Competitive inhibition of SGLT2 by tofogliflozin or phlorizin induces urinary glucose excretion through extending splay in cynomolgus monkeys

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Sodium-glucose cotransporter 2 (SGLT2) is specifically expressed in renal proximal tubules and plays an important role in renal glucose reabsorption (14, 39). Since SGLT2 inhibition is expected to have a high potential to induce urinary glucose excretion (UGE) with low safety concerns (10, 32, 41), several SGLT2 inhibitors are currently being developed in human clinical studies (3, 8).

The substantial role that SGLT2 plays in renal glucose handling in rodents has been demonstrated by the increased UGE in SGLT2 knockout mice (36), the improvement of hyperglycemic conditions in db/db mice with SGLT2 deletion (13), and the improvement in hyperglycemic conditions accompanied by markedly increased UGE in diabetic rats treated with SGLT2 inhibitors (10, 16, 18, 32).

In humans, genetic and functional analyses of familial renal glucosuria (FRG), a disorder caused by mutations of SLC5A2 (SGLT2), have revealed the predominant role that SGLT2 plays in the handling of glucose by the kidney (2). FRG affects key parameters of glucose titration studies, including the tubular transport maximum for glucose (TmG), the threshold of renal glucose excretion, and splay (5). Patients with FRG type A exhibit increased UGE with a low threshold and TmG, but with normal splay, suggesting that a loss in the amount of functional SGLT2 contributes to their increased UGE. In contrast, patients with FRG type B exhibit increased UGE with a low threshold and exaggerated splay, but with a normal TmG, suggesting that reduced affinity of SGLT2 for glucose due to a missense mutation contributes to their increased UGE (1, 28, 29). Of note, the term “splay” has been generally defined as “the deviation from a linear relationship between the filtered and reabsorbed glucose encountered during glucose titrations” (22) or “the difference between the actual and theoretical thresholds” (5). However, there have been few reports on the quantitative evaluation on the magnitude of splay (22, 31).

From observations of the glucose titration curves of FRG mentioned above, SGLT inhibitors are thought to induce UGE in vivo by two inhibition mechanisms. One is the suppression of TmG through noncompetitive inhibition of SGLT2 activity, and the other is the extension of splay through competitive inhibition. Although there are a few reports from glucose titration studies in dogs (20, 33) and rats (16) that examine the effect of SGLT2 inhibitors on TmG and the threshold of glucosuria, there is no report to quantitatively examine their effects on splay.

Moreover, with the method previously used to determine threshold in rats and humans using nonlinear regression between blood glucose and UGE (18, 26, 30), the fitted GluER line lacks the splay curve, implying that the threshold value estimated in their studies may vary from the actual threshold, which would also affect the accuracy of the estimated splay value.

Considering the importance of splay as an indicator of the homogeneity of nephron function in patients with chronic progressive renal diseases (22, 27, 34) and in certain types of FRG patients (1, 28, 29), we believe that it is also necessary to evaluate the effect of SGLT2 inhibitors on splay, to predict their efficacy precisely.

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Recently, DeFronzo et al. (6) reported that dapagliflozin, a selective SGLT2 inhibitor, produces its glucosuric effect in type 2 diabetes (T2D) patients and healthy subjects by reducing the TmG and threshold for glucosuria together with a reduction of splay (6). However, they started the glucose titration from a normal plasma glucose concentration with a single dose of dapagliflozin, suggesting the human glucose titration study had several limitations, such as a narrow range of plasma glucose and dapagliflozin concentrations, which may affect the accuracy of the estimated drug’s effect on TmG, threshold, and splay.

Nonhuman primates are more closely related to humans than are rodents. Therefore, they are currently considered the primary model for evaluating diabetic drugs (4, 17, 37). In fact, cynomolgus monkeys have been used to evaluate the efficacy and toxicity of a second-generation antisense nucleotide targeting human SGLT2 (42). In that report, 13-wk administration of ISIS 388626 reduced the expression of SGLT2 mRNA in the kidneys and promoted UGE in cynomolgus monkeys, suggesting that using cynomolgus monkeys is relevant for predicting the effects of SGLT2 inhibition in humans.

However, there are no previous reports about the molecular cloning of nonhuman primate SGLT1 or SGLT2 or about the in vitro and in vivo activities of SGLT inhibitors in nonhuman primates, indicating that the properties of cynomolgus monkey SGLT1 and SGLT2 and their contribution to renal glucose handling have yet to be fully characterized.

In this study, we first cloned the SGLT1 and SGLT2 cDNAs of cynomolgus monkeys and expressed cSGLT1 and cSGLT2 in COS-7 cells. We then confirmed the properties of tofogli- flozin, a highly selective SGLT2 inhibitor in humans (24, 32), and phlorizin, a nonselective SGLT1/2 inhibitor (25), in the inhibition of cSGLT1 and cSGLT2. Next, we evaluated the effect of these SGLT inhibitors on renal glucose handling in cynomolgus monkeys with a glucose titration study, in which TmG, glucosuria threshold, and splay were quantitatively estimated using a newly introduced method for fitting the titration curve.

MATERIALS AND METHODS

Chemicals

Tofoglioflozin ([1S, 3'R, 4'S, 5'S, 6'R]-6-[(4-ethylpheny1)methyl]-3', 4', 5', 6'-tetrahydro-6'-[hydroxymethyl]-spiro[sobenzofuran-1(3H), 2'-(2H) pyran]-3', 4', 5'-triol) was synthesized in our laboratories at Chugai Pharmaceutical. Phlorizin and α-methyl-D-glucopyranoside (AMG) were purchased from Sigma-Aldrich (St. Louis, MO), and α-methyl-D-[1-14C]glucopyranoside ([14C]AMG) was purchased from General Electronic (Tokyo, Japan). Glucose solutions (20 and 50%) were purchased from Fuso Pharmaceutical Industries (Osaka, Japan) and Otsuka Pharmaceutical Factory (Tokushima, Japan), respectively. Tofoglioflozin was dissolved at 0.34 mg/ml in lactated Ringer solution and diluted serially. Phlorizin was dissolved at 1.93 mg/ml in lactated Ringer solution and diluted serially. Glucose solution (20 or 50%) was diluted with purified water to make concentrations of 10, 30, or 40%.

Animals

Male cynomolgus monkeys (Macaca fascicularis) were purchased from Hamri (Tsukuba, Japan). The animals were kept under a 12:12-h light-dark cycle (lights on 7:00 AM-7:00 PM) with controlled room temperature (20–26°C) and humidity (35–75%) and were allowed ad libitum access to a certified primate diet (5048; LabDiet, St. Louis, MO), fruit as supplementary food, and water. No cynomolgus monkeys were euthanized specifically for the experiment in this study. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical under the approval of the company’s Institutional Animal Care and Use Committee and also in compliance with the “Act on Welfare and Management of Animals” in Japan. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, a nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs (http://www.aalac.org/).

We conducted the experiments using cynomolgus monkeys, adhering to the principles stated in the US National Research Council’s Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used.

Molecular Cloning of Cynomolgus Monkey SGLT1 and SGLT2

In vitro inhibition studies using cells expressing cynomolgus monkey SGLT1 (cSGLT1) and cynomolgus monkey SGLT2 (cSGLT2) were performed as follows by a method reported previously (32). Cynomolgus monkey SGLT1 and SGLT2 cDNA was amplified by RT-PCR from total RNA isolated from the kidney of a cynomolgus monkey. The sequences of the PCR primers used were 5′-CGCTGC-CACCATGGACAGTA-3′ and 5′-CTAGTGGGAAATAACACACTC-3′ for cSGLT1 and 5′-CGCTGCACCATGGACAGTA-3′ and 5′-CCACCTCTGTAGGCTGTG-3′ for cSGLT2. Experimental conditions for PCR with KOD Plus (Toyobo, Osaka, Japan) were as follows: 94°C for 2 min; 35 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 3 min. Expression plasmids containing cSGLTs were prepared by ligating amplified cDNA fragments into the multimlcnnoning site of pcDNA3.1(-) (Life Technologies In vitrogen, Grand Island, NY). Transfection of pcDNA3.1(-) containing cSGLT1 or cSGLT2 cDNA fragments or empty vector (pcDNA3.1(-)) was transfected into African green monkey SV40-transfected kidney fibroblast cells (COS-7) obtained from the American Type Culture Collection (ATCC, Manassas, VA), and the cells transiently expressing each cSGLT were used for the AMG uptake assay.

Inhibition Assay of AMG Uptake in COS-7 Cells Transiently Expressing cSGLT1/2

For the AMG uptake assay, cells expressing each cSGLT were cultured in 96-well plates for 2 or 3 days and washed twice with sodium-free buffer containing 140 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES/Tris (pH 7.4). The cells were then incubated in sodium-free buffer or sodium buffer containing 140 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES/Tris (pH 7.4) with 1 mM AMG mixture (nonradiolabeled AMG and [14C]AMG) at 37°C for 45 min. Sodium-dependent AMG uptake was calculated by subtracting the radioactivity detected in cells incubated in the sodium-free buffer from the radioactivity detected in the cells incubated in the sodium buffer. IC50 values of the SGLT inhibitors were calculated with the empirical four-parameter model fitting of XLfit (IDBS, Guildford, UK). To measure Ki, Vmax, and IC50 values, the AMG uptake assays were performed in sodium buffer or sodium-free buffer containing various concentrations of AMG. Ki, Vmax, and IC50 values of inhibitors were calculated from Lineweaver-Burk plots. Ki, Vmax, IC50, and IC50 values were indicated as mean values of two independent experiments.

Glucose Titration Study in Cynomolgus Monkeys

Study design. Three male cynomolgus monkeys, 4–6 yr of age and weighing 3.5 to 5 kg, were used and allocated to treatment using a randomized block design. Two monkeys were allocated to receive all six treatments consisting of vehicle, tofoglioflozin (13.3 or 133 ng/ml),...
and phlorizin (133, 1,333, or 13,333 ng/ml) treatment. The other monkey was given four treatments consisting of vehicle, two dose levels of tofogliflozin (13.3 and 133 ng/ml), and phlorizin (13,333 ng/ml) treatment. For each animal, the titration experiments with drug and glucose infusion were conducted over ~6 mo, and the mean interval between two titration studies for each animal was 32 days (11–68 days).

**Surgical procedure.** Animals were fasted overnight before each experiment. A preanesthetic was given as an intramuscular injection of atropine sulfate (0.15 mg/animal), and anesthetic induction was performed with an intramuscular injection of ketamine (0.5 mg/animal). Intubated animals were maintained with isoflurane inhalation at a minimum alveolar concentration (MAC) of 1–3%. Isoflurane (Acoma Medical Industry, Tokyo, Japan) according to vital signs, at a minimum alveolar concentration (MAC) of 1–3%. Isoflurane (Acoma Medical Industry, Tokyo, Japan) according to vital signs, including heart rate, blood pressure, respiratory rate, oxygen saturation (Spo2), and rectal temperature. The respiratory rate was maintained spontaneously or controlled using a respirator (PRO-45VA; Acoma Medical Industry) at 20–30 breaths/min. Vital signs were monitored using a bedside monitor (BP-88S; Omron Colin, Tokyo, Japan). Heart rates were maintained at 130–160 beats/min. Mean blood pressure was maintained at ~40 mmHg. Spo2 was maintained at >95%. Rectal temperature was maintained at 36.5–37.5°C. An indwelling venous catheter (V1) was placed in the left saphenous vein for infusion of drugs or Ringer solution, and another indwelling venous catheter (V2) was placed in the right saphenous vein for infusion of glucose solution. An indwelling venous needle was placed in the left cephalic vein for blood sampling. A Foley catheter was placed in the bladder for urine sampling.

After completion of the study, the bladder catheter and venous needles were removed, and bleeding was prevented by bandages and application of pressure. A tracheal extubation was performed after recovery of the swallowing reflex, and animals were carefully monitored until complete recovery from anesthesia.

**Infusion protocol of SGLT1 inhibitor and glucose.** The infusion of tofogliflozin or phlorizin solution was started at 2 ml/kg (bolus) and continued at 5 ml·kg⁻¹·h⁻¹ through the left saphenous vein catheter (V1). The concentrations of tofogliflozin and phlorizin solution infused were determined on the basis of pharmacokinetic parameters obtained from separate pharmacokinetic studies (data not shown) so as to maintain a target plasma concentration of 13.3 or 133 ng/ml for tofogliflozin and 133, 1,333, or 13,333 ng/ml for phlorizin. The infusion rate to achieve a target plasma concentration of 133 ng/ml tofogliflozin was 226 μg/kg (bolus) and 53.5 μg·kg⁻¹·h⁻¹ (constant), and the infusion rate to achieve a target plasma concentration of 13,333 ng/ml phlorizin was 1.9 mg/kg (bolus) and 965 μg·kg⁻¹·h⁻¹ (constant).

After 120 min of tofogliflozin or phlorizin infusion, infusion of 10% glucose solution through the left saphenous vein catheter (V2) was started at 5 ml·kg⁻¹·h⁻¹ and increased in a stepwise manner (20, 30, 40, and 50%) at 60-min intervals to raise the plasma glucose concentrations to above 8 mg/ml. Thereafter, the infusion rate of 50% glucose solution was increased to 10 ml·kg⁻¹·h⁻¹ to achieve ~10 mg/ml plasma glucose within 60 min. A blood sample (0.5 ml) was collected every 20 min with a heparinized syringe; the plasma glucose level of the sample was checked with a plasma-glucose monitoring system (Accu-Chek Aviva; Roche Diagnostics, Tokyo, Japan), and then a plasma sample was obtained by centrifugation to determine plasma glucose, creatinine, and tofogliflozin or phlorizin concentrations.

Urine was collected at 30-min intervals beginning 60 min after the start of drug infusion and at 20-min intervals after glucose infusion by injecting 10 ml saline into the bladder catheter to flush urine into preweighed polyethylene sample tubes. The weight of the sampled urine plus sample tube was recorded, and urine volume was determined by subtracting the weight of the preweighed sample tube from sampled urine and urine weight, with the specific gravity of sampled urine taken as 1. Urine and plasma samples were stored at −80°C until use.

**Analysis**

Plasma tofogliflozin concentrations were measured with an LC-MS/MS system (HPLC Shimadzu 20A, Shimadzu, Kyoto, Japan; mass spectrometry: API-4000 AB Sciex, Foster City, CA). Plasma phlorizin concentrations were measured with an liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (HPLC: Acuity UPLC, Waters, Milford, MA; mass spectrometry: API-3200, AB Sciex).

Plasma glucose and urinary glucose concentrations were measured by the hexokinase/G-6-PDH method (L-Type Glu 2; Wako Pure Chemical Industries, Osaka, Japan) with an automatic analyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan). Creatinine concentrations of plasma and urine were measured by the creatininas/HMMPS method (L-Type Creatinine M; Wako Pure Chemical Industries) with an automatic analyzer.

**Calculations**

The following parameters were calculated: creatinine clearance (ml/min) = (urine creatinine; mg/ml) × (urine excretion rate; ml/min)/(plasma creatinine; mg/ml); glucose clearance (ml/min) = (urine glucose; mg/ml) × (urine excretion rate; ml/min)/(plasma glucose; mg/ml); and fractional excretion of glucose (FEG) = glucose clearance/creatinine clearance × 100 (%) (20). This calculation method was based on the formula used to calculate the FEG in a clinical study with another SGLT2 inhibitor (7). The same formula was used to calculate the percent inhibition of RGR in a clinical study with dapagliflozin (15).

Glucose filtration rates (GluFR), glucose excretion rates (GluER), and glucose reabsorption rates (GluRR) were determined by the following formulas: GluFR = (plasma glucose; mg/ml) × (creatinine clearance; ml/min); GluER = urinary glucose excretion (mg)/sampling period (min); and GluRR = GluFR (mg/min) − GluER (mg/min).

The titration curves were plotted as GluFR vs. GluER or GluRR. Glucose reabsorption rates were fitted by the following single exponential equation developed by Martin (21) as follows:

\[ \text{GluRR}(x) = x_0 + \left( \frac{T_mG}{m} - x_0 \right) \times \left( 1 - \exp\left[ -k(x - x_0) \right] \right) \]  

where \( x \) is glucose filtration rate, \( x_0 \) is the threshold indicating the GluFR at which glucose first appears in the urine, and \( k \) is a coefficient of glucosuric effect.

We defined the threshold (\( x_0 \)) as the GluFR at which the measured FEG exceeded 5% in each experiment for the first time. \( T_mG \) is defined as the mean GluRR at a GluFR >90 mg/min for each experiment and is designated as observed \( T_mG \). This definition is based on the saturated GluRR observed at a GluFR >90 mg/min in the vehicle treatment of this study.

Splay values (S1 and S2 regions illustrated in Fig. 1) were determined from the following formula, in which the first term is the area under the GluER curve ranging from \( x_0 \) to \( T_mG \), and the second term is the area between the GluER curve and the \( (x - T_mG) \) line from \( T_mG \) to positive infinity:

\[ \text{Splay} = \int_{x_0}^{T_mG} \text{GluER}(x) \, dx + \int_{T_mG}^{\infty} \left\{ \text{GluER}(x) - (x - T_mG) \right\} \, dx \]

\[ = \left( T_mG - x_0 \right) / k - 1 / 2 \times \left( T_mG - x_0 \right)^2 \]

where \( x_0 \) and \( k \) are the parameters determined in the above-mentioned formula (Eq. 1). The glucose transport rate through cSGLT2 in vivo at \( x_0 \) was estimated from Michaelis-Menten equation for competitive inhibition as follows.
SGLT2 INHIBITION EXTENDS SPLAY BY LOWERING THRESHOLD

Fig. 1. Schematic diagram of the splay area in the glucose titration curve.

\[
V_{x_b} = V_{\text{max}} \times \left[ \frac{[S]}{[S] + K_m} \times \left( 1 + \frac{[\text{SGLT}]/K_i)}{[\text{SGLT}] + [K_i]} \right) \right]
\]

where \([S]\) represents plasma glucose levels at \(x_b\) in the above-mentioned titration curve, and \([\text{SGLT}]\) represents the estimated free SGLT inhibitor concentration corrected to reflect the protein-binding properties (32, 40). The plasma glucose levels at \(x_b\) are the actual ones from the above-mentioned titration study using cynomolgus monkeys with two or three replications for each treatment. \(V_{\text{max}}\) and \(K_m\) values of cSGLT2 and \(K_i\) values of inhibitors were calculated from a Lineweaver-Burk plot of the AMG uptake assay. This estimation was based on the equipotent affinity of AMG and D-glucose to human SGLT2 (39).

All parameters were calculated with Microsoft Excel 2007 (Microsoft, Redmond, WA), and GluER was fitted with Prism 5.0 (GraphPad Software, San Diego, CA). The coefficient of determination was calculated from the following formula:

\[
R^2 = 1 - \frac{\text{RSS} (\text{residual sum of squares})}{\text{TSS} (\text{total sum of squares})}
\]

Statistical Analysis

Data are presented as means ± SD or SE. To examine the effects of drug treatment upon the parameters (FEG at 2.5–3.5 mg/ml plasma glucose, observed TmG, threshold, and splay) in the titration study, statistical analyses were performed by randomized block ANOVA. Post hoc comparisons were performed using Tukey’s honest significant difference (HSD) test (JMP 9.02; SAS Institute Japan, Tokyo, Japan).

RESULTS

In Vitro Characterization of Cynomolgus Monkey SGLT1 and SGLT2

The amino acid sequences deduced from the cloned cSGLT1 and cSGLT2 cDNA sequences were perfectly matched to the predicted amino acid sequences of rhesus monkey SGLT1 (XM_001112212) and SGLT2 (XM_001113206), respectively, in the RefSeq protein product database. The nucleotide sequence homologies in cynomolgus monkey, rodent, rhesus monkey, and human SGLTs are shown in Table 1. The amino acid sequence similarities between cSGLT1/2 against rhesus monkey and human SGLT1/2 were higher than those of cSGLT1/2 against rodent SGLT1/2.

The inhibitory activity of tofogliflozin or phlorizin against cSGLT2 was examined in COS-7 cells overexpressing cSGLT2 by evaluating sodium-dependent AMG uptake. Substantial sodium-dependent AMG uptake was confirmed in COS-7 cells overexpressing cSGLT1/2 above background levels (Fig. 2), and fold-change values in AMG uptake for cSGLT1/2 relative to the background levels seen in cells with a transfected vector were 186 and 307 times, respectively, indicating that cSGLT1/2 are functionally active, as shown in a similar protocol (25).

Analysis using Lineweaver-Burk plots showed that both compounds inhibited AMG uptake in a substrate-competitive manner (Fig. 3), and the \(K_i\) value of phlorizin for cSGLT2 inhibition was 26.1 ± 6.1 nM. Tofogliflozin inhibited cSGLT2 more strongly than did phlorizin: The \(K_i\) value of tofogliflozin for cSGLT2 inhibition was 5.0 ± 0.6 nM. In addition, from the Lineweaver-Burk plot (Fig. 3), \(K_m\) and \(V_{\text{max}}\) values of cSGLT2 were 1.63 ± 0.17 mM and 0.97 ± 0.09 nmol/h, respectively. The \(V_{\text{max}}\) value of cSGLT2 was not altered by either tofogliflozin or phlorizin (phlorizin at 40 and 80 nM: 1.08 ± 0.12 and 1.08 ± 0.41 nmol/h; tofogliflozin at 6 and 12 nM: 0.97 ± 0.26 and 0.99 ± 0.22 nmol/h, respectively).

The IC\(_{50}\) values of phlorizin and tofogliflozin against cSGLT2 were 35.8 ± 4.9 and 8.9 ± 0.5 nM, respectively (Table 2). In addition, tofogliflozin was more selective than phlorizin: the selectivity of tofogliflozin toward cSGLT2 was 1,000 times that toward cSGLT1, and the IC\(_{50}\) values of tofogliflozin and phlorizin against cSGLT1 were 8,875 ± 390 and 309 ± 81 nM, respectively (Table 2). These results suggest that tofogliflozin inhibits SGLT2 more selectively than phlorizin does in cynomolgus monkeys, as well as in rodents and humans (32).

Glucose Titration Study in Cynomolgus Monkeys

In both tofogliflozin and phlorizin treatments, plasma drug concentrations were maintained at a stable level during the entire study. Briefly, actual plasma drug concentrations of tofogliflozin were maintained at 132 and 142% of the targeted plasma concentrations of 13.3 and 133 ng/ml, respectively (Fig. 4A). On the other hand, actual plasma drug concentrations of phlorizin were maintained at 72, 69, and 43% of the targeted plasma concentrations of 133, 1,333, and 13,333 ng/ml, respectively (Fig. 4B).

The individual data of body weight, treatment, TmG, and creatinine clearance are shown in Table 3. During the course of the repeated titration experiments over ~6 mo, there were no remarkable changes in body weight or creatinine clearance (Table 3).

Table 1. Nucleotide sequence homology of cynomolgus monkey SGLT1 and SGLT2 against other species.

<table>
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<th>Species</th>
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The percentages of homology of sodium/glucose transporter SGLT1/2 among species were calculated from the nucleotide global homology program in Genetyx, ver. 10.0.3.
The plasma glucose levels continuously increased from ~0.5 to 10 mg/ml with increasing glucose load (Fig. 5, A and B). At the final sampling point, plasma glucose levels for each treatment were as follows: vehicle, 9.030 ± 0.432 mg/ml; tofogliflozin (13.3 ng/ml), 9.300 ± 0.664 mg/ml; tofogliflozin (133 ng/ml), 8.938 ± 0.566 mg/ml; phlorizin (133 ng/ml), 10.303 ± 0.323 mg/ml; phlorizin (1,333 ng/ml), 8.825 ± 0.235 mg/ml; and phlorizin (13,333 ng/ml), 9.112 ± 0.449 mg/ml.

GluER increased with increasing plasma glucose levels (Fig. 5, C and D). In both tofogliflozin and phlorizin treatment, compared with the vehicle treatment, a clear increase in GluER was observed under normoglycemic conditions. Interestingly, these differences in GluER between vehicle and the inhibitor treatments tended to disappear under hyperglycemic conditions.

The creatinine clearance values in all treatments were maintained at a stable level of ~15 ml/min during the experiment (Fig. 5, E and F). The creatinine clearance values in this study were similar to those in conscious cynomolgus monkeys (38).

The time course of FEG is shown in Fig. 6, A and B. In both tofogliflozin and phlorizin treatments, a dose-dependent increase in FEG was observed even under normal plasma glucose levels before and after the start of glucose infusion. Then FEG increased to ~50% in both tofogliflozin and phlorizin treatments. Although FEG in the vehicle treatment group was negligible before and until 60 min after the start of glucose infusion, FEG in the vehicle treatment group began to increase when the plasma glucose levels exceeded 2 mg/ml and gradually increased up to 50% at the end of the titration study, when the plasma glucose levels were ~10 mg/ml.

From FEG in the vehicle treatment in this experiment (Fig. 6, A and B), the threshold of glucose reabsorption in cynomolgus monkeys was assumed to be ~2.5–3.5 mg/ml, which is similar to that in rats (23). Accordingly, we compared the FEG at plasma glucose levels ranging from 2.5 to 3.5 mg/ml to estimate the maximum inhibitory effects of SGLT inhibitors on RGR. The FEG at 2.5–3.5 mg/ml plasma glucose was significantly influenced by treatment with SGLT inhibitors (drug treatment effect $F_{S,8} = 51.5544$, $P < 0.0001$). The FEG at 2.5–3.5 mg/ml plasma glucose increased in a dose-dependent manner in both tofogliflozin and phlorizin treatments compared with vehicle treatment (13.3 ng/ml tofogliflozin, $P = 0.0002$; 133 ng/ml tofogliflozin, $P < 0.0001$; 1,333 ng/ml phlorizin, $P = 0.0014$; 13,333 ng/ml phlorizin, $P < 0.0001$; vs. vehicle treatment; Tukey’s HSD test; Fig. 6C).

The representative relationships between the calculated GluFR and GluER and between GluFR and GluRR for cynomolgus monkey number 2 are shown in Fig. 7. Compared with vehicle treatments, both tofogliflozin and phlorizin treatment began to induce GluER at lower GluFR levels (Fig. 7, A and B). With vehicle treatment, the GluRR increased with increasing GluFR, but levels began to plateau when GluFR was ~90 mg/min. In contrast, although the GluRR with both tofogliflozin treatment (13.3 and 133 ng/ml) and phlorizin treatment (1,333 and 13,333 ng/ml) also increased depending on the GluFR, no clear plateau in GluRR was observed, even when the GluFR exceeded 90 mg/min (Fig. 7, C and D). From these titration curves, we determined the TmG and threshold of UGE according to the criteria mentioned in MATERIALS AND METHODS. Splay values were then calculated according to Eq. 2.

The TmG values observed in all experiments are listed in Table 3 and summarized in Table 4. Although a marginal difference was detected with ANOVA (drug treatment effect $F_{S,8} = 3.8039$, $P = 0.0463$), a post hoc comparison detected no significant differences in the TmG values between SGLT2 inhibitor and vehicle treatment (13.3 ng/ml tofogliflozin, $P = 0.9945$; 133 ng/ml tofogliflozin, $P = 0.7405$; 133 ng/ml phlorizin, $P = 0.2255$; 1,333 ng/ml phlorizin, $P = 0.9860$; 13,333 mg/ml phlorizin, $P = 0.6285$; vs. vehicle treatment; Tukey’s HSD test; Fig. 8A).

The mean threshold value with vehicle treatment was 37.01 ± 3.96 mg/min, at which the mean plasma glucose level was 2.54 ± 0.50 mg/ml. The threshold was significantly influenced by treatment with SGLT inhibitors (drug treatment effect $F_{S,8} = 24.1356$, $P = 0.0001$). The threshold values observed with tofogliflozin 13.3 and 133 ng/ml treatment were respectively decreased to ~36 and 27% of vehicle treatment (13.3 ng/ml tofogliflozin, $P = 0.0016$; 133 ng/ml tofogliflozin, $P = 0.0007$; vs. vehicle treatment, Tukey’s HSD test). Although no significant difference in the threshold was detected between 133 ng/ml phlorizin and vehicle treatment ($P = 0.9574$), with phlorizin at higher doses (1,333 and 13,333 ng/ml) the threshold was significantly decreased to ~53 and 11%, respectively.

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Fig. 2. Functional validation in COS-7 cells expressing cynomolgus monkey (c) sodium-glucose transporter 1/2 (SGLT1/2). Na$^+$-dependent α-methyl-D-glucopyranoside (AMG) uptake was compared in COS-7 cells expressing cSGLT1 (A) or cSGLT2 (B) with an empty vector (MOCK). Values are means ± SD.  

The time course of FEG is shown in Fig. 6, A and B. In both tofogliflozin and phlorizin treatments, a dose-dependent increase in FEG was observed even under normal plasma glucose levels before and after the start of glucose infusion. Then FEG increased to ~50% in both tofogliflozin and phlorizin treatments. Although FEG in the vehicle treatment group was negligible before and until 60 min after the start of glucose infusion, FEG in the vehicle treatment group began to increase when the plasma glucose levels exceeded 2 mg/ml and gradually increased up to 50% at the end of the titration study, when the plasma glucose levels were ~10 mg/ml.
using the observed TmG (Table 3) and each threshold (data not shown). Then, we calculated the splay area in the titration curve according to Eq. 2. The mean splay area of vehicle treatment was 640 ± 157 mg²/min². The splay was influenced by treatment with an SGLT inhibitor (drug treatment effect $F_{5,8} = 9.1088$, $P = 0.0037$). There were significant increases in the splay values with 13.3 ng/ml tofogliflozin ($P = 0.0148$), 133 ng/ml tofogliflozin ($P = 0.0057$), and 13,333 ng/ml phlorizin ($P = 0.0038$) treatments compared with vehicle treatment (Tukey’s HSD test; Fig. 8C).

Next, to confirm the validity of the method, we constructed the fitting equations for GluRR according to Eq. 1 by using the pooled GluER and GluFR data with the observed TmG of the vehicle treatment and the mean threshold of each treatment. The measured GluRR values were well predicted from GluFR by using the fitting equations, with a coefficient of determination ranging from 0.8547 to 0.9342 (Fig. 9, A–F). The parameters of the equation and the coefficients of determination are summarized in Table 4.

Finally, to assess the relationship between SGLT2 inhibition and glucose excretion in vivo, we estimated the transport rates at the threshold of glucosuria ($V_{xb}$) by assigning the actual plasma glucose concentration at $x_b$, as the substrate concentration, and by assigning $V_{max}$ and $K_m$ values of cSGLT2, $K_i$ values of each SGLT2 inhibitor and the estimated free SGLT inhibitor to the Michaelis-Menten equation with competitive inhibition, as shown in Eq. 3. The actual plasma glucose levels (means ± SD) at $x_b$ for each treatment were as follows: vehicle, 14.01 ± 4.79 mM ($n = 3$); 13.3 ng/ml tofogliflozin, 5.80 ± 0.65 mM ($n = 3$); 133 ng/ml tofogliflozin, 6.75 ± 3.28 mM ($n = 3$); 133 ng/ml phlorizin, 11.85 ± 0.57 mM ($n = 2$); 1,333 ng/ml phlorizin, 7.92 ± 2.91 mM ($n = 2$); and 13,333 ng/ml phlorizin, 0.98 ± 0.68 mM ($n = 3$). The estimated $V_{xb}$ value of the vehicle treatment group was similar to the $V_{max}$ value of the vehicle treatment group was similar to the $V_{max}$ value of cSGLT2 for AMG uptake in COS-7 cells (0.86 ± 0.02 nmol/h). The $V_{xb}$ values of the tofogliflozin and phlorizin treatment groups decreased in a dose-dependent manner (Fig. 10).

**DISCUSSION**

In this study, we examined the inhibition kinetics and activity of tofogliflozin, an SGLT2-specific inhibitor, and phlorizin, a SGLT1/2 nonspecific inhibitor, against cynomolgus monkey SGLT1/2 in vitro. We then clarified the in vivo effects of tofogliflozin and phlorizin on TmG, threshold, and splay, the main parameters of renal glucose reabsorption.

First of all, we cloned cSGLT1/2 cDNAs and investigated the inhibitory effects of tofogliflozin and phlorizin in cells overexpressing cSGLT1/2. As expected, tofogliflozin and phlorizin competitively inhibited the uptake of substrates by

<table>
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<th>IC$\text{_{50}}$ values of phlorizin and tofogliflozin against cynomolgus monkey SGLT1/2</th>
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<td>Tofogliflozin</td>
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<td>8.9 ± 0.5</td>
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Values are means ± SD from 2 independent experiments.

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Fig. 3. Lineweaver-Burk plots for inhibition of cSGLT2 by tofogliflozin and phlorizin. Na$^+$-dependent and -independent AMG uptake was measured with COS-7 cells expressing cSGLT2 in the presence or absence of tofogliflozin (A) or phlorizin (B) with various concentrations of AMG (s). Na$^+$-dependent AMG uptake velocity ($v$) was calculated and used for the Lineweaver-Burk plots against $1/s$. $[I]$ indicates concentration of tofogliflozin or phlorizin. Experiments were performed 2 times, independently.
cSGLT2, which is consistent with results found for human, mouse, and rat SGLT2 (32). The IC50 of tofogliflozin against cSGLT2 was similar to that against human SGLT2 and lower than against both mouse and rat SGLT2s. These differences in inhibitory activity among species may reflect the higher similarity of the cynomolgus monkey SGLT1/2 amino acid sequence to human SGLT1/2 than to rodent SGLT1/2 (Table 1).

Although it had been generally believed that SGLT2 mediated 90% of RGR in humans (3, 39), recent clinical studies with SGLT2 inhibitors have shown only 30–50% inhibition of RGR, provoking debate on the mechanisms underlying this discrepancy (12, 19, 35). In this glucose titration study using cynomolgus monkeys, both tofogliflozin and phlorizin increased the FEG at 2.5–3.5 mg/ml plasma glucose up to 50%, suggesting that the maximum inhibitory effects of these SGLT inhibitors on RGR in cynomolgus monkey are nearly the same as those in rats (23). A recent human glucose titration study with the selective SGLT2 inhibitor dapagliflozin also showed 50–70% inhibition of RGR (6). Our results suggest that the contribution of SGLT2 to RGR in cynomolgus monkeys is comparable to that in other species, including rats and humans.

We have newly introduced a detailed fitting method for the titration curve to evaluate the threshold and splay with the

![Fig. 4. Actual plasma drug concentration in cynomolgus monkeys. Tofogliflozin (A) or phlorizin (B) was administered by bolus (iv) followed by continuous infusion. The concentrations indicated (13.3–13,333 ng/ml) are target plasma concentrations. Values are means ± SE; n = 2–3.](image)

Table 3. Individual data for body weight, treatment, TmG, and creatinine clearance for each cynomolgus monkey

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TmG, tubular transport maximum for glucose; TOFO, tofogliflozin; PHZ, Phlorizin.
equation for glucose reabsorption (Eq. 1) that Martin (21) proposed. To our knowledge, this is the first report of a splay analysis based on Martin’s equation using this glucose titration technique.

In previous reports using nonlinear regression as the method for determining threshold (18, 26, 30), the fitted GluER line lacks the splay curve, implying that the threshold value estimated in those studies may vary from the actual threshold, which would also affect the accuracy of the estimated splay value. To avoid this variability in the estimation, in our approach we used the experimentally determined TmG and threshold values to estimate coefficient $k$. In this way, we could successfully express the splay curve using three parameters, TmG, threshold, and $k$, with a good coefficient of determination. As a result, we were able to estimate the area of splay, and the splay was expressed by

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Fig. 5. Time course of plasma glucose, glucose excretion rate, and creatinine clearance of cynomolgus monkeys. A and B: time course of plasma glucose before and after glucose loading and infusion with tofogliflozin (TOFO; A) or phlorizin (PHZ; B). C and D: time course of glucose excretion rate (GluER) before and after glucose loading and infusion with TOFO (C) or PHZ (D). E and F: time course of creatinine clearance before and after glucose loading and infusion with TOFO (E) or PHZ (F). After 120 min of TOFO or PHZ infusion, infusion (5 ml·kg$^{-1}$·h$^{-1}$) of glucose solution (10%) was started (time 0) and increased in a stepwise manner (20, 30, 40, and 50%) at 60-min intervals followed by 60-min infusion of 50% glucose at 10 ml·kg$^{-1}$·h$^{-1}$ with the constant infusion of TOFO or PHZ. The concentrations indicated (13.3–13,333 ng/ml) are target plasma concentrations. Values are means ± SE; $n = 2–3$. 

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a simple equation using the three parameters mentioned above.

It is notable that the decreased threshold levels and extended splay were quite similar between the tofogliflozin and phlorizin treatments. Based on the actual plasma concentrations of tofogliflozin (Fig. 4A) and its protein-binding properties (32), we estimated the unbound tofogliflozin concentrations to be 10 nM at 13.3 ng/ml (actual mean concentration = 18 ng/ml) and 113 nM at 133 ng/ml (actual mean concentration = 189 ng/ml).

Considering the IC50 values of tofogliflozin against cSGLT1 and cSGLT2 (8,875 nM cSGLT1; 8.9 nM cSGLT2) calculated from its inhibitory activity on the uptake of AMG (Table 2), the unbound concentrations of tofogliflozin mentioned above are relevant concentrations for inhibiting 50 and 100% of cSGLT2 activity, but scarcely affect cSGLT1 activity.

Similarly, based on the actual plasma concentrations of phlorizin (Fig. 4B) and its protein-binding properties (40), we estimated the unbound phlorizin concentrations to be 698 nM

Fig. 6. Effects of TOFO or PHZ on the fractional excretion of glucose in cynomolgus monkeys. A and B: time course of the fractional excretion of glucose (FEG) before and after glucose loading and infusion with TOFO (A) or PHZ (B). After 120 min of TOFO or PGZ infusion, infusion (5 ml·kg⁻¹·h⁻¹) of glucose solution (10%) was started (time 0) and increased in a stepwise manner (20, 30, 40, and 50%) at 60-min intervals followed by 60-min infusion of 50% glucose at 10 ml·kg⁻¹·h⁻¹ with the constant infusion of TOFO or PHZ. C: FEG at 2.5–3.5 mg/ml plasma glucose of cynomolgus monkeys infused with TOFO or PHZ. The concentrations indicated (13.3–13,333 ng/ml) are target plasma concentrations. Values are means ± SE; n = 2–3. **P < 0.01 vs. vehicle treatment by Tukey’s honest significant difference (HSD) test.

Fig. 7. Glucose filtration rate (GluFR) plotted against GluER (A and B) or against GluFR and glucose reabsorption rate (GluRR; C and D) in cynomolgus monkey 2 treated with vehicle, TOFO, or PHZ. Infusion of vehicle and TOFO (A and C) or PHZ (B and D) is shown. Symbols represent individual values.
Determination in glucose titration curves

Transporter for glucose, implying that SGLT2 inhibitors mainly inhibit renal glucose reabsorption in a competitive manner, which is consistent with the in vitro inhibition kinetics of the two compounds in this study and in other species (25, 32). In particular, as the plasma glucose levels of cynomolgus monkeys were

\[
\text{GluFR} \quad \text{increased, implying that both tofogliflozin and phlorizin inhibit renal glucose reabsorption in a competitive manner, which is consistent with the in vitro inhibition kinetics of the two compounds in this study and in other species (25, 32).}
\]

This connection, Ferrannini and Solini (8) suggested that SGLT2 inhibitors predominantly reduce the affinity of the transporter for glucose, implying that SGLT2 inhibitors mainly inhibit renal glucose reabsorption in a competitive manner, which is consistent with the in vitro inhibition kinetics of the two compounds in this study and in other species (25, 32).

In contrast to FEG with 133 ng/ml tofogliflozin treatment, greater FEG (~30%) was evident with 13,333 ng/ml phlorizin treatment under hyperglycemic conditions after afternoon fasting. The concentrations indicated (13.3–13,333 ng/ml) were target plasma concentrations. Observed TmG was calculated by averaging the glucose filtration rates under hyperglycemic conditions (GluFR >90 mg/min). Threshold was defined as the GluFR at which the measured fractional excretion of glucose exceeded 5% for the first time in each experiment. Splay values were determined from Eq. 2. Values are means ± SE; n = 2–3. *P < 0.05, **P < 0.01 vs. vehicle treatment by Tukey’s HSD test.
expand splay rather than decrease TmG, which was supported by their recent findings in urinary glucose absorption and excretion in T2D patients with chronic kidney disease (9).

Furthermore, our estimation of the transport rates at the threshold of glucosuria ($V_{xb}$) (Fig. 10) suggests a close relationship between the in vitro kinetic parameters of SGLT inhibitors and the three critical in vivo factors (TmG, threshold, and splay) in renal glucose reabsorption as follows.

First, the unchanged TmG values in tofogliflozin and phloretin treatments in vivo are consistent with their negligible
effects on the $V_{\text{max}}$ values of cSGLT2 in the in vitro AMG uptake assay. Next, in the vehicle treatment group, the nearly identical values of $V_{\text{obs}}$ and in vitro $V_{\text{max}}$ suggest that transport activity can be saturated at plasma glucose levels around the threshold. However, as the $V_{\text{max}}$ of cSGLT2 in COS-7 cells would be influenced with its protein expression levels, the in vitro $V_{\text{max}}$ values are independent of in vivo $V_{\text{max}}$ values, indicating the limitations to the above-mentioned interpretation on the relationship between $V_{\text{obs}}$ and in vitro $V_{\text{max}}$. Nevertheless, the plasma glucose level at the threshold ($x_b$) of vehicle treatment was $\sim 14$ mM, about ninefold of the $K_m$ value of cSGLT2 (1.63 mM), suggesting that SGLT2 was almost saturated at $x_b$ of vehicle treatment.

In addition, dose-dependent decrease in $V_{\text{obs}}$ in both the SGLT2 inhibitor treatment groups (Fig. 10) suggests that a decrease in threshold coincided with reduced transport activity by SGLT2 inhibitors. From Eq. 2, the changes in threshold are expected to mainly contribute to the splay extension in this study because the TmG is unchanged. Therefore, the reduced transport rate of substrates at the lower plasma glucose concentration created by SGLT2 inhibitors is closely related to the splay extension that occurs as the threshold of UGE decreases.

In contrast, noncompetitive SGLT2 inhibitors may reduce the apparent $V_{\text{max}}$, which may lead to reduced TmG. Although a noncompetitive SGLT2 inhibitor will not affect the $K_m$ value, the apparent transport rate at a lower plasma glucose concentration will be reduced when the apparent $V_{\text{max}}$ is reduced, which may decrease the threshold of UGE. As a result, it is expected from Eq. 2 that a reduction in both TmG and $x_b$ will reduce splay.

In distinct contrast to our results, DeFronzo et al. (6) have recently reported that dapagliflozin exerts its glucosuric effect in T2D patients and healthy subjects by reducing both TmG and splay. At present, we assume the reasons for the discrepancy between dapagliflozin and tofogliflozin in terms of the effects on the TmG and splay are as follows. First, dapagliflozin may inhibit human SGLT2 with different kinetics from ordinary competitive inhibitors, which is suggested by the remarkably slow dissociation of dapagliflozin from human SGLT2, even with high (100 mM) glucose concentration, in contrast to the rapid dissociation of phlorizin (11). Second, the titration study with dapagliflozin was conducted at plasma glucose levels of between $\sim 5$ mM (0.9 mg/ml) and 30 mM (5.4 mg/ml), whereas those of our experiments were from $\sim 0.5$ mg/ml to nearly 10 mg/ml. The relatively narrow range of plasma glucose levels in the titration study with dapagliflozin might lead to underestimating the TmG value, especially after dapagliflozin treatment. The reduced TmG may also affect the degree of splay extension, because the TmG value itself is a critical determinant of splay area in the glucose titration curves (Fig. 1).

There are several limitations in this study. First, although we used a randomized block design to test the efficacy of tofogliflozin and phlorizin as much as possible and to minimize the number of animals, the number of cynomolgus monkeys for each treatment was relatively small. Second, although the GluRR in treatment with both tofogliflozin (13.3 and 133 ng/ml) and phlorizin (1,333 and 13,333 ng/ml) showed no clear plateau within the GluFR ranges in this study, we defined the observed TmG as the mean GluRR at GluFR $\geq$ 90 mg/min, which may influence the accuracy of observed TmG. Nevertheless, it is evident that neither tofogliflozin nor phlorizin showed any remarkable inhibition of TmG. Third, the effect of anesthesia on the actual filtered glucose remains to be assessed, although the creatinine clearance levels were not reduced compared with values reported in the literature, suggesting that the filtered glucose load in proximal tubules would be maintained normally due to the autoregulation of glomerular filtration. Finally, we used creatinine clearance to estimate GFR, which may affect the accuracy of the estimated GluFR.

In conclusion, we determined the inhibitory activity of tofogliflozin and phlorizin in cells expressing cSGLT1 or cSGLT2 and suggest that the contribution of SGLT2 in RGR in cynomolgus monkeys is similar to the contribution in humans and rodents and that the competitive inhibition of SGLT2 exerts a glucosuric effect by mainly extending splay without affecting TmG. Further studies are required before these results can be extrapolated to diabetic patients.

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DISCLOSURES

All authors are employees of Chugai Pharmaceutical Co., Ltd. or its affiliate company, Chugai Research Institute for Medical Science, Inc.

AUTHOR CONTRIBUTIONS

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