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Short communication

In vitro characterization of luseogliflozin, a potent and competitive sodium glucose co-transporter 2 inhibitor: Inhibition kinetics and binding studies

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ABSTRACT

In this study, we evaluated an inhibition model of luseogliflozin on sodium glucose co-transporter 2 (SGLT2). We also analyzed the binding kinetics of the drug to SGLT2 protein using [^{3}H]-luseogliflozin. Luseogliflozin competitively inhibited human SGLT2 (hSGLT2)-mediated glucose uptake with a Ki value of 1.10 nM. In the absence of glucose, [^{3}H]-luseogliflozin exhibited a high affinity for hSGLT2 with a Kd value of 1.3 nM. The dissociation half-time was 7 h, suggesting that luseogliflozin dissociates rather slowly from hSGLT2. These profiles of luseogliflozin might contribute to the long duration of action of this drug.

Sodium glucose co-transporters (SGLTs) are a class of proteins expressed on the membranes of cells in various tissues. SGLT2 is mainly localized in the S1 and S2 segments of the renal proximal tubules and mediates the reabsorption of filtered glucose via the glomeruli. Recently, clinical studies have confirmed the efficacy of SGLT2 inhibitors in improving glycemic control (1–3), and several SGLT2 inhibitors are currently available for the treatment of type 2 diabetes (T2DM). Luseogliflozin ((1S)-1,5-anhydro-1-[5-(4-ethoxybenzyl)-2-methoxy-4-methylphenyl]-1-thio-D-glucitol hydrate) (Lusefi™, Taisho Pharmaceutical Co., Ltd.) is a novel and specific SGLT2 inhibitor, and is commercially available as a pharmaceutical agent to treat T2DM. In a previous study, luseogliflozin was demonstrated to inhibit sodium-dependent glucose uptake in cells stably expressing human SGLT2 (hSGLT2), with an IC_{50} value of 2.26 nM (4). In preclinical animal models of diabetes, luseogliflozin was shown to enhance urinary glucose excretion and reduce the severity of hyperglycemia (5). Although the IC_{50} value of luseogliflozin for SGLT2 *in vitro* was similar to that of commercially available SGLT2 inhibitors that have been used to treat T2DM, clinical studies in Japanese patients with T2DM have shown that once-daily administration of luseogliflozin, even at a very low dose (0.5 mg), leads to significant improvement of the HbA1c levels (1). In healthy Japanese subjects, luseogliflozin-induced dose-dependent increases in urinary glucose excretion were observed for at least 48 h after a single administration of 1–25 mg (6).

In the present study, we investigated the SGLT2 inhibition kinetics of luseogliflozin against sodium-dependent glucose uptake in Chinese hamster ovary-K1 cells stably expressing hSGLT2 to evaluate an inhibition model for glucose transport. We employed the [^{3}H]-luseogliflozin–SGLT2 binding assay to investigate whether luseogliflozin might bind specifically to the SGLT2 protein expressed on the membranes of cells.

Methods to prepare cells stably expressing hSGLT2 and glucose uptake study were described in a previous study (5). The cells were incubated in 75 μL of sodium buffer (140 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM Tris, pH 7.2–7.4) containing 1, 2, 4, 8 or 16 mM of the methyl-α-D-glucopyranoside (α-MG) mixture ([^{14}C]-α-MG: PerkinElmer, Tokyo and unlabeled α-MG: Sigma–Aldrich, St. Louis, MO, USA) and 1, 2 or 4 nM of luseogliflozin (Taisho Pharmaceutical Co., Ltd., Saitama) or dimethyl sulfoxide vehicle at 37 °C for 60 min; each of the incubations was performed in triplicate.

Binding assays were performed using a modification of the method of Grempler (7). For the saturation binding experiments, cell membranes (60 μg/well) were incubated with 1.3–368.8 nM [^{3}H]-luseogliflozin (Quotient Bioresearch Ltd., Cardiff, UK) in assay

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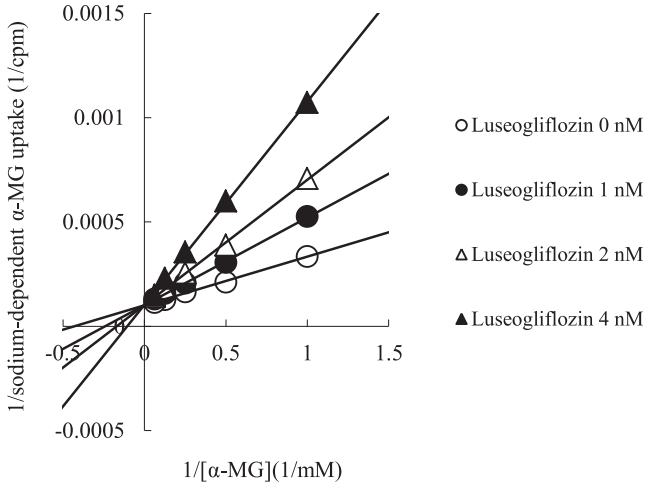

Fig. 1. Kinetic analysis of the inhibition of hSGLT2 by luseogliflozin. Cells stably expressing hSGLT2 were incubated in sodium buffer containing various concentrations of an α-MG mixture ([^{14}C]-α-MG and unlabeled α-MG) and luseogliflozin at 37 °C for 60 min. Sodium-dependent α-MG uptake was calculated by subtracting radioactivity (cpm) in the absence of sodium from radioactivity in the presence of sodium. The inhibition pattern was analyzed by Lineweaver–Burk plots. The data points represent the mean of three wells from a single representative experiment.

buffer [10 mM HEPES (pH 7.4), 137 mM NaCl] in the absence and presence of glucose (20 mM, Wako Pure Chemical Industries Ltd., Osaka), respectively, for 6 h at 25 °C in 96-well plates. For the association experiments, membranes were incubated with 5.2 and 19 nM of [^{3}H]-luseogliflozin in the absence and presence of glucose (20 mM), respectively, for 0.25, 1, 3, 6, 9 and 12 h at 25 °C. For the dissociation experiments, 12 h after association, 760 and 1970 nM of unlabeled luseogliflozin, approximately 100 times the amount of [^{3}H]-luseogliflozin, in the absence and presence of glucose, respectively, were added, followed by incubation for 2, 4, 7.67, 18.5 and 30 h at 25 °C. The approximate Kd values calculated from the saturation binding experiments were used as the concentrations for [^{3}H]-luseogliflozin in the presence or absence of glucose. Nonspecific binding was determined with 50 μM of phlorizin (Sigma–Aldrich). The data were analyzed using GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA).

In this study, we investigated the inhibitory kinetics of luseogliflozin for SGLT2, to elucidate the mechanism of SGLT2 inhibition. According to the Lineweaver–Burk analysis (Fig. 1), luseogliflozin competitively inhibited SGLT2-mediated sodium-dependent glucose uptake, and calculation based on Dixon plots yielded a Ki value of 1.10 nM (95% confidence interval: 1.01–1.18 nM).

Specific binding of [^{3}H]-luseogliflozin to the membranes of hSGLT2-expressing cells was saturated at 50 nM of [^{3}H]-luseogliflozin (Fig. 2A). Scatchard analysis of the binding of luseogliflozin to

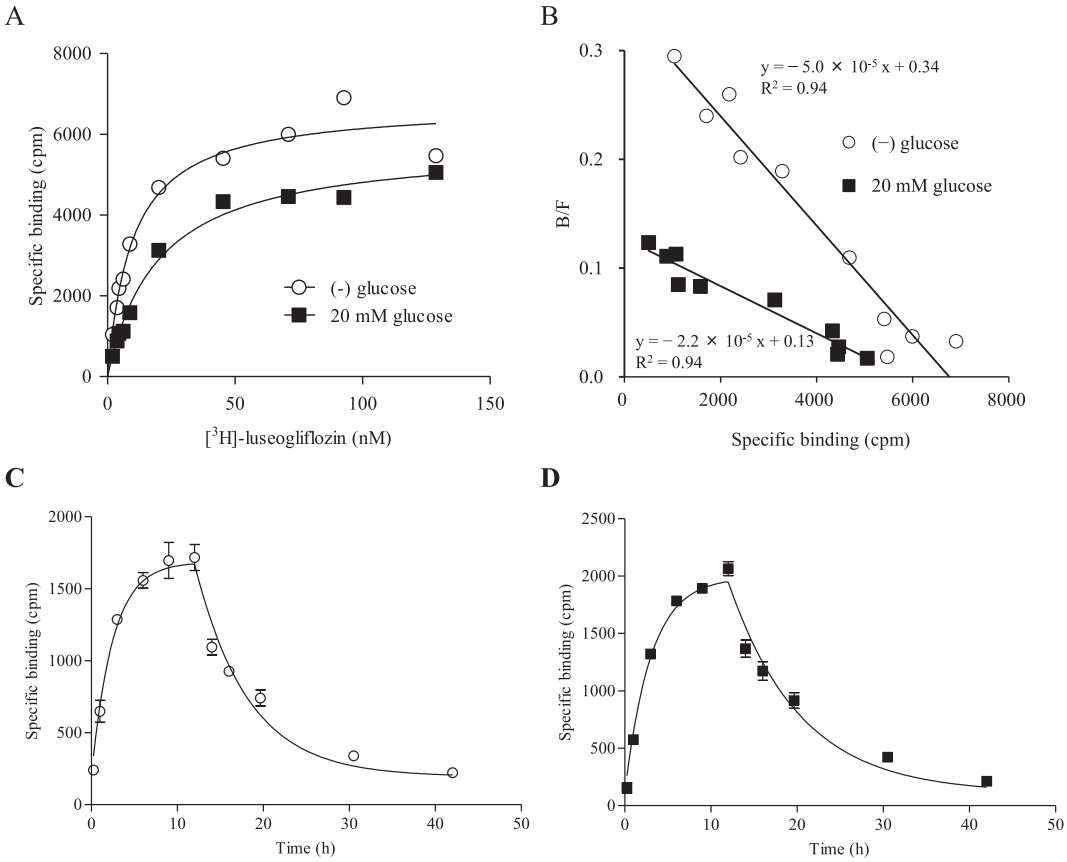

Fig. 2. Analysis of the binding kinetics of [^{3}H]-luseogliflozin to hSGLT2 protein. A and B) Saturation curve and Scatchard plot of [^{3}H]-luseogliflozin binding to hSGLT2. Membranes of hSGLT2 cells were incubated with [^{3}H]-luseogliflozin in an assay buffer in the absence and presence of glucose (20 mM) for 6 h at 25 °C. The data points represent the mean of two wells from a single representative experiment. C and D) Association and dissociation curves of [^{3}H]-luseogliflozin binding to hSGLT2 in the absence and presence of glucose. For the association time-course experiments, membranes were incubated with [^{3}H]-luseogliflozin [5.2 nM (C) and 19 nM (D)] for 0.25–12 h at 25 °C. For the dissociation time-course experiments, after 12 h incubation with [^{3}H]-luseogliflozin, unlabeled luseogliflozin was added in excess [760 nM (C) or 1970 nM (D)] and incubated for 2–30 h at 25 °C. Data represent the mean ± SEM of three experiments.

Table 1
Kinetic binding data of [^{3}H]-luseogliflozin for hSGLT2 in the absence and presence of 20 mM glucose.

	(−) glucose	20 mM glucose
	Mean ± SE	Mean ± SE
Kd (nM)	1.3 ± 0.13	7.0 ± 0.70
Kon ($M^{-1} \cdot min^{-1}$)	$1.4 \times 10^6 \pm 1.5 \times 10^5$	$2.4 \times 10^5 \pm 6.9 \times 10^3$
Koff (min^{-1})	$1.8 \times 10^{-3} \pm 5.4 \times 10^{-5}$	$1.7 \times 10^{-3} \pm 1.6 \times 10^{-4}$
Dissociation half-time (min)	420 ± 10	430 ± 39

Each of the values was calculated from the association and dissociation curves. Data represent the mean ± SEM of three experiments.

hSGLT2 in Fig. 2B shows the linear regression models, suggesting that [^{3}H]-luseogliflozin binds to one site on hSGLT2 protein in the absence and presence of glucose (20 mM). The time-course of the association and dissociation for specific binding of [^{3}H]-luseogliflozin to the membranes of cells stably expressing hSGLT2 is shown in Fig. 2C and D. The association for specific binding of [^{3}H]-luseogliflozin approached a plateau after approximately 12 h. After addition of unlabeled luseogliflozin at approximately 100 times the amount of [^{3}H]-luseogliflozin, the radioligand gradually dissociated from hSGLT2, with almost complete dissociation occurring by approximately 30 h in both the absence and presence of glucose (20 mM). The values of Kon were 1.4×10^6 $M^{-1} \cdot min^{-1}$ and 2.4×10^5 $M^{-1} \cdot min^{-1}$ in the absence and presence of glucose (20 mM), respectively. The value of Kon in the presence of glucose was lower than that in the absence of glucose, consistent with the competitive inhibition model of luseogliflozin. On the other hand, the values of Koff in both the absence and presence of glucose were in the same order (1.8×10^{-3} min^{-1} and 1.7×10^{-3} min^{-1}, respectively). Then, the Kon and Koff values were used to calculate the dissociation half-time and Kd values. In the absence and presence of glucose (20 mM), the dissociation half-time were 420 and 430 min, respectively. Based on the Kon and Koff values, [^{3}H]-luseogliflozin exhibited a high affinity for SGLT2 in the absence of glucose, with a Kd value of 1.3 nM. In the presence of glucose, the affinity for SGLT2 was slightly lower, with a Kd value of 7.0 nM (Table 1).

Luseogliflozin, a structurally novel C-5-thioglucoside, is a potent inhibitor of SGLT2 with an IC_{50} value of 2.26 nM, and exhibits a 1765-fold selectivity for hSGLT2 as compared to hSGLT1 (5). Recently, several SGLT2 inhibitors have been developed for the control of hyperglycemia in patients with T2DM. Sergliflozin (O-glucoside) developed earlier and tofogliflozin (*O*-spiroketal C-glucoside) developed later have been shown to be competitive SGLT2 inhibitors (8,9). Thus, the present study demonstrates that the mechanism of SGLT2 inhibition by a C-5-thioglucoside, luseogliflozin, is similar to that of the O- and C-glucosides.

Recent report indicates that there is a possibility that sugar moiety of a selective SGLT2 inhibitor, dapagliflozin, binds in the glucose binding site (10). The Kd value of luseogliflozin for SGLT2 in the presence of a high glucose concentration (20 mM) was larger than that in the absence of glucose, which suggested that luseogliflozin was a competitive SGLT2 inhibitor. Furthermore, the Kd value in the absence of glucose was the same as the Ki value of SGLT2 inhibition in the inhibitory kinetics study.

Our study also revealed that the dissociation half-time of luseogliflozin–hSGLT2 binding was approximately 7 h. On the other hand, phlorizin, a non-selective SGLT inhibitor, has been reported to show short-acting inhibition, with a half-time of phlorizin–hSGLT2 binding of 24 s (11). Recently, empagliflozin, a novel C-glucoside, was shown as a slow-dissociating SGLT2 inhibitor with a half-time of empagliflozin–hSGLT2 binding of approximately 60 min (7). Although the reasons for these differences in the rates of dissociation of the inhibitors have not yet been determined, the dissociation of luseogliflozin from hSGLT2 appears to be much slower than that of phlorizin or empagliflozin.

In a previous study, we demonstrated that luseogliflozin was present at an approximately 35-fold concentration in the kidney as compared to that in the plasma at 4 h after oral administration in rats, indicating that appreciable amounts of luseogliflozin are distributed to the target organ (4). In healthy Japanese subjects, luseogliflozin-induced dose-dependent increases in urinary glucose excretion were observed for at least 48 h after a single administration at all doses tested, although the mean plasma concentrations of luseogliflozin had decreased to 2–3% of maximum plasma level or lower (6). These results are in agreement with the much slower dissociation of the luseogliflozin–hSGLT2 complex. It is thought that there is a possibility of luseogliflozin binding to SGLT2 in the kidney after the disappearance of the drug from the plasma.

It still remains unclear whether luseogliflozin binds to extracellular or intracellular site of SGLT2. Our previous study demonstrates that luseogliflozin shows moderate Caco-2 cell permeability (4) and might accumulate in the proximal tubular cells. However, previous report indicates that phlorizin and a specific SGLT2 inhibitor, TA-3404, inhibit SGLT2 by acting from the extracellular site of the plasma membrane (12). Based on these findings, it seems that, like phlorizin, luseogliflozin inhibits SGLT2 by acting from the extracellular side. Additional experiments are required to resolve this question.

In conclusion, luseogliflozin is a highly potent and competitive inhibitor of SGLT2, and specifically binds to SGLT2. Furthermore, the results of the present study indicated that the dissociation of luseogliflozin from SGLT2 occurs very slowly. The results suggest that the binding kinetics of luseogliflozin to SGLT2 localized in the S1 and S2 segments of the renal proximal tubules may contribute to the sustained pharmacological effect of this drug.

Conflicts of interest

All authors are employees of Taisho Pharmaceutical Co., Ltd., and there are no known conflicts of interest associated with this publication.

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