Acta Pharmaceutica Sinica B 2013;3(2):97–101

Institute of Materia Medica, Chinese Academy of Medical Sciences
Chinese Pharmaceutical Association

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com

ORIGINAL ARTICLE

A cell-based fluorescent glucose transporter assay for SGLT2 inhibitor discovery

Yi Huan, Linyi Li, Quan Liu, Shuainan Liu, Zhufang Shen*

State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Diabetes Research Center, Institute of Materia Medica, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100050, China

Received 11 October 2012; revised 7 January 2013; accepted 23 January 2013

KEY WORDS

SGLT2 inhibitor;
NBDG;
Type 2 diabetes mellitus;
Kidney

Abstract The sodium/glucose cotransporter 2 (SGLT2) is responsible for the majority of glucose reabsorption in the kidney, and currently, SGLT2 inhibitors are considered as promising hypoglycemic agents for the treatment of type 2 diabetes mellitus. By constructing CHO cell lines that stably express the human SGLT2 transmembrane protein, along with a fluorescent glucose transporter assay that uses 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]2-deoxyglucose (2-NBDG) as a glucose analog, we have developed a nonradioactive, cell-based assay for the discovery and characterization of SGLT2 inhibitors.

*Corresponding author. Tel./fax: +86 10 8317 2669.
E-mail address: shenzhf@imm.ac.cn (Zhufang Shen).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

Production and hosting by Elsevier

2211-3835 © 2013 Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.apsb.2013.02.001

1. Introduction

Previous research has shown that about 180 g of glucose is filtered through human kidney glomeruli every day, and more than 95% is reabsorbed in the S1 and S2 segments of the proximal tubule. Two sodium/glucose cotransporters (SGLT 1 and 2) are believed to be responsible for this reuptake. Based on pharmacokinetic studies with several species, SGLT1 is a high-affinity glucose transporter (K_m 0.3–0.5 mM) with a 2:1 sodium:glucose coupling ratio, and is mainly located in the S3 segment of renal tubule, although there is some expression in the digestive tract. SGLT2 is a low-affinity glucose transporter (K_m 5–6 mM) with a 1:1 sodium:glucose coupling ratio and a distribution along the S1 and S2 segments of the kidney tubule[1–2].

As a result, these Na^+/glucose cotransporters, especially SGLT2, are responsible for most of the glucose adaptive reabsorption and play an important role in body glucose homeostasis. On the other hand, abnormal expression and dysfunction of SGLT2 has been found in the onset and progression of diabetes, and may contribute to the chronic hyperglycemia and glucotoxicity leading to β-cell dysfunction[3–4]. By decreasing glucose reabsorption in kidney and increasing urinary glucose excretion, SGLT2 inhibitors can normalize the blood glucose level and decrease glucotoxicity to organs and tissues, including pancreatic islet cells and the microvasculature, and can be considered as hypoglycemic agents for type 2 diabetes mellitus. These agents may also prevent the development of diabetic complications[5–8].

Phlorizin had been identified as an inhibitor for both SGLT1 and SGLT2, and several phlorizin derivatives have been developed to specifically inhibit SGLT2. Dapagliflozin and Canagliflozin were the first-in-class oral SGLT2 inhibitors, which have been proven to be effective when used alone or in combination with available antidiabetic drugs in Phase III clinical trials[9,10]. Although the risk–benefit profile has not been fully characterized, several clinical trials are currently underway[11,12].

Screening approaches for the discovery of SGLT2 inhibitors have been based on sodium-dependent glucose uptake measurements, and generally included radiolabeled substrates and SGLT2-expressing host cells[13,14]. In this study we introduce a cell-based fluorescence assay for sodium-dependent glucose uptake that can be used to identify SGLT2 inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

Trizol Reagent, Dulbecco's modified Eagle's medium (DMEM)/F12, lipofectamine 2000, and 2-NBDG were purchased from Invitrogen (USA). Cell culture plates were from Corning-Costar (USA). Fetal bovine serum (FBS) was a product of Biochrome AG (Germany). A first-strand cDNA synthesis kit was purchased from Vigorous (Beijing, China). A SYBR green PCR kit (SYBR Premix Ex Taq^{TM}) was obtained from Takara (Japan). Phlorizin was purchased from Sigma-Aldrich (USA).

2.2. Cell culture and plasmid transfection

Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection and propagated in DMEM/F12 supplemented with 10% FBS and 1% penicillamine-streptomycin at 37 °C in 5% CO_2 and humidified air.

To establish human SGLT2 (hSGLT2) expression and activity in cell membranes, we inserted the hSGLT2 full-length cDNA (NCBI reference sequence: NM_003041.3) into the plasmid vector Peak13CD5L–GFP to construct an SGLT2 expression plasmid with a fluorescent expression marker (GFP), Peak13CD5L–hSGLT2–GFP. For cell-based glucose uptake assays the GFP cassette was removed, yielding the Peak13CD5L–hSGLT2 expression plasmid. In transient transfection experiments the cells were seeded in 6-well plates for 24 h, reaching 50–60% confluence, and then the transfection mixture containing 3 μg plasmid DNA and 4.5 μg lipofectmine 2000 was added to the cell culture medium. After incubating for 48 h, the cells were harvested for experimental assays.

2.3. Fluorescence microscopy

The Peak13CD5L–hSGLT2–GFP plasmid was transiently transfected into CHO cells, and after 48 h the cells were visualized and photographed on the confocal laser microscope (Leica TCS SP2, Germany) with filters for green fluorescence protein.

2.4. Stable cell line construction

CHO cells transfected with the Peak13CD5L–hSGLT2 plasmid were selected after 48 h of culture by adding 1.2–2.0 μg/mL of puromycin to the medium. Non-transfected CHO cells cultured in the same medium served as the negative control. After 3–5 days of culture, the negative control cells died of puromycin toxicity and the transfected cells were harvested and diluted for monoclone culture in 96-well plates for at least 10 days.

After propagation of the monoclone cells to stable cell lines, total RNA was isolated using Trizol Reagent, followed by reverse transcriptase reactions using a first-strand cDNA synthesis kit. The quantitative real-time polymerase chain reaction (PCR) was used to identify hSGLT2-expressing cell lines using the SYBR green PCR kit. The primers for hSGLT2 used in RT-PCR were: 5′–GCAGGCTCGGCACCAGAGATG–3′ (forward), and 5′–TGGACCACAAGCCAACGCCA–3′ (reverse). To ensure stable transfection, the RT-PCR-identified cell line was further cultured in puromycin-containing medium and passaged for five or more generations, and confirmed by RT-PCR as needed.

2.5. Sodium-dependent glucose uptake assay

Choline buffer, prepared for cell incubations under Na^+-free conditions, contained 140 mM choline chloride, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, and 10 mM HEPES (pH = 7.4, adjusted with 2.5 M Tris). Sodium buffer contained 140 mM NaCl instead of choline chloride and was used for cell incubations to measure NBDG and glucose uptake in the presence of sodium.

For the Na^+/glucose cotransporter assay we used stably transfected cells with the Peak13CD5L–hSGLT2 plasmid. Cells were harvested and seeded into the poly-lysine pre-coated 96 well plates at 40,000–60,000 cells per well. After 10-h adherent growth, the cells were washed with 200 μL choline buffer and serum-starved for at least 1 h. The buffer was replaced with 50 μL of sodium buffer containing vehicle (DMSO), SGLT2 inhibitor (Phlorizin) or a candidate SGLT2

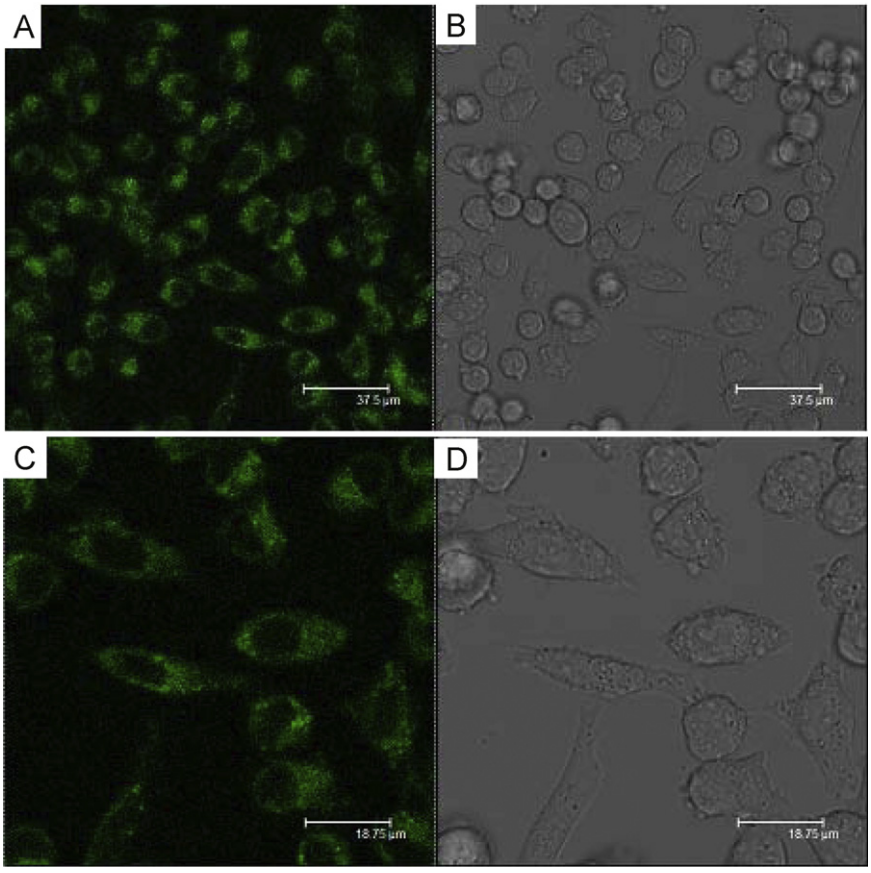

Figure 1 Fluorescent image of hSGLT2–GFP fusion protein expression in CHO cells. The fluorescence of the hSGLT2–GFP fusion protein was obtained by a confocal laser microscope 2 days after transfection of the Peak13CD5L–hSGLT2–GFP plasmid into CHO cells. Both the fluorescent images (A and C) and the bright field images (B and D) were taken for two selected fields. The scale bar in the images is 37.5 µm and 18.75 µm.

inhibitor for a 10-min incubation, to which was added NBDG (50 µM final concentration) or *D*-glucose (5 mM final concentration) in 50 µL of sodium buffer. The concentration of candidate compounds was usually set at 10^{-6} M. After being incubated at 37 °C for 60 min, the buffer was discarded and the cells were rinsed three times in choline buffer and released and lysed with 50 µL cold lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 40 mM KCl, 20 mM Tris, pH 7.4). The fluorescent intensity of the cell lysate was detected on a Bioteck Synergy 2 microplate reader (Excitation = 488 nm, Emission = 520 nm). The amount of NBDG taken up by the cells in 60 min was determined from a standard curve of 100 pM to 100 µM of NBDG in lysis buffer.

2.6. *Statistical analysis*

All values are presented as mean ± S.E.M. The Sigma Plot software was used for data analysis.

3. Results

3.1. *Optimized human SGLT2 transporter expression in CHO cells*

It has been reported previously that several host cells could be used to express SGLT2 recombinant protein, including HEK293, xenopus oocytes, CHO and COS-7 cells. In this study, both HEK293 and CHO cells were successfully transfected with our plasmid constructs and expressed high levels of recombinant hSGLT2 protein. CHO cells were selected for further characterization due to their better adhesion properties and greater stability to repeated buffer changes during the glucose uptake assays. Compared with the previous plasmids that we have used for hSGLT2 expression, the CHO cells transfected with Peak13CD5L–hSGLT2–GFP express a high level of hSGLT2–GFP fluorescent fusion protein in the cell membrane (Fig. 1).

3.2. *Identification of stable cell lines by RT-PCR*

To identify stable hSGLT2-expressing cells, RT-Real Time PCR analysis was carried out with puromycin-resistant CHO monoclone cells. Relative gene expression was determined by calculating the $2^{-\Delta\Delta CT}$ value. With comparison to expression of hSGLT2 in non-transfected CHO cells, multiple cell lines were identified in which the expression level of hSGLT2 was 200-fold greater than that of the negative control (Fig. 2).

3.3. *Optimized incubation buffer for the fluorescent glucose uptake assay*

To measure the sodium-dependent glucose uptake activity of hSGLT2 and the inhibitory potency of candidate inhibitors,

several buffer recipes were tested which differed in the concentration of NBDG, with or without supplemented glucose. Under these conditions NBDG uptake was measured in the presence or absence of phlorizin (Table 1). Results indicated that the sodium buffer with 25–100 μM of NBDG, supplemented with 5 mM of glucose was suitable for the fluorescent glucose uptake assay. Under these conditions the IC_{50} for phlorizin was approximately 10^{-6} M (Fig. 3).

3.4. *Concentration-dependent inhibition of hSGLT2 by phlorizin*

To identify the practicability of this fluorescent glucose uptake assay for SGLT2 inhibitor discovery, we measured the inhibition of NBDG uptake by phlorizin in the concentration range 10^{-7} to 10^{-5} M. Results showed that NBDG uptake decreased as the concentration of phlorizin increased (Table 2) and the potential concentration-dependent inhibition ability is shown in Fig. 4.

4. Discussion

Recently, the SGLT family members, and most importantly SGLT2, have been considered as important contributors to the pathophysiology of diabetes mellitus. Several analogs of phlorizin that act as SGLT2 inhibitors are currently in phase II clinical trials. Current *in vitro* methods to screen for effective SGLT2 inhibitors utilize radioactive substrates, with their inherent risks. NBDG is a fluorescent analog of glucose which is used in our laboratory for the direct measurement of glucose uptake[15] and for assessing hepatic glucose production in mice during hyperinsulinemic euglycemic clamp[16]. In the current study we utilized the fluorescent glucose analog NBDG to measure sodium-dependent glucose transport by SGLT2 and to establish an assay for the identification of SGLT2 inhibitors. Although nonradioactive assays have been described by others[17], we successfully applied this approach with a recombinant hSGLT2-expressing CHO cell line.

We found that the sodium buffer with 5 mM glucose was much more effective than the buffer without glucose, which can be explained in part in that the concentration of glucose in

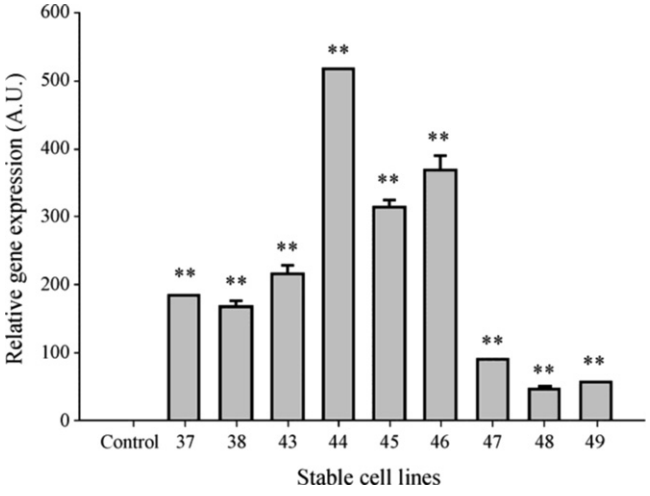

Figure 2 Relative gene expression in stably transfected hSGLT2 cells. Several stable cell lines (shown as Arabic numbers) were examined for human SGLT2 gene expression by real-time quantitative PCR. The results are shown as arbitrary units (A.U.) of relative quantitation values ($2^{-\Delta\Delta CT}$), using non-transfected CHO cells as a control calibrator and β-actin as reference gene. Each bar represents the mean±S.E.M. of triplicate determinations. $^{**}P<0.01$ *vs.* control.

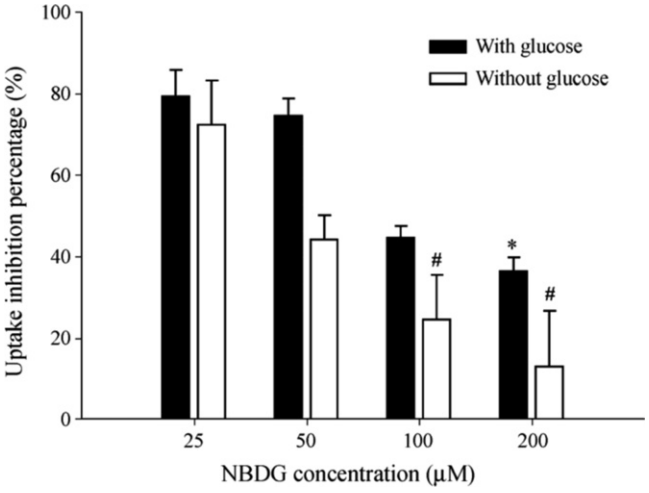

Figure 3 Inhibition of NBDG uptake by phlorizin in the optimized incubation buffer. Inhibition of NBDG uptake by phlorizin (10^{-6} M) was measured in Sodium buffer with (black bar) or without (white bar) 5 mM glucose. The concentrations of NBDG in the buffer were set at 25, 50, 100 and 200 μM. Data are shown as mean±S.E.M. in three parallel experiments. $^{*}P<0.05$ *vs.* data measured in Sodium buffer with 25 μM NBDG and 5 mM glucose. $^{\#}P<0.05$ *vs.* data measured in Sodium buffer with 25 μM NBDG in the absence of 5 mM glucose.

Table 1 NBDG uptake by cells with different incubation mixtures.

Concentration of NBDG (μM)	Sodium buffer with glucose		Sodium buffer without glucose	
	Phlorizin (−)	Phlorizin (+)	Phlorizin (−)	Phlorizin (+)
25	10.76±2.14	2.23±1.54*	18.51±3.50	5.14±2.03*
50	39.34±11.41	10.08±4.24	35.37±0.61	19.77±2.10*
100	59.01±3.82	32.75±1.17*	70.06±2.45	53.00±7.80
200	110.66±4.88	70.45±2.79*	149.90±8.56	130.33±20.26

Values were represented as mean±S.E.M. of three experiments.
$^{*}P<0.05$, NBDG uptake of phlorizin group *vs.* no phlorizin group under the same incubation conditions.

Table 2 NBDG uptake by the cells with different concentrations of phlorizin.

Concentration (M)	NBDG uptake
Control	21.60±0.50
10^{-7}	17.53±0.69*
10^{-6}	16.12±0.54*
10^{-5}	5.19±1.94*

Values were represented as mean±S.E.M. of three experiments.
*$P<0.05$, NBDG uptake with phlorizin *vs*. control.

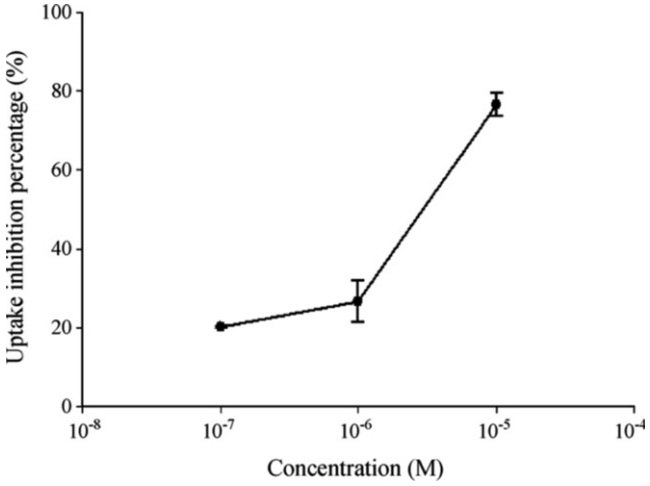

Figure 4 Concentration-dependent inhibition of SGLT2 by phlorizin. In the NBDG uptake assay the concentrations of phlorizin ranged from 10^{-7} to 10^{-5} M. Sodium buffer with 25 μM NBDG and 5 mM glucose was used in all assays. Data are shown as mean±S.E.M. in three parallel experiments.

this buffer approaches the physiological concentration in cells, and falls in the range reported for the K_m of SGLT2. In our study, phlorizin, an established SGLT2 inhibitor, showed good inhibitory ability and potential in the incubation buffer under a wide range of NBDG concentrations. For large scale screening experiments we recommend using sodium buffer with 50 μM NBDG and 5 mM glucose supplement, based on the stability and reliability of the results obtained herein.

Our SGLT2 expression plasmid yielded a high level of expression and localization of SGLT2 in the plasma membrane CHO cells, even in transiently transfected cells. Furthermore, the stable cell line clones can not only simplify the assay by bypassing the transfection step, but also maintain the consistency of results in subsequent screening assays. This method is currently being used in our laboratory in screening for SGLT2 inhibitors, and the stable cell line will be used for high-throughput screening in the future.

Acknowledgments

This study was supported by Beijing Natural Science Foundation (No. 7102118) and the Key Project of the National Twelfth-Five Year Research Program of China and National S&T Major Special Project on Major New Drug Innovation (No. 2012ZX09301002-004). The Plasmid Peak13CD5L-GFP used in the experiment was a kindly gift from Professor Chengyu Jiang. Compounds used for screening were provided by the Chemistry Synthesis laboratories in our institute.

References

1. Turner RJ, Silverman M. Sugar uptake into brush border vesicles from normal human kidney. *Proc Natl Acad Sci USA* 1997;**74**:2825–9.
2. Hummel CS, Lu C, Loo DD, Hirayama BA, Voss AA, Wright EM. Glucose transport by human renal Na^+/*D*-glucose cotransporters SGLT1 and SGLT2. *Am J Physiol Cell Physiol* 2011;**300**:14–21.
3. Washburn WN. Development of the renal glucose reabsorption inhibitors: a new mechanism for the pharmacotherapy of diabetes mellitus type 2. *J Med Chem* 2009;**52**:1785–94.
4. Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J. Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. *Diabetes* 2005;**54**:3427–34.
5. Abdul-Ghani MA, DeFronzo RA. Inhibition of renal glucose reabsorption: a novel strategy for achieving glucose control in type 2 diabetes mellitus. *Endocr Pract* 2008;**14**:782–90.
6. Liang Y, Arakawa K, Ueta K, Matsushita Y, Kuriyama C, Martin T, et al. Effect of canagliflozin on renal threshold for glucose, glycemia, and body weight in normal and diabetic animal models. *PLoS One* 2012;**7**:e30555.
7. Action to Control Cardiovascular Risk in Diabetes Study Group, Gerstein HC, Miller ME, Byington RP, Goff DC Jr, Bigger JT, et al. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med* 2008;**358**:2545–59.
8. DeFronzo RA, Davidson JA, Del Prato S. The role of the kidneys in glucose homeostasis: a new path towards normalizing glycaemia. *Diabetes Obes Metab* 2012;**14**:5–14.
9. Anderson SL, Marrs JC. Dapagliflozin for the treatment of type 2 diabetes. *Ann Pharmacother* 2012;**46**:590–8.
10. Devineni D, Morrow L, Hompesch M, Skee D, Vandebosch A, Murphy J, et al. Canagliflozin improves glycaemic control over 28 days in subjects with type 2 diabetes not optimally controlled on insulin. *Diabetes Obes Metab* 2012;**14**:539–45.
11. Suzuki M, Honda K, Fukazawa M, Ozawa K, Hagita H, Kawai T, et al. Tofogliflozin, a potent and highly specific sodium/glucose cotransporter 2 inhibitor improves glycemic control in diabetic rats and mice. *Pharmacol Exp Ther* 2012;**341**:692–701.
12. Tahara A, Kurosaki E, Yokono M, Yamajuku D, Kihara R, Hayashizaki Y, et al. Pharmacological profile of ipragliflozin (ASP1941), a novel selective SGLT2 inhibitor, *in vitro* and *in vivo*. *Naunyn Schmiedeberg's Arch Pharmacol* 2012;**385**:423–36.
13. Ikari A, Suketa Y. Expression of GFP-tagged low affinity Na^+-dependent glucose transporter in Xenopus oocytes and CHO cells. *Jpn J Physiol* 2002;**52**:395–8.
14. Castaneda F, Kinne RKA. 96-well automated method to study inhibitors of human sodium-dependent *D*-glucose transport. *Mol Cell Biochem* 2005;**280**:91–8.
15. Zou CH, Wang YJ, Shen ZF. 2-NBDG as a fluorescent indicator for direct glucose uptake measurement. *J Biochem Biophys Methods* 2005;**64**:207–15.
16. Liu SN, Liu Q, Sun SJ, Jiang Q, Peng J, Shen ZF. The application of 2-NBDG as a fluorescent tracer for assessing hepatic glucose production in mice during hyperinsulinemic euglycemic clamp. *Acta Pharm Sin B* 2012;**2**:403–10.
17. Blodgett AB, Kothinti RK, Kamyshko I, Petering DH, Kumar S, Tabatabai NM. A fluorescence method for measurement of glucose transport in kidney cells. *Diabetes Technol Ther* 2011;**13**:743–51.