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Characterization of a fluorescent glucose derivative 1-NBDG and its application in the identification of natural SGLT1/2 inhibitors

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Abstract

Glucose is an important energy source for cells. Glucose transport is mediated by two types of glucose transporters: the active sodium-coupled glucose cotransporters (SGLTs), and the passive glucose transporters (GLUTs). Development of an easy way to detect glucose uptake by the cell can be valuable for research. 1-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-1-deoxy-D-glucose (1-NBDG) is a newly synthesized fluorescent glucose analogue. Unlike 2-NBDG, which is a good substrate of GLUTs but not SGLTs, 1-NBDG can be transported by both GLUTs and SGLTs. Thus, 1-NBDG is useful for the screening of SGLT1 and SGLT2 inhibitors. Here we further characterized 1-NBDG and compared it with 2-NBDG. The fluorescence of both 1-NBDG and 2-NBDG was quenched under alkaline conditions, but only 1-NBDG fluorescence could be restored upon neutralization. HPLC analysis revealed that 2-NBDG was decomposed leading to loss of fluorescence, whereas 1-NBDG remained intact in a NaOH solution. Thus, after cellular uptake, 1-NBDG fluorescence can be detected on a plate reader simply by cell lysis in a NaOH solution followed by neutralization with an HCl solution. The fluorescence stability of 1-NBDG was stable for up to 5 h once cells were lysed; however, similar to 2-NBDG, intracellular 1-NBDG was not stable and the fluorescence diminished substantially within one hour. 1-NBDG uptake could also be detected at the single cell level and inhibition of 1-NBDG uptake by SGLT inhibitors could be detected by flow cytometry. Furthermore, 1-NBDG was successfully used in a high-throughput cell-based method to screen for potential SGLT1 and SGLT2 inhibitors. The SGLT inhibitory activities of 67 flavonoids and flavonoid glycosides purified from plants were evaluated and several selective SGLT1, selective SGLT2, as well as dual SGLT1/2 inhibitors were identified. Structure-activity relationship analysis revealed that glycosyl residues were crucial since the aglycon showed no SGLT inhibitory activities. In addition, the sugar inter-linkage and their substitution positions to the aglycon affected not only the inhibitory activities but also the selectivity toward SGLT1 and SGLT2.

Keywords: Antidiabetic, Glucose transporters, 1-NBDG, 2-NBDG, SGLT inhibitors

1. Introduction

Glucose is the main energy source for humans, and its transport and metabolism are closely associated with diseases, such as diabetes and cancer. There are two types of glucose transporters: the facilitative glucose transporters (GLUTs) which are responsible for passive transport of glucose across plasma membranes,

and the sodium-coupled glucose cotransporters (SGLTs) which are responsible for active transport of glucose into cells. Both SGLTs and GLUTs are good targets for the development of antidiabetic drugs and/or anticancer drugs [1,2].

SGLT1, a high-affinity, low capacity transporter, plays an important role in glucose absorption in the intestine. SGLT2, a low-affinity, high-capacity transporter expressed exclusively in the S1 and S2 segments of the renal proximal tubule, is responsible for

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reabsorption of more than 90% of glucose in the kidney. SGLT1 is also expressed in the distal S3 segment of the renal proximal tubule and responsible for reabsorbing the remaining glucose [3]. Selective SGLT2 inhibitors have become a new class of antidiabetic drugs which reduce blood glucose levels via inhibition of glucose reabsorption in the kidney, a unique mechanism different from that of other antidiabetic drugs. Four SGLT2 inhibitors (canagliflozin, dapagliflozin, empagliflozin and ertugliflozin) have been approved in the United States and other countries, and three additional SGLT2 inhibitors (ipragliflozin, luseogliflozin and tofogliflozin) are approved in Japan for the treatment of type 2 diabetes in monotherapy or combination therapy. SGLT2 inhibitors can not only decrease blood glucose levels, but also reduce body weight and blood pressure, and may also decrease the risk of diabetic nephropathy. Furthermore, unlike other antidiabetic drugs, SGLT2 inhibitors do not cause hypoglycemia [2]. A dual inhibitor LX4211 (sotagliflozin), which targets both SGLT2 in the kidney and SGLT1 in the GI track and kidney but does not cause severe diarrhea, is under development for the treatment of type 1 and type 2 diabetes [4].

SGLT1 and SGLT2 are active glucose transporters that can transport glucose into cells even under a low extracellular glucose condition, which is common in tumor microenvironment. It has been reported that SGLT1 and SGLT2 are overexpressed in cancer cells and they are potential targets for anticancer therapy [2].

Radioactive glucose analogues are frequently used as substrates for glucose uptake assays, e.g., ^{14}C -1-O-methyl- α -D-glucopyranoside (^{14}C -AMG) for SGLTs and [^3H]-2-deoxy-D-glucose for GLUTs. However, the use of radioactive isotopes is costly and has safety and waste disposal issues. Fluorescent glucose analogues, such as 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-D-glucose (2-NBDG), provide non-radioactive alternatives and have also become valuable research tools for monitoring glucose uptake in living cells [5]. 2-NBDG is phosphorylated at the C-6 position but cannot be metabolized further and is decomposed to a non-fluorescent derivative inside *Escherichia coli* cells [6]. Although 2-NBDG can be transported by GLUTs, it is a poor substrate for SGLTs [7]. We synthesized a fluorescent glucose analogue 1-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-1-deoxy-D-glucose (1-NBDG) and demonstrated that, unlike 2-NBDG, 1-NBDG was a good substrate for SGLTs and could be a good replacement for ^{14}C -AMG. Using 1-NBDG, a non-radioactive cell-based method for the screening of SGLT1 and SGLT2 inhibitors was developed [7].

Here we further characterized and compared 1-NBDG with 2-NBDG, investigated their stability in various conditions and evaluated the potential use in flow cytometric analysis for the detection of glucose uptake in single cells. Furthermore, we also developed a high-throughput cell-based method using 1-NBDG to identify SGLT1 and SGLT2 inhibitors. Natural resources are rich in highly diversified structures that can be potential therapeutic agents. For example, SGLT2 inhibitors currently on the market are C-glucoside derivatives of phlorizin, a natural product originally isolated from the bark of apple trees. Many natural SGLT inhibitors have been identified from traditional herbal medicine as potential candidates for further development of new hypoglycemic agents [8,9]. To search for new pharmacophores, the SGLT inhibitory activities of 67 flavonoids and flavonoid glycosides purified from plants were evaluated using the non-radioactive method we established and structure-activity relationship analysis was also conducted.

2. Materials and methods

2.1. Chemicals

1-NBDG was synthesized as previously described [7] and 2-NBDG was purchased from Invitrogen. Phlorizin and choline chloride were purchased from Sigma. 1-NBDG and 2-NBDG stock solutions were prepared in deionized water. Phlorizin and purified natural products were dissolved in DMSO.

2.2. Cell culture and transfection

COS-7 cells were cultured in low-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. Transfection was performed using Lipofectamine 2000 (Invitrogen) as previously described [7]. Oral squamous cell carcinoma UPCI:SCC131 (abbreviated as SCC131) cells were grown in Minimal Essential Medium (MEM) with Earle's salts supplemented with 10% FBS and MEM nonessential amino acids [10]. Antibiotics (100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B) were added to all the culture media except for transfection.

2.3. Determination of the stability of 1-NBDG and 2-NBDG under alkaline, acidic conditions or at different pH

1-NBDG or 2-NBDG was diluted in 0.2 N NaOH, 0.2 N HCl or 20 mM Tris buffers with pH values of

6.8, 7.5, 8.0, 8.8 and 9.8. Fluorescence was detected in a black 96-well plate using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices) at an excitation wavelength of 458 nm and an emission wavelength of 528 nm (458/528 nm) for 1-NBDG and 475/550 nm for 2-NBDG.

2.4. HPLC analysis

Mightysil RP-18 GP column was used for HPLC analysis and the mobile phase was a gradient of water and acetonitrile from 100% water at 4 min to 100% acetonitrile at 30 min with a flow rate of 1 mL/min. Ten microliters of samples were injected and detected by a UV/Vis detector (set at 475 nm).

2.5. Determination of intracellular stability of 1-NBDG and 2-NBDG

SCC131 cells were seeded into 24-well plates at 1×10^5 cells/well and subjected to glucose uptake assay the following day. Cells were rinsed twice with 500 μ L of warm choline buffer, followed by incubation with 200 μ L of 160 μ M 1-NBDG or 2-NBDG in sodium buffer for 2 h [7]. Cells were then washed twice with warm choline buffer, kept in 500 μ L PBS at 37 °C in the CO₂ incubator for 0–5 h and lysed in 600 μ L of a neutral buffer containing 1% sodium deoxycholate, 1% NP-40, 40 mM KCl and 20 mM Tris–HCl, pH 7.5 [11]. One hundred-microliter aliquots of cell lysates were transferred to a black 96-well plate for the detection of fluorescence intensity.

2.6. Detection of glucose uptake by flow cytometry

Exponentially growing cells in 6-well plates were rinsed twice with 1 mL of warm choline buffer and then incubated with 500 μ L of 100 μ M 1-NBDG or 2-NBDG in sodium buffer or choline buffer for 30 min at 37 °C. Cells were then washed 3 times with warm choline buffer and harvested by trypsinization. After centrifugation, cells were resuspended in cold PBS and subjected to flow cytometric analysis by FACSCalibur (BD Biosciences, San Jose, CA) with an excitation wavelength of 488 nm for FL1 channel. Phlorizin was used to inhibit glucose transport via hSGLT1. Flowing Software 2 was used for quantitative analysis.

2.7. Cell-based screening method for SGLT1 and SGLT2 inhibitors using 1-NBDG

COS-7 cells were transfected with pcDNAhSGLT1 or pcDNAhSGLT2 as previously described [7] and plated into 96-wells the following day at $3\text{--}5 \times$

10^4 cells/well. Cells were allowed to attach overnight and reach 100% confluence, then subjected to glucose uptake assay in sodium buffer containing test compounds and 100 or 160 μ M 1-NBDG at 37 °C for 1.5 h (SGLT1) or 2 h (SGLT2). Cells in 96-wells were rinsed with 150 μ L of warm choline buffer and then incubated with 50 μ L of sodium buffer containing 1-NBDG and test compounds. After incubation, cells were washed twice with cold choline buffer, examined under microscope to check cell conditions, then lysed in 75 μ L of 0.2 N NaOH, and neutralized with 75 μ L of 0.2 N HCl. Lysates (100 μ L) were transferred to a black 96-well plate for fluorescence detection. Phlorizin was used as a positive control for SGLT inhibitor and 1-NBDG in choline buffer was used to measure sodium-independent glucose uptake via GLUTs. Cells in sodium buffer without 1-NBDG were used to measure auto-fluorescence (cell background).

2.8. Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was assessed using the two-sided Student's *t*-test and *P*-values less than 0.05 were considered statistically significant (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001).

3. Results

3.1. Stability of 1-NBDG and 2-NBDG fluorescence under alkaline and acidic conditions

Since 2-NBDG is a poor substrate for SGLTs, we have synthesized a novel fluorescent glucose analogue 1-NBDG and have demonstrated that 1-NBDG is a substrate for SGLTs and a good substitute of ¹⁴C-AMG for the screening of SGLT inhibitors. Cellular uptake of 1-NBDG can be examined by fluorescence microscopy. The fluorescence intensity can also be quantified after cell lysis using a plate reader [7]. When ¹⁴C-AMG was used for glucose uptake assay via SGLTs, NaOH solution was frequently used to lyse cells following the uptake assay [12]. However, we found that 1-NBDG fluorescence was dramatically quenched in NaOH solution but was restored after neutralization. Thus, an optimal lysis solution system (cell lysis in 0.2 N NaOH followed by neutralization with an equal volume of 0.2 N HCl) was used in various assays to quantify 1-NBDG transport [7]. Here we further tested the stability of 1-NBDG fluorescence in strong alkaline and acidic solutions over time and compared it with 2-NBDG. As shown in Fig. 1A, 1-NBDG fluorescence intensity was not changed much

after incubation in 0.2 N NaOH for up to 30 min followed by neutralization. In contrast, the fluorescence intensity of 2-NBDG decreased dramatically to $33.2 \pm 2.88\%$, $22.6 \pm 0.55\%$ and $15.3 \pm 0.53\%$ after 5, 10 and 30 min in 0.2 N NaOH respectively followed by neutralization. 1-NBDG or 2-NBDG was also incubated in 0.2 N HCl for 0–30 min, followed by neutralization with 0.2 N NaOH. The fluorescence of both 1-NBDG and 2-NBDG was relatively stable in 0.2 N HCl and the intensity was even slightly increased after 30 min in the acidic solution (Fig. 1B). The fluorescence of 1-NBDG and 2-NBDG in 20 mM Tris buffers with pH values of 6.8, 7.5, 8.0, 8.8 and 9.8 was also compared, and the intensity was diminished with increasing pH, but 1-NBDG was more stable with intensity decreased to $42.8 \pm 0.62\%$ at pH 9.8 relative to pH 6.8 (Fig. 1C) while the decrease of 2-NBDG fluorescence was more drastic and the relative intensity was $43.6 \pm 0.48\%$ at pH 8.8 and $23.1 \pm 0.02\%$ at pH 9.8 (Fig. 1D).

3.2. Chemical stability of 1-NBDG and 2-NBDG under alkaline conditions

To compare chemical stability, 10 μM of 1-NBDG or 2-NBDG solution in water or in 0.2 N NaOH for

30 min was subjected to HPLC analysis. Chromatograms shown in Fig. 2 revealed that the retention times of 1-NBDG peaks before (Fig. 2A, peak *a*) and after alkali treatment (Fig. 2B, peak *b*) remained the same (variation of retention time $< 2\%$); whereas, the peaks of α - and β -anomers of 2-NBDG (Fig. 2C, peaks *c* and *d*) disappeared after alkali treatment and a new peak with a longer retention time (Fig. 2D, peak *e*) emerged instead. The results suggested that 2-NBDG was decomposed into a nonfluorescent derivative after alkali treatment.

Altogether, these results indicated that 1-NBDG is relatively stable under alkaline conditions and its fluorescence is less affected by variations of pH. Our results also confirmed those reported by Blodgett et al. [11] that alkaline solutions are not suitable for the detection of 2-NBDG fluorescence and neutral lysis buffers should be used to lyse cells following 2-NBDG uptake assays.

3.3. Comparison of 1-NBDG and 2-NBDG stability inside the cell

Fluorescence stability of 1-NBDG in SCC131 cells, which exhibited both sodium-dependent and

Fig. 1. The stability of 1-NBDG and 2-NBDG under alkaline, acidic conditions, or at different pH. A-B. Fluorescence intensity of 1-NBDG or 2-NBDG upon neutralization following treatment in 0.2 N NaOH (A) or 0.2 N HCl (B) for 0–30 min. C-D. Fluorescence intensity of 1-NBDG (C) or 2-NBDG (D) at different pH. The final concentration of 1-NBDG or 2-NBDG was 0.1 μM . Each experiment was performed in triplicate. Data are presented as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. HPLC analysis of 1-NBDG and 2-NBDG after alkali treatment. Profile of 10 μ M 1-NBDG in H₂O (A) and 0.2 N NaOH for 30 min (B). Profile of 10 μ M 2-NBDG in H₂O (C) and 0.2 N NaOH for 30 min (D). Retention times of peaks a–e are indicated.

sodium-independent 1-NBDG uptake, was determined following 2 h of uptake and 2-NBDG was included for comparison. Cells were kept in PBS for 0–5 h, and then lysed in a neutral buffer containing detergents [11]. As illustrated in Fig. 3A, the fluorescence intensity of 1-NBDG was reduced to $64.2 \pm 7.36\%$ within 30 min, $22.3 \pm 3.37\%$ after 1 h, $11.1 \pm 1.77\%$ after 2 h, $10.7 \pm 2.39\%$ after 3 h, and totally diminished after 5 h in the cells ($t_{1/2} = 38$ min). 2-NBDG was slightly more stable and the fluorescence intensity was decreased to $73.6 \pm 7.69\%$ within 30 min, $51.1 \pm 5.17\%$ after 1 h, $27.2 \pm 2.06\%$ after 2 h, $17.5 \pm 2.04\%$ after 3 h, and only $9.25 \pm 2.37\%$ remained after 5 h ($t_{1/2} = 61$ min).

The stability of fluorescence in the neutral lysis buffer was also evaluated. Cells were lysed immediately following 2 h-incubation with 1-NBDG or 2-NBDG and the fluorescence intensity was measured from 0 to 5 h and the results indicated that fluorescence of both 1-NBDG and 2-NBDG was very stable in this neutral lysis buffer (Fig. 3B).

3.4. Detection of 1-NBDG and 2-NBDG uptake by flow cytometry

It has been reported that 2-NBDG uptake by cells can be detected by flow cytometry at the single cell level [13]. We then tested whether 1-NBDG uptake could be detected by flow cytometry in the parental COS-7 cells or COS-7 cells transiently overexpressing hSGLT1. As shown in Fig. 4A, 1-NBDG or 2-NBDG uptake was detected in COS-7 cells, but

2-NBDG showed better fluorescence intensity. Flow cytometric histogram of COS-7 cells overexpressing hSGLT1 (hSGLT1/COS-7) showed a peak of 1-NBDG uptake in sodium buffer. In the presence of 1 μ M of phlorizin, the peak was shifted back and overlapped with that in choline buffer, indicating hSGLT1-specific 1-NBDG uptake in sodium buffer (Fig. 4B). In contrast, flow cytometric analysis of hSGLT1/COS-7 cells incubated with 2-NBDG showed no differences in sodium buffer, choline buffer, or sodium buffer in the presence of phlorizin (Fig. 4C), confirming our previous results that 2-NBDG was a poor substrate for SGLTs [7]. Quantitative analysis revealed that, in hSGLT1/COS-7 cells, the normalized geomean of fluorescence intensity (NGFI) of 1-NBDG was significantly increased in sodium buffer relative to choline buffer, but 1-NBDG uptake in sodium buffer was significantly suppressed by phlorizin. The NGFI of hSGLT1/COS-7 cells in choline buffer or in sodium buffer with phlorizin was similar to that in the parental COS-7 cells, indicative of SGLT-independent 1-NBDG uptake. However, no significant differences in 2-NBDG uptake were observed among all the groups (Fig. 4D).

3.5. Validation of a high-throughput cell-based method for the screening of SGLT inhibitors using 1-NBDG

1-NBDG was used as a fluorescent glucose analogue to conduct glucose uptake assay in COS-

Fig. 3. Stability of 1-NBDG and 2-NBDG fluorescence. A. Stability of 1-NBDG and 2-NBDG fluorescence inside the cell. SCC131 cells were incubated with 1-NBDG or 2-NBDG for 2 h, washed and incubated in PBS for 0–5 h before lysed in a neutral lysis buffer. B. Stability of 1-NBDG and 2-NBDG fluorescence in cell lysate. SCC131 cells were incubated with 1-NBDG or 2-NBDG for 2 h, lysed in the neutral lysis buffer after washes, and incubated at room temperature for 0–5 h before the fluorescence intensity measurement. Each experiment was performed in triplicate. Data are presented as mean $\pm$ SEM of three independent experiments. ** $P < 0.01$, *** $P < 0.001$.

7 cells transiently transfected with pcDNAhSGLT1 and CHO–K1 cells stably expressing hSGLT2 for the screening of SGLT1 and SGLT2 inhibitors, respectively [7]. Here we demonstrated that it was also feasible to use COS-7 cells transiently transfected with pcDNAhSGLT2 (hSGLT2/COS-7) for the screening of SGLT2 inhibitors. The method was also extended from 24-well format [7] to 96-well format suitable for high-throughput screening.

The signal and noise ratio was determined first. The ratios of 1-NBDG uptake in sodium buffer vs.

choline buffer in the parental COS-7 cells and hSGLT1/COS-7 or hSGLT2/COS-7 cells are listed in Table 1. After cell lysis, 1-NBDG was in a 0.1 N NaCl solution which caused a low background reading at 458/528 nm. After subtracting the background of NaCl solution, the ratios of 1-NBDG uptake in sodium buffer vs. choline buffer were 4.56 and 2.20 in hSGLT1/COS-7 and hSGLT2/COS-7 cells, respectively. However, after subtracting cell background due to autofluorescence, the ratios were increased to 7.25 and 3.37, respectively, which were significantly higher than those obtained by simply subtracting the background of NaCl solution. The ratios were approximately 1 regardless of whether NaCl or cell background was subtracted in COS-7 cells, confirming the absence of sodium-dependent 1-NBDG uptake in the parental COS-7 cells. These results demonstrated good signal/noise ratios in both hSGLT1/COS-7 and hSGLT2/COS-7 cells for effective evaluation of inhibitory activities toward SGLT1 and SGLT2.

3.6. Identification of natural flavonoids with inhibitory activities toward SGLT1 and SGLT2

The validated method using 1-NBDG was applied to test 195 crude methanol extracts from approximately 60 species of plants in the Lauraceae family in search of novel natural SGLT inhibitors and two potent natural SGLT1 and SGLT2 inhibitors were identified from the leaves of *Cinnamomum macrostemon* (Yang et al., manuscript in preparation). The IC_{50} values of these two compounds (I and II) were in the nanomolar range and comparable to phlorizin, the most potent natural SGLT inhibitor with IC_{50} of 110 ± 10.2 nM for SGLT1 and 9.65 ± 1.83 nM for SGLT2 in our assay system.

Since compounds I and II are kaempferol glycosides (Fig. 5), the SGLT inhibitory activities of 7 simple flavonoids and 60 flavonoid glycosides isolated from plants were evaluated in this study. The basic skeletons of these compounds are flavonols (1–45) (Table 1), flavones (46–56), dihydroflavonols (57–63), and dihydroflavones (64–67) (Table 2) (Fig. 6). Compounds were tested at a concentration of 50 μ M. 1-NBDG uptake between 40% and 70% was considered moderate inhibition and lower than 40% was considered strong inhibition. The results of 1-NBDG uptake are summarized in Tables 2 and 3. In the assay of SGLT1 inhibitory activity, both flavonol O-glycosides 14 and 15 displayed a strong SGLT1 inhibitory activity with 17.5% and 33.1% uptake via hSGLT1, respectively, while flavonol O-glycosides 6 and 11 showed a moderate SGLT1 inhibitory activity with 52.9% and 57.0% uptake,

Fig. 4. Detection of 1-NBDG and 2-NBDG uptake by flow cytometry. A. Flow cytometric profiles of COS-7 cells after incubation without (Control) or with 100 μ M 1-NBDG or 2-NBDG in sodium buffer for 30 min. B–C. Flow cytometric profiles of hSGLT1/COS-7 cells after incubation with 100 μ M 1-NBDG (B) or 2-NBDG (C) in sodium buffer, choline buffer or sodium buffer with phlorizin (Ph) for 30 min. Experiments were performed three times and representative results are shown in A–C. D. Quantitative flow cytometric analysis of 1-NBDG and 2-NBDG uptake. Geomean of fluorescence intensity was normalized with that of untreated cells to obtain normalized geomean of fluorescence intensity (NGFI). Results are presented as mean $\pm$ SEM of three independent experiments. ** $P < 0.01$, *** $P < 0.001$.

respectively. In the assay of SGLT2 inhibitory activity, both flavone C-dioside 52 and dihydroflavonol *O*-glycoside 60 were potent SGLT2 inhibitors with 35.9% and 37.1% uptake via hSGLT2, respectively, while the flavonol *O*-glycosides 8, 12, 13, and 41, and the flavone C-glycoside 54 exhibited a moderate SGLT2 inhibitory activity with % uptake ranging from 55.9% (8) to 68.4% (12). Two flavonol *O*-glycosides, 7 and 34, were found to inhibit both SGLT1 and SGLT2. Compound 34 moderately inhibited 1-NBDG uptake via both hSGLT1 and hSGLT2 (58.7% and 69.1% respectively). Compound 7 [K-3-*O*-(6-*O*-glc)-glc] strongly inhibited SGLT2 (0.70% uptake) in addition to moderate inhibition against SGLT1 (58.7% uptake) (Table 2). The IC_{50} values of 7 toward SGLT2 and SGLT1 were

$4.52 \pm 1.06 \mu$ M ($n = 3$) and $76.0 \pm 3.87 \mu$ M ($n = 3$), respectively, with a SGLT2 selectivity (SGLT1 IC_{50} /SGLT2 IC_{50}) of 16.8. These results indicated that compounds possessing selective SGLT1 inhibition (14), dual inhibition (7), and selective SGLT2 inhibition (52 and 60) could be candidates for further development.

3.7. Structure-activity relationship analysis

Structure-activity relationship (SAR) of these assayed flavonoids was analyzed on a basis of the 1-NBDG uptake data shown in Tables 2 and 3. Compounds I and II are kaempferol glycosides with potent SGLT inhibitory activities. Their aglycon kaempferol (1), however, had no inhibitory activity,

Table 1. The ratios of 1-NBDG uptake in sodium buffer vs. in choline buffer.

Fluorescence ratio	1-NBDG uptake in Na buffer/1-NBDG uptake in Ch buffer		
	COS-7 ($n = 2$)	hSGLT1/COS-7 ($n = 3$)	hSGLT2/COS-7 ($n = 5$)
NaCl bkg subtracted	0.98 ± 0.00	4.56 ± 0.09	2.20 ± 0.20
Cell bkg subtracted	1.03 ± 0.18	$7.25 \pm 0.25^{**}$	$3.37 \pm 0.35^*$

Data are presented as mean $\pm$ SEM of 2–5 independent experiments. Significant differences were observed between the ratios calculated from subtracting the NaCl background (bkg) and subtracting the cell bkg. * $P < 0.05$, ** $P < 0.01$.

Fig. 5. Potent SGLT1/2 inhibitors, compounds I and II and those verified in this study.

suggesting the glycon part to be crucial. Among kaempferol monoosides (2–6), only kaempferol rhamnoside (6) showed a moderate inhibitory activity against SGLT1 (~50% inhibition at 50 μ M).

Interestingly, compound II, a 2''- β -D-glucosyl derivative of 6, showed much greater inhibitory activities toward both SGLT1 and SGLT2 (100% inhibition at 50 μ M) (Yang et al., manuscript in

Table 2. SGLT inhibitory activities of 45 flavonols and flavonol glycosides at 50 μ M.

No.	Name ^a	% Uptake via		No.	Name ^a	% Uptake via	
		hSGLT1	hSGLT2			hSGLT1	hSGLT2
1	Kaempferol (K)	142 $\pm$ 10.0	155 $\pm$ 1.03	24	Q-3-O-rha ^p	89.8 $\pm$ 0.08	103 $\pm$ 5.90
2	K-3-O-gal ^p	96.2 $\pm$ 4.13	121 $\pm$ 6.27	25	Q-3-O-(2-O-ara ^p)-rha ^p	112 $\pm$ 1.80	111 $\pm$ 8.04
3	K-3-O-glc ^p	93.1 $\pm$ 6.24	104 $\pm$ 5.55	26	Q-3-O-(2-O-rha ^p)-gal ^p	113 $\pm$ 3.19	102 $\pm$ 10.1
4	K-3-O-ara ^f	90.9 $\pm$ 5.63	122 $\pm$ 9.54	27 ^b	Q-3-O-(6-O-rha ^p)-gal ^p	96.0 $\pm$ 8.82	106 $\pm$ 7.00
5	K-3-O-ara ^p	92.5 $\pm$ 5.91	93.2 $\pm$ 4.63	28	Q-3-O-(2-O-rha ^p)-glcUA ^p	111 $\pm$ 2.83	96.0 $\pm$ 8.32
6	K-3-O-rha ^p	52.9 $\pm$ 3.53	107 $\pm$ 2.15	29	Q-3-O-(2-O-rha ^p)-rha ^p	96.0 $\pm$ 8.64	99.2 $\pm$ 9.58
7	K-3-O-(6-O-glc ^p)-glc ^p	58.7 $\pm$ 2.47	0.70 $\pm$ 0.19	30	Q-3-O-[2-O-(2-O-rha ^p)-ara ^p]-rha ^p	71 $\pm$ 8.77	92.4 $\pm$ 5.67
8	K-7-O-rha ^p ,3-O-(6-O-glc ^p)-glc ^p	95.2 $\pm$ 5.72	55.9 $\pm$ 2.52	31	Q-3-O-(2,6-di-O-rha ^p)-gal ^p	99.5 $\pm$ 8.79	119 $\pm$ 6.75
9	K-3-O-(2-O-api ^f)-rha ^p	79.5 $\pm$ 3.50	119 $\pm$ 8.53	32 ^b	Q-3-O-(6-O-rha ^p ,2-O-xyl ^p)-glc ^p	91.7 $\pm$ 5.64	91.1 $\pm$ 3.78
10	K-3-O-(2-O-rha ^p)-gal ^p	92.5 $\pm$ 4.65	99.9 $\pm$ 5.99	33 ^b	Isorhamnetin (IsoR)	169 $\pm$ 5.03	128 $\pm$ 13.4
11	K-3-O-(2-O-rha ^p)-rha ^p	57.0 $\pm$ 5.75	112 $\pm$ 1.58	34	IsoR 3-O-(6-O-cou ^z)-glc ^p	58.7 $\pm$ 5.79	69.1 $\pm$ 8.25
12 ^b	K-3-O-(6-O-rha ^p)-glc ^p	86.4 $\pm$ 6.53	68.4 $\pm$ 7.29	35	IsoR 3-O-(2,6-di-O-rha ^p)-gal ^p	119 $\pm$ 3.37	84.0 $\pm$ 7.69
13	K-3-O-(2-O-api ^f ,6-O-rha ^p)-glc ^p	90.2 $\pm$ 7.20	64.3 $\pm$ 4.53	36	IsoR 3-O-glc ^p	98.0 $\pm$ 1.95	77.4 $\pm$ 6.65
14	K-3-O-(2,6-di-O-rha ^p)-gal ^p	17.5 $\pm$ 4.09	118 $\pm$ 4.40	37	IsoR 3-O-neohesperidoside	104 $\pm$ 2.06	86.4 $\pm$ 9.55
15 ^b	K-3-O-(6-O-cou)-glc ^p	33.1 $\pm$ 0.85	84.7 $\pm$ 7.95	38	IsoR 3-O-rha ^p	74.4 $\pm$ 4.40	82.5 $\pm$ 1.00
16	K-3-O-(2,4-di-O-cou ^f)-rha ^p	146 $\pm$ 11.9	261 $\pm$ 18.6	39 ^b	IsoR 3-O-(2,6-di-O-rha ^p)-glc ^p	106 $\pm$ 2.58	107 $\pm$ 7.33
17	K-3-O-(2-O-rha ^p)-glcUA ^p	132 $\pm$ 7.26	146 $\pm$ 23.3	40 ^b	IsoR 3-O-(6-O-rha ^p)-gal ^p	78.3 $\pm$ 3.30	78.4 $\pm$ 1.93
18	Quercetin (Q)	138 $\pm$ 8.83	158 $\pm$ 10.7	41 ^b	IsoR 3-O-(6-O-rha ^p)-glc ^p	78.6 $\pm$ 5.81	67.2 $\pm$ 4.89
19	Q-3-O-ara ^f	105 $\pm$ 1.07	105 $\pm$ 4.81	42	Rhamnetin 3-O-glc ^p	95.5 $\pm$ 1.83	109 $\pm$ 9.05
20	Q-3-O-ara ^p	102 $\pm$ 2.45	96.4 $\pm$ 4.17	43	Rhamnetin 3-O-rha ^p	72.8 $\pm$ 2.65	151 $\pm$ 2.40
21 ^b	Q-3-O-gal ^p	108 $\pm$ 3.24	106 $\pm$ 16.3	44 ^b	Myricetin 3-O-ara ^f	122 $\pm$ 5.93	150 $\pm$ 7.18
22	Q-3-O-glc ^p	101 $\pm$ 1.20	95.1 $\pm$ 4.27	45 ^b	Myricetin-3-O-rha ^p	84.4 $\pm$ 3.68	98.1 $\pm$ 14.6
23	Q-3-O-glcUA ^p	122 $\pm$ 10.8	141 $\pm$ 19.2				

gal: β -D-galactosyl; glc: β -D-glucosyl; rha: α -L-rhamnosyl; ara: α -L-arabinosyl; glcUA: β -D-glucuronyl; xyl: β -D-xylosyl; api: β -D-apiosyl; cou: (*E*)-*p*-coumaroyl.

Data are presented as mean $\pm$ SEM of at least three independent experiments.

^a *f* and *p* stand for furanosyl and pyranosyl in the intermediate, while furanoside and pyranoside in the terminal.

^b 12: Kaempferol-3-O-rutinoside, nicotiflorin; 15: Tilioside; 21: Hyperoside; 27: Quercetin-3-O-robinoside; 32: Quercetin-3-O-(2G-xyl)^p-rutinoside; 33: 3'-O-Methylquercetin; 39: Typhaneoside; 40: Isorhamnetin-3-O-robinobioside; 41: Isorhamnetin-3-O-rutinoside; 44: Betmidin; 45: Myricitrin.

Fig. 6. Four subgroups of flavonoids and structures and activities of flavonol 3-O-rhamnosides **6**, **24**, **38**, **43** and **45**. Kaempferol glycoside **6** showed the best inhibitory activity (~50% inhibition) toward SGLT1 among these compounds. Additional hydroxyl groups and methoxy groups in rings A and B may reduce the SGLT1 inhibitory activity (**6** vs. **24**, **38** and **45**). Besides, methylation of the 3'-OH in ring B may slightly enhance the SGLT2 inhibitory activity (**38** vs. **24**).

preparation). Kaempferol-3-O- β -D-glucoside (**3**) did not inhibit SGLT1 and SGLT2. In contrast, compound **7** [K-3-O-(6-O-glc)-glc] showed significant inhibitory activities at 50 μ M, especially toward

SGLT2 (SGLT1~40%, SGLT2 ~99%). Compound **12** (K-3-O-(6-O-rha)-glc) differs from **7** only at the terminal sugar residue (rhamnosyl vs. glucosyl) and is also an isomer of **II**, differing both in the sugar

Table 3. SGLT inhibitory activities of flavones (**46**–**56**), dihydroflavonols (**57**–**63**), and dihydroflavones (**64**–**67**) at 50 μ M.

No.	Name ^a	% Uptake via		No.	Name ^a	% Uptake via	
		hSGLT1	hSGLT2			hSGLT1	hSGLT2
46	Apigenin-4'-O-glc ^p (A-4'-O-glc ^p)	102 $\pm$ 3.12	144 $\pm$ 9.39	57	(+)-Dihydro-K	153 $\pm$ 4.00	180 $\pm$ 10.1
47^b	A-6-C-glc ^p	100 $\pm$ 4.58	75.8 $\pm$ 1.03	58^b	(+)-Dihydro-Q	113 $\pm$ 9.58	143 $\pm$ 13.7
48^b	A-8-C-glc	92 $\pm$ 5.96	84.3 $\pm$ 9.47	59	(2R,3R)-Dihydro-Q 7-O-glc ^p	123 $\pm$ 5.34	121 $\pm$ 5.88
49	A-6,8-di-C-glc ^p	101 $\pm$ 4.46	72.1 $\pm$ 3.81	60	(2R,3R) Dihydro-Q 3-O-rha ^p	94.5 $\pm$ 4.37	37.1 $\pm$ 2.99
50^b	A-6-C-(2-O-glc ^p)-glc ^p	100 $\pm$ 5.71	93.1 $\pm$ 0.45	61	(2S,3R)-Dihydro-Q 3-O-rha ^p	108 $\pm$ 4.66	84.6 $\pm$ 9.35
51^b	A-6-C-(2-O-rha ^p)-glc ^p	108 $\pm$ 5.32	118 $\pm$ 4.74	62	(2R,3S)-Dihydro-Q 3-O-rha ^p	91.4 $\pm$ 0.81	89.6 $\pm$ 12.0
52	A-6-C-glc ^p -8-C-xyl ^p	99.5 $\pm$ 5.48	35.9 $\pm$ 1.81	63	(2S,3S)-Dihydro-Q 3-O-rha ^p	87.5 $\pm$ 2.82	81.3 $\pm$ 5.69
53^b	7-O-Methyl apigenin	173 $\pm$ 3.30	178 $\pm$ 19.8	64	Pinocembrin 7-O-glc ^p	124 $\pm$ 1.42	116 $\pm$ 15.3
54^b	Luteolin 6-C-glc ^p	81.0 $\pm$ 3.63	60.6 $\pm$ 1.79	65	Naringenin	149 $\pm$ 2.72	137 $\pm$ 5.20
55^b	Luteolin 6-C-(2-O-cou ^E)-glc ^p	84.4 $\pm$ 3.78	75.0 $\pm$ 7.02	66	Naringenin 7-O-glc ^p	116 $\pm$ 2.72	122 $\pm$ 19.4
56^b	Luteolin 6-C-(2-O-rha ^p)-glc ^p	98.2 $\pm$ 4.66	103 $\pm$ 7.03	67	Eriodictyol 7-O-glc ^p	N/A (cell loss)	49.3 (n = 1)

gal: β -D-galactosyl; glc: β -D-glucosyl; rha: α -L-rhamnosyl; ara: α -L-arabinosyl; glcUA: β -D-glucuronyl; xyl: β -D-xylosyl; api: β -D-apiosyl; cou: (*E*)-*p*-coumaroyl.

Data are presented as mean $\pm$ SEM of at least three independent experiments except **67** which caused severe cell loss.

^a f and p stand for furanosyl and pyranosyl in the intermediate, while furanoside and pyranoside in the terminal.

^b **47**: Isovitexin; **48**: Vitexin; **50**: Isovitexin 2''-O-glc^p; **51**: Isovitexin 2''-O-rha^p; **53**: Genkwanin; **54**: Isoorientin; **55**: Isoorientin 2''-cou^E; **56**: Isoorientin 2''-O-rha^p; **58**: Taxifolin.

linkage and sequence [glc (6 → 1) rha vs. rha (2 → 1) glc, II]. However, it only showed some activity against SGLT2 at 50 μ M. Whereas the diside 11 differing from 12 by replacement of the intermediate glucosyl in 12 by rhamnosyl displayed moderate SGLT1 inhibitory activity. These data suggest that the aglycon kaempferol (1) cannot exert SGLT inhibitory activities and 3-O-glycosylation is essential for SGLT inhibitory activities. Among kaempferol monoosides, the rhamnoside shows better SGLT1 inhibition than glucoside, galactoside, and arabinoside. Kaempferol disides are more potent than the monoosides. In addition, those possessing glucosyl as the terminal residue show both SGLT1 and SGLT2 inhibitory activities, especially to SGLT2.

Intriguingly, replacement of terminal glucosyl residue by *E-p*-coumaroyl group (15) also showed SGLT1 inhibitory activity. Comparing 10 [K-3-O-(2-O-rha)-gal] with 14 [K-3-O-(2,6-di-O-rha)-gal] revealed that an addition of 6-O-rhamnosyl unit at galactosyl residue may increase the SGLT1 selectivity and inhibitory activity. While compound 31, a

3'-hydroxylated derivative of 14, did not show any inhibitory activity toward SGLT1, suggesting that the 3'-hydroxyl group at the flavonols will decrease the inhibitory activity. Compounds 6, 24, 38, 43, and 45 are all 3-O-rhamnosyl flavonoids and only compound 6, the monohydroxylated one (4'-OH) in the B-ring, showed significant inhibitory activity (~50% inhibition) toward SGLT1 (Fig. 6). Thus, additional hydroxyl substituents in the B ring weaken the SGLT1 inhibitory activity, although 3'-methoxy group may slightly enhance SGLT2 inhibitory activity (24 vs. 38; 31 vs. 35; 27 vs. 40) (Table 2).

7-O-Glycosylation on flavonols may also affect inhibitory activities toward SGLTs. Compound 8 [K-7-O-rha,3-O-(6-O-glc)-glc] had a 7-O-rhamnosyl residue more than compound 7, leading to much less inhibitory activity against SGLT2 than 7 (44.1% vs. 99.3%, 50 μ M) in addition to almost no inhibitory activity toward SGLT1 (Table 2).

The effect of C-glycosylation at C-6 or C-8 position of flavones, the 3-dehydroxylated flavonols, was also analyzed. Among the apigenin C-glycosides 47–52, the diside 52 (A-6-C-glc-8-C-xyl) had better SGLT2

(A) Flavonols/Dihydroflavonols

(B) Flavones

Fig. 7. Structure-activity relationship analysis of flavonols/dihydroflavonols (A) and flavones (B), drawn from assay results of 67 flavonoids. IA: inhibitory activity; one arrow: increase or decrease; two arrows: marked increase or decrease.

inhibitory activity than the dioside 49 (A-6-C-glc-8-C-glc) (35.9% uptake by 52 vs. 72.1% uptake by 49), indicating C-8 xylosyl to be a better substituent for such activity than C-8 glucosyl residue. For the isomeric monoosides 47 and 48, C-glucosidation at C-6 position showed a slightly better SGLT2 inhibitory activity than at C-8 position. Furthermore, 49 with an additional C-6-glc compared to 48, also had a slightly better SGLT2 inhibitory activity (Table 3). Isoorientin, i.e. luteolin-6-C- β -D-glucoside (54) or 3'-hydroxy-apigenin 6-C- β -D-glucoside, showed ~40% inhibition of SGLT2 and ~20% inhibition of SGLT1 at 50 μ M, better than 47 (A-6-C-glc). This phenomenon is different from that of kaempferol vs. quercetin 3-O-glycosides. That is 3'-hydroxylation at flavones increases inhibitory activity against both SGLTs (Table 3). Likewise, dihydroflavone 67, the 3'-hydroxylated 66, might be much more active than 66; however, it was the result of one experiment since 67 caused cell loss. As flavones lack C-3 substitution, their interaction sites on SGLTs should be different from flavonols, causing different inhibitory activity as stated above.

Dihydroflavonol 3-O-monoosides (59–63), the Δ^2 -saturated flavonol glycosides, also showed weak or no inhibitory activity against SGLTs except for 60, the (2R,3R)-isomer among four stereomers (60–63), which showed good SGLT2 inhibitory activity (~63% inhibition). This indicates that stereochemistry at the C-2 and C-3 positions of dihydroflavonol glycosides plays a critical role toward the interaction between ligands and hSGLT2.

A summary of SAR analyses stated above is illustrated in Fig. 7.

4. Discussion

We demonstrated here that the fluorescence of 1-NBDG and 2-NBDG is quenched under alkaline conditions. Unlike 2-NBDG which is decomposed in NaOH, the fluorescence of 1-NBDG can be restored after neutralization. This characteristic of 1-NBDG provides an easy way to lyse cells following glucose uptake assay and an advantage when determination of protein content in the lysate is required such as kinetic studies. A large volume of lysate, which is required due to low protein concentration, in 0.1 N NaCl (equal volume of 0.2 N NaOH and 0.2 N HCl) will not interfere with common protein assays, such as Bradford assay, while large volumes of lysates in lysis buffers containing detergents will interfere with protein assay results.

More and more studies have shown that cancer cells may also overexpress SGLT1 or SGLT2 other than GLUTs [1,2]. Therefore, 1-NBDG could be a

better choice to detect glucose transport via both GLUTs and SGLTs. However, the kinetics of 1-NBDG transport through GLUTs and SGLTs require further studies.

The intracellular stability of 1-NBDG ($t_{1/2}$ = 38 min) is not as good as 2-NBDG ($t_{1/2}$ = 61 min). Although the fluorescence stability of 1-NBDG in cell lysate was good enough for experimental examinations, like 2-NBDG, the use of 1-NBDG in the study of glucose metabolism in live cells is limited due to its poor intracellular stability. We speculate that 1-NBDG can also be phosphorylated at C-6 and may consequently become metabolized or decomposed to nonfluorescent compounds. Thus, the same as 2-NBDG, fluorescence intensity of 1-NBDG reflects a dynamic equilibrium of generation and decomposition of its fluorescent metabolite inside the cell. Nevertheless, short-term (e.g., 5–10 min) monitoring of 1-NBDG uptake in live cells is technically feasible as in the case of 2-NBDG [5].

We also demonstrated that 1-NBDG uptake could be detected at the single cell level by flow cytometry, however, the fluorescence signal of 1-NBDG was not as intense as 2-NBDG possibly due to the deviation of the optimal excitation wavelength (458 nm) of 1-NBDG [7] from that frequently used for the FL1 channel of flow cytometer (488 nm), whereas the optimal excitation wavelength of 2-NBDG is 475 nm which is close to 488 nm. Furthermore, since 1-NBDG is less stable inside the cell, it may in part account for the lower fluorescence signal of 1-NBDG. Nevertheless, inhibition of 1-NBDG uptake by SGLT inhibitors was clearly detected by flow cytometry.

We have further expanded the 1-NBDG uptake assay system from 24-well to 96-well format for high-throughput screening to search for novel SGLT1 and SGLT2 inhibitors and have successfully identified two kaempferol glycosides from the leaves of *C. macrostemon* as potent natural SGLT1/2 inhibitors comparable to phlorizin (Yang et al., manuscript in preparation). Natural products have been used in treatment of diabetes for a long time. Flavonoids, a huge group of polyphenolic compounds abundant in plants, have numerous biological activities, and their potential use for the treatment of diabetes has been reported [8,9,14]. Flavonoids are suitable for SAR analysis due to huge diversity of structures and substitutions. Since kaempferol glycosides belong to the family of flavonoid glycosides, the 1-NBDG uptake assay system was used to evaluate 67 purified flavonoids and flavonoid glycosides and several selective SGLT1 inhibitors, selective SGLT2 inhibitors as well as dual SGLT1/2 inhibitors were identified. These

results were further subjected to SAR analysis. As summarized in Fig. 7, for flavonols and dihydroflavonols, glycosyl residues attached to the 3-hydroxyl group is important for the inhibitory activities toward SGLT1 and SGLT2. Besides, glucosyl group as the terminal residue at 3-O position in diosides makes great contribution to both SGLT1 and SGLT2 inhibitory activities. On the other hand, 7-O-glycosides and additional hydroxyl groups in the B ring may reduce the inhibitory activities. Moreover, saturation of 2,3 double bond also affects the inhibitory activities. As for flavones, a xylosyl rather than glucosyl group at C-8 and a glucosyl group at C-6 rather than at C-8 show better SGLT2 inhibitory activity. Unlike flavonols, 3',4'-dihydroxyl substitution shows better SGLT inhibitory activities than 4'-hydroxyl flavone glucoside. For all the flavonoid glycosides, the sugar inter-linkage and their substitution positions to the aglycon influence not only the inhibitory activities but also the selectivity toward SGLT1 and SGLT2. We hope that the potency and selectivity of SGLT inhibitors identified in this study could be increased by structure modification based on our SAR findings. The modified SGLT inhibitors may have better SGLT inhibitory effect, and may also possess anticancer activity especially to cancer cells overexpressing SGLT1 and/or SGLT2, either alone or in combination with other chemotherapeutic drugs.

Conflicts of interest

The authors declare no conflict of interest.

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