract while being eliminated.

Carboxyl group-terminated PAMAM dendrimer–arbutin conjugates (PAMAM–ARB) were synthesized according to Scheme 2. ω-Amino triethylene glycol-bound arbutin (Am-ARB), which is a precursor of the conjugate, was prepared in a similar manner to that used for Am-PRZ in our previous study [8]. The ω-amino triethylene glycol linker was introduced into 3′-phenolic hydroxyl groups of arbutin. The arbutin unit was successfully immobilized on the surface of PAMAM dendrimers by coupling the amino groups of Am-ARB with the EDC-activated carboxyl groups of the dendrimers (Run 4, Table 1). On an average, 12.5% of applied Am-ARB was bound to the dendrimer surface. As a reference, γ-PGA–arbutin conjugate (PGA–ARB) with a binding degree close to that of PAMAM–ARB-4/32 for arbutin was prepared through the same coupling as in the final step of the procedure for the synthesis of PAMAM–ARB (Run 5, Table 1).

We first estimated the inhibitory effect of arbutin and Am-ARB on SGLT1-mediated D-glucose uptake. As shown in Table 3, in vitro uptake experiments showed that the glucoside-equivalent IC_{50} value of intact arbutin was 1.0 mM on an average; this was approximately 30 times that of intact phloridzin. As expected from the *Km* value, the inhibitory effect of arbutin was not strong. The inhibitory effect on SGLT1-mediated D-glucose uptake was further reduced when arbutin was modified with ω-amino triethylene glycol. D-Glucose uptake by BBMVs was constant even when Am-PRZ was used in the uptake experiment at a concentration of 2 mM as a glucoside equivalent, which was the maximal concentration examined here. The introduction of the linker into the hydroxyl groups on the aromatic ring of arbutin probably influenced the affinity of the glucose moieties of arbutin for the binding site on SGLT1, as observed in the case of phloridzin.

Table 3
IC_{50} of arbutin and its derivatives against SGLT1-mediated D-glucose uptake.

Inhibitors	IC_{50} (mM)	
	Glucoside equivalent	Dendrimer equivalent
Arbutin	1.0	–
Am-ARB[a]	>2.0	–
PAMAM–ARB-4/32[b]	0.15	0.038
PGA–ARB-15%[c]	>2.0	–

(Mean of 4–5 experiments).
[a] ω-Amino triethylene glycol-bound arbutin.
[b] Carboxyl group-terminated PAMAM dendrimer–arbutin conjugate.
[c] Poly(γ-glutamic acid)–arbutin conjugate.

It has been predicted that arbutin bound to macromolecules is not absorbed through the intestinal membrane because of increased molecular weights, even though the original drug is a substrate of SGLT1. Therefore, PAMAM–ARB is expected to be a SGLT1 inhibitor if glucoside-derived intramolecular glucose moieties still recognize the binding site on SGLT1. As shown in Table 3, the glucoside-equivalent IC_{50} value of PAMAM–ARB-4/32 was estimated to be 0.15 mM, which was close to that of PAMAM–PRZ-4/32, with the same glucoside content. On the other hand, a similar phenomenon was not observed when PAMAM dendrimers were substituted with γ-PGA (only 20% inhibition in D-glucose uptake by BBMVs was observed when 2 mM of PGA–ARB-15% as a glucoside equivalent was used in the uptake experiment).

The in vivo experiments described in our previous article were subsequently carried out to examine the potential of PAMAM–ARB-4/32 as an oral anti-diabetic drug that suppresses the increase in blood glucose levels [8]. Since the conjugate is expected to inhibit glucose transport through SGLT1, it should be administered before meals. Arbutin or its dendritic conjugate was given orally to fasted rats at 10 min before oral administration of D-glucose aqueous solution, which was given instead of meals. The changes in the blood glucose concentration were monitored as shown in Fig. 2 and Table 4. When D-glucose was administered orally at a dose of 500 mg/kg without the pre-administration of any inhibitor (control), a rapid increase in the blood glucose concentration was observed. After the glucose concentration reached the maximal level at 30 min, it gradually decreased and returned to the initial value within 120 min. The area of glucose elevation was $3.62 \times 10^3 \pm 0.45 \times 10^3$ mg·min/dL. Arbutin did not significantly suppress the increase in the blood glucose levels at the dose used here, presumably because of its low affinity for SGLT1. When the same dose of PAMAM–ARB-4/32 as an arbutin equivalent was administered prior to D-glucose, the blood glucose concentration was remarkably suppressed during the 120-min experiment. The area of glucose elevation was $1.98 \times 10^3 \pm 0.16 \times 10^3$ mg·min/dL, which was significantly lower than that in the control experiment. No suppression of the blood glucose level was observed when the PAMAM dendrimer itself or a physical mixture of intact arbutin and the dendrimer was given to rats before D-glucose administration. It was likely that conjugation with PAMAM dendrimers enabled arbutin to show its activity in in vivo conditions.

We were truly surprised that the arbutin activity remarkably increased through conjugation with carboxyl group-terminated

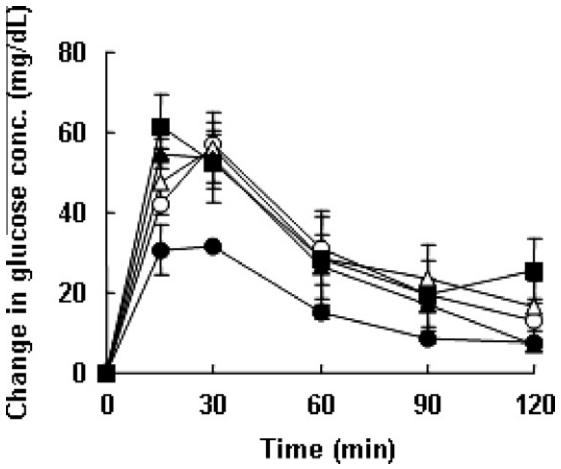


Fig. 2. Concentration–time profiles of glucose in blood after oral administration of D-glucose. Water (○), arbutin (Δ), PAMAM–ARB-4/32 (●), PAMAM dendrimers (▲), or a physical mixture of arbutin and PAMAM dendrimers (■) was given orally 10 min before D-glucose administration. The doses of arbutin and PAMAM–ARB-4/32 were adjusted to 0.125 mmol/kg as an arbutin equivalent. The dose of PAMAM dendrimers was adjusted to 0.0313 mmol/kg that was equal to the dose of PAMAM–ARB-4/32 as a dendrimer equivalent. Data were represented as the change in glucose concentration before and after D-glucose administration. Each value represents mean ± se of 3–4 experiments.

Table 4
Hyperglycemic effect after oral administration of D-glucose with and without arbutin or its dendritic conjugate (n = 3–4).

Inhibitors	Dose (mmol/2.5 mL/kg)[a]	Area of glucose elevation (mg·min/dL)[b]
Control[c]	0	$3.62 \times 10^3 \pm 0.45 \times 10^3$
Arbutin	0.125	$3.81 \times 10^3 \pm 0.84 \times 10^3$[g]
PAMAM–ARB-4/32[d]	0.125	$1.98 \times 10^3 \pm 0.16 \times 10^3$[h]
PAMAM dendrimers[e,f]	0	$3.44 \times 10^3 \pm 0.71 \times 10^3$[g]
Physical mixture of arbutin and PAMAM dendrimers[f]	0.125	$3.94 \times 10^3 \pm 1.0 \times 10^3$[g]

[a] mmol as an arbutin equivalent/2.5 mL of water/kg of rat body weight.
[b] The area between the curve for the change in the glucose concentration versus time and a horizontal line (mean ± se).
[c] Water was given orally before D-glucose administration (2.5 mL/kg).
[d] Carboxyl group-terminated PAMAM dendrimer–arbutin conjugates with an arbutin content of 4/32.
[e] Carboxyl group-terminated PAMAM dendrimers were given orally before D-glucose administration (2.5 mL/kg).
[f] Dose of PAMAM dendrimers was adjusted to that of PAMAM–ARB-4/32 as a dendrimer equivalent (0.0313 mmol/2.5 mL/kg).
[g] Not significant difference from control.
[h] Statistically significant difference from control.

PAMAM dendrimers. It seems that PAMAM–ARB has potential as an oral anti-diabetic drug that inhibits D-glucose absorption via SGLT1, which no one has succeeded in developing yet [27]. We should prove the non-permeability of the conjugate with molecular weight of ca. 10 kDa through the intestinal membranes, although these macromolecules are not generally delivered to the systemic circulation. This would ensure the usefulness of our polymer technology as a novel strategy for drug design. The arbutin content, dendrimer generation, length of the linker, and terminal functional groups of dendrimers probably influence the inhibitory effect of dendritic conjugates bearing arbutin on SGLT1-mediated D-glucose uptake. Analysis of the complex mechanism responsible for the inhibition of D-glucose uptake by PAMAM–ARB at the molecular level may be also required.

4. Conclusions

Dendritic conjugates bearing either phloridzin or arbutin via a ω-amino triethylene glycol linker were synthesized. When the linker was bound to the 4′-phenolic hydroxyl groups of phloridzin to yield a precursor of the conjugate, the in vitro inhibitory effect on SGLT1-mediated D-glucose uptake by BBMVs was reduced to about 10% that of the original. The inhibitory effect was recovered by immobilizing the precursor on the surface of the carboxyl group-terminated PAMAM dendrimers. We considered that the aglycon part of phloridzin bound to the dendrimers was not always required for SGLT1 inhibition. The validity of this hypothesis was suggested by the results obtained with the PAMAM dendrimer–arbutin conjugates whose in vitro inhibitory effect was similar to that of the PAMAM dendrimer–phloridzin conjugates. The former also significantly suppressed the increase in the blood glucose level after oral administration of D-glucose in rats, while intact arbutin barely affected the D-glucose-induced hyperglycemic effect. It seems that the dendritic conjugate bearing arbutin without the toxic aglycon part is a good candidate as an oral anti-diabetic drug.

Acknowledgments

This work was financially supported in part by a Grant-in-aid for scientific research (No. 21590051) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan (MEXT). The authors thank Meiji Seika Co., Ltd. for gifting γ-PGA.

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