Tofogliflozin, a potent and highly specific sodium/glucose cotransporter 2 inhibitor, improves glycemic control in diabetic rats and mice

Masayuki Suzuki, Kiyofumi Honda, Masanori Fukazawa, Kazuharu Ozawa, Hitoshi Hagita, Takahiro Kawai, Minako Takeda, Tatsuo Yata, Mio Kawai, Taku Fukuzawa, Takamitsu Kobayashi, Tsutomu Sato, Yoshiki Kawabe, Sachiya Ikeda


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MI:  myo-inositol
SGLT: sodium/glucose cotransporter
SMIT: sodium/myo-inositol transporter
T2D: type 2 diabetes mellitus
UGE: urinary glucose excretion

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Abstract

Sodium/glucose cotransporter 2 (SGLT2) is the predominant mediator of renal glucose reabsorption, and is an emerging molecular target for the treatment of diabetes. We identified a novel potent and selective SGLT2 inhibitor, tofogliflozin (CSG452), and examined its efficacy and pharmacological properties as an antidiabetic drug. Tofogliflozin competitively inhibited SGLT2 in cells over-expressing SGLT2, and $K_i$ values for human, rat, and mouse SGLT2 inhibition were 2.9, 14.9, and 6.4 nM, respectively. The selectivity of tofogliflozin toward human SGLT2 versus human SGLT1, SGLT6 and SMIT1 was the highest among the tested SGLT2 inhibitors under clinical development. Furthermore, no interaction with tofogliflozin was observed in any of a battery of tests examining glucose-related physiological processes such as glucose uptake, glucose oxidation, glycogen synthesis, hepatic glucose production, glucose-stimulated insulin secretion, and glucosidase reactions. A single oral gavage of tofogliflozin increased renal glucose clearance and lowered blood glucose level in ZDF rats. Tofogliflozin also improved postprandial glucose excursion in a meal tolerance test with GK rats. In db/db mice, 4-week tofogliflozin treatment reduced glycated hemoglobin and improved glucose tolerance in the oral glucose tolerance test 4 days after the final administration. No blood glucose reduction was observed in normoglycemic SD rats treated with tofogliflozin. These findings demonstrate that tofogliflozin inhibits SGLT2 in a specific manner, lowers blood glucose levels by increasing renal glucose clearance, and improves pathological conditions of type 2 diabetes with a low hypoglycemic potential.
Introduction

According to the World Health Organization, 346 million people worldwide have diabetes mellitus, and this number is expected to more than double by the year 2030 (World Health Organization, 2011). Approximately 90% of all patients with diabetes mellitus have type 2 diabetes mellitus (T2D), a progressive metabolic disease characterized by chronic hyperglycemia due to insulin resistance and impaired insulin secretion from the pancreatic β-cells. T2D is associated with a high incidence of both macrovascular (cardiovascular disease) and microvascular complications (nephropathy, retinopathy, and neuropathy), and patients with T2D have a higher cardiovascular and all-causes mortality than do people without diabetes.

Although the benefit of intensive glycemic control to reduce the increased risk of cardiovascular disease in people with T2D is still controversial, intensive glycemic control is required for the prevention of diabetes-related microvascular complications in these patients. Many drugs are currently available for the clinical treatment of T2D. In most cases, however, the current treatment options are not sufficient in themselves to prevent disease progression, resulting in treatment with combinations of drugs or with insulin therapy. In addition, antidiabetic drugs can cause undesirable side effects such as hypoglycemia, body weight gain, gastric symptoms, and fluid retention. There is, therefore, a high unmet medical need for novel and potent drugs for glycemic control with a good safety and tolerability profile.

Glucose exists in free form in the plasma and is filtered freely through the glomerulus. In healthy individuals, the kidney retrieves glucose from the filtrate, and the urine is therefore practically glucose free. Glucose reabsorption is mediated by sodium/glucose cotransporters, namely high-affinity sodium/glucose cotransporter 1 (SGLT1) and low-affinity sodium/glucose cotransporter 2 (SGLT2) in the proximal tubules of the kidney (Kanai et al., 1994). Therefore, the induction of urinary glucose excretion (UGE) by the inhibition of SGLTs is thought to be a strategy to control blood glucose levels. Indeed, the hyperglycemia in a diabetic animal model was improved with the induction of UGE by treatment with phlorizin (Kahn et al., 1991).
SGLT2 is specifically expressed in the proximal tubules of the kidney. In humans, mutations of the SGLT2 gene resulting in defective transport activity cause familial renal glucosuria (Kleta et al., 2004; Magen et al., 2005; Santer et al., 2003). Despite UGE occurring in individuals with familial renal glucosuria, these people are generally asymptomatic and have normal blood glucose levels and a normal glucose tolerance.

SGLT1, on the other hand, transports glucose and galactose not only in the renal tubules but also in the small intestine (Wright et al., 2011). In infants, mutations of the SGLT1 gene resulting in defective transport activity cause diarrhea and dehydration (glucose–galactose malabsorption) due to the reduction of both glucose and galactose absorption in the small intestine, and patients with these mutations also show mild renal glucosuria (Wright et al., 2001).

These genetic disorders suggest that SGLT2 plays a dominant role in renal glucose reabsorption and that SGLT1 inhibition could cause gastrointestinal symptoms. Therefore selective SGLT2 inhibition, because of its potency to induce UGE and its low safety concerns, is an attractive target for the development of a next-generation antidiabetic drug. Currently several SGLT2 inhibitors with various degrees of selectivity toward SGLT2 versus SGLT1 selectivity are being tested in clinical trials (Musso et al., 2011). Since a high safety profile is required for any drug used for life-long treatment of T2D, long-term experience will be needed to examine whether this emerging class of drugs can safely fulfill the unmet medical needs of T2D.

Recently we identified a potent and highly selective SGLT2 inhibitor, tofogliflozin (Sato et al., 2010). The small number of patients with familial renal glucosuria limits confidence in the lower safety concern which is normally applied to long-term SGLT2 inhibition by virtue of the benign condition of the patient population, making intensive and multidimensional profiling of this emerging class of drugs of value in drug development, especially for T2D.

In the current study, we examined the pharmacological profiles of tofogliflozin (CSG452), both in vitro and in vivo, including evaluation not only of its selectivity toward other SGLTs but also of its effect on glucose-related physiological processes such as glucose uptake, glucose oxidation, glycogen synthesis, hepatic glucose production, glucose-stimulated insulin secretion, and
glucosidase reactions. We found that tofogliflozin was highly specific to SGLT2—its selectivity toward SGLT2 versus other SGLT members was the highest among the SGLT2 inhibitors we tested—and that it improved T2D pathological conditions by lowering blood glucose levels through the inhibition of renal glucose reabsorption, with a low risk of hypoglycemia.
Methods

Chemicals

Tofogliflozin ((1S, 3'R, 4'S, 5'S, 6'R)-6-[(4-ethylphenyl)methyl]-3',4',5',6'-tetrahydro-6'-(hydroxymethyl)-spiro[isobenzofuran-1(3H), 2'[2H] pyran]-3',4',5',6'-triol) (Sato et al., 2010; Fig. 1), dapagliflozin (Meng et al., 2008), canagliflozin (Nomura et al., 2010), ipragliflozin (Tahara et al., 2011), empagliflozin (Grempler et al., 2012b), luseogliflozin (Kakinuma et al., 2010), PF-04971729 (Mascitti et al., 2011) were all synthesized in our laboratories at Chugai Pharmaceutical Co.

Phlorizin, α-methyl-D-glucopyranoside (AMG), myo-inositol (MI), and fructose were purchased from Sigma-Aldrich (St. Louis, MO, USA); α-methyl-D-[14C]-glucopyranoside ([14C]-AMG), myo-[3H]-inositol ([3H]-MI), [14C]-fructose, [14C]-2-deoxy-glucose ([14C]-2DG), [3H]-2-deoxy-glucose ([3H]-2DG), and [14C]-glucose were purchased from General Electronic Company (Tokyo, Japan). Voglibose was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada)

Animals

Male SD rats (CD/Crl) and ZDF rats (ZDF/Crl-Lepr\textsuperscript{fa}; fa/fa) were purchased from Charles River Laboratories Japan (Yokohama, Japan). Male db/db mice (BKS.Cg-+ Lepr\textsuperscript{db}/+ Lepr\textsuperscript{db}/Jcl) and GK rats (GK/Jcl) were purchased from Clea Japan (Tokyo, Japan). Male Wistar rats (Slc:Wistar) were purchased from Japan SLC (Shizuoka, Japan). These animals were housed under a 12-h/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 diet of laboratory chow (Purina5008 pellets [PMI Nutrition International LLC, St. Louis, MO, USA] for ZDF rats; CE-2 pellets [Clea Japan] for other animals) and water. All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical Co.
**SGLT inhibition**

Expression plasmids containing human SGLTs (hSGLT1, hSGLT2, hSGLT3, hSGLT4, hSGLT5, hSGLT6, and hSMIT1), rat SGLTs (rSGLT1 and rSGLT2), and mouse SGLTs (mSGLT1 and mSGLT2) were prepared by the ligation of fragments amplified from Human Small Intestine Marathon-Ready cDNA, Human Kidney Marathon-Ready cDNA (Clontech Laboratories, Mountain View, CA, USA) or cDNA fragments prepared from the kidney or small intestine of Wistar rat or db/db mouse with primers designed from published sequences (GenBank accession numbers: NM000343, NM003041, AJ133127, AK131200, NM001042450, NM052944, AK092248, NM001107229, NM022590, BC003845, AY033886), into the multi-cloning site of pcDNA3.1(−) (Life Technologies Corporation [Invitrogen], Grand Island, NY, USA). The expression plasmids containing hSGLT1, hSGLT2, hSGLT4, hSGLT6, hSMIT1, mSGLT1, or mSGLT2 cDNA fragment were transfected into Chinese hamster ovary-K1 cells (CHO; American Type Culture Collection [ATCC]), and the expression plasmid containing the hSGLT3 cDNA fragment was transfected into human embryonic kidney cells (HEK293H; Invitrogen). Clones stably expressing each SGLT were used for the AMG or MI uptake assay. The expression plasmids containing hSGLT5, rSGLT1, or rSGLT2 cDNA fragment were transfected into African green monkey SV40-transfected kidney fibroblast cells (COS-7; ATCC), and the cells transiently expressing each SGLT were used for the AMG or fructose uptake assay.

For the AMG, MI, or fructose uptake assay, the cells expressing each SGLT were cultured in 96-well plates for 2 or 3 days and washed twice with sodium-free buffer (140 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES/Tris pH7.4). The cells were then incubated in sodium-free buffer or sodium buffer (140 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES/Tris pH7.4) each containing 1mM AMG (mixture of non-radiolabeled AMG and [³¹⁴C]-AMG), 0.1 mM MI (mixture of non-radiolabeled MI and [³H]-MI), 0.1 mM fructose (mixture of non-radiolabeled fructose and [¹⁴C]-fructose) at 37°C for 45 min. Sodium-dependent AMG, MI,
or fructose 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. IC$_{50}$ values of inhibitors were calculated with the empirical 4-parameter model fitting of XLfit (IDBS, Guildford, UK) and are indicated as mean ± S.D. of 3 to 12 independent experiments. For measurement of $K_i$ values, the AMG uptake assays were performed in sodium buffer or sodium-free buffer containing various concentrations of AMG. $K_i$ values of inhibitors were calculated from Lineweaver-Burk plots.

**Effects on cellular functions relating to glucose metabolism**

Glucose uptake was evaluated by 2DG uptake assay in XM13A1 cells, a temperature-sensitive SV40 large T-antigen immortalized cell line derived from human skeletal muscle (Lonza, Basel, Switzerland), differentiated L6 rat skeletal muscle cells (L6 cells; ATCC), and mouse adipocytes (3T3-L1 cells; ATCC) according to previously described methods (Bosch et al., 2004; Koivisto et al., 1991), in the presence or absence of tofogliflozin with or without stimulation with 100 or 1000 nM insulin (Sigma-Aldrich) using $[^3]$H-2DG as a substrate.

Glycogen synthesis was evaluated in XM13A1 cells according to the method of Anand et al. (2010). Briefly, after overnight serum starvation in starvation medium (Ham’s F-10 Medium containing 0.2% bovine serum albumin [BSA; Sigma-Aldrich] and 10 mM HEPES pH7.2–7.5) cells were incubated in starvation medium plus 175 kBq/mL $[^1]$C-glucose with or without 1 μM insulin in the presence or absence of tofogliflozin at 37°C for 2 h. Glycogen in the lysate of the cells was precipitated by ethanol, and insulin-stimulated glycogen synthesis was calculated by subtracting the radioactivity detected in glycogen of non-insulin-stimulated cells from that in insulin-stimulated cells.

Glucose oxidation was evaluated in XM13A1 cells. After overnight serum starvation, cells were incubated with $[^1]$C-glucose with or without tofogliflozin at 37°C for 4 h. $[^1]$C-CO$_2$ content in air exhausted from the culture medium was trapped on a LumaPlate-96 (PerkinElmer, Waltham, MA, USA) and glucose oxidation activity was calculated.
Hepatic glucose production was evaluated in rat primary hepatocytes isolated from the liver of SD rats with standard methods (Andersen et al., 1999). The hepatocytes were cultured in a medium containing 1.25% BSA, 5% fetal bovine serum, 20 nM insulin, and 20 mM glucose. The cells were then incubated at 37°C for 2 h in a buffer containing 0.5 nM glucagon (Peptide Institute, Osaka, Japan) in the presence or absence of tofogliflozin. Glucagon-induced glucose production was calculated by measuring glucose concentration in the medium with an Amplex Red Glucose Assay Kit (Invitrogen).

Glucose-stimulated insulin secretion (GSIS) was evaluated in pancreatic islets isolated from Wistar rats with standard methods (Iwakura et al., 2005). After the incubation of islets with 3.3 or 16.7 mM glucose in the presence or absence of tofogliflozin at 37°C for 1 h, insulin concentrations in the media were measured and GSIS was calculated from the difference between the insulin concentrations in the supernatant of the 3.3 and 16.7 mM glucose-stimulated islets.

**Glucosidase inhibition**

Inhibition of human α-amylase activity was measured with an iodine-starch reaction method (Amylase-Test Wako; Wako Pure Chemical Industries, Osaka, Japan). The reaction mixture containing α-amylase (Biodesign International, Saco, ME, USA) and the substrate solution with or without tofogliflozin was incubated at room temperature for 5 min, and the α-amylase activity was measured.

Activities of rat intestinal glucosidases were measured by using extracts of rat intestinal acetone powder (Sigma-Aldrich) as partially purified glucosidases based on a previously reported method (Nishioka et al., 1998). Partially purified glucosidases from XPEMB1 cells (human skin fibroblast cells; ATCC) were used to measure the activities of human lysosomal glucosidases (Wisselaar et al., 1989). Each of 4-methylumbelliferyl α-D-glucopyranoside (Sigma-Aldrich), 4-methylumbelliferyl β-D-glucopyranoside (Sigma-Aldrich), 4-methylumbelliferyl β-D-galactopyranoside (Sigma-Aldrich), 4-methylumbelliferyl α-D-mannopyranoside (Sigma-Aldrich), and 4-methylumbelliferyl β-D-mannopyranoside (Sigma-Aldrich) was used as a substrate for the
reaction of $\alpha$-glucosidase, $\beta$-glucosidase, $\beta$-galactosidase, $\alpha$-mannosidase, and $\beta$-mannosidase, respectively (Hosli, 1977). A reaction mixture containing partially purified glucosidases, 250 mM sodium citrate pH 4.2, and 40–160 $\mu$M substrate, with or without tofogliflozin, was incubated at room temperature for 1–3 h. Enzyme activity was detected by measuring 4-methylumbelliferone concentration in the reaction mixture in terms of fluorescence (excitation wavelength 360 nm/emission wavelength 450 nm).

**Binding on various receptors, channels, and transporters**

Selectivity of tofogliflozin to various molecular targets (71 receptors, 5 ion channels, 3 transporters) was examined by Cerep (Celle l’Evescault, France) using standard *in vitro* radioligand binding assays. Tofogliflozin was tested at 10 $\mu$M. All studies were internally controlled with reference ligands, and further details of the methodologies for each assay can be found at http://www.cerep.fr (Supplemental Table 1).

**Renal glucose clearance and blood glucose lowering effects in diabetic rats (ZDF rats)**

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle (0.5% CMC; 5 mL/kg) was orally administered to non-fasted ZDF rats (male, 10 weeks of age). Blood samples were collected from the tail vein immediately before administration (0 h) and at 1, 2, 4, 6, 8, 12, and 24 h after administration, and blood glucose levels and concentrations of tofogliflozin in the plasma were measured. Urine samples were collected from the rats’ metabolic cages every 4 h from immediately after administration to 12 h after administration, and the urine volume and the urinary glucose concentrations were measured. Renal glucose clearance was determined by dividing the amount of urinary glucose excretion for each urine collection period (4-h cumulative urine sample) by the blood glucose area under the curve (Glu AUC) for each respective 4-h period (Glu AUC$_{0-4h}$, Glu AUC$_{4-8h}$, or Glu AUC$_{8-12h}$). These Glu AUCs were determined by using the trapezoidal rule. Blood and urinary glucose...
glucose concentrations were measured by the hexokinase method (Autosera S GLU; Sekisui Medical Co., Tokyo, Japan). Plasma tofogliflozin concentrations were measured with an LC-MS/MS system (high performance liquid chromatography [Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA]; tandem quadrupole mass spectrometer [API 4000; Applied Biosystems, Foster City, CA, USA]). Pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-24}, t_{1/2}) were obtained from the quantitative values of each rat by using WinNonlin ver.4.1 (Pharsight, St. Louis, MO, USA).

**Blood glucose lowering effects in diabetic mice (db/db mice)**

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle (0.5% CMC; 5 mL/kg) was orally administered to non-fasted db/db mice (male, 9 weeks of age). Blood samples were collected from the tail vein immediately before administration (0 h) and at 1, 2, 4, 6, 8, 10, 12, and 24 h after administration, and blood glucose levels and plasma tofogliflozin concentrations were measured as described above.

**Postprandial blood glucose lowering effects in diabetic rats (GK rats)**

Voglibose (0.1, 0.3, or 1 mg/kg), tofogliflozin (1, 3, or 10 mg/kg), or vehicle (0.5% CMC; 5 mL/kg) was orally administered to overnight-fasted GK rats (male, 9 weeks of age) 5 min before oral administration of a meal mixture prepared by the suspension of 42 g of custom diet (composition [g/100 g diet] was as follows: 53.8 g equal volumes of maltose and dextrin, 19.2 g casein, 13.2 g olive oil, 4.2 g corn oil, 1.0 g safflower oil, 4.6 g mineral mixture, 2.3 g vitamin mixture, 0.2 g L-cysteine, 0.1 g D.L-methionine, and 1.4 g cellulose [Oriental Yeast Co., Ltd., Tokyo, Japan]) in sufficient water for a final concentration of 0.35 g/mL. Blood samples were collected from the tail vein 10 min before the meal loading and at 15 and 30 min and 1, 2, 3, and 4 h after the meal loading, and the blood glucose levels were determined as described above.
Blood glucose lowering effects and improvement of glucose intolerance by long-term administration in diabetic mice (db/db mice)

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle (0.5% CMC; 5 mL/kg) was orally administered to db/db mice (male, 8 weeks of age) once daily between 17:00 and 19:00 for 28 days. Body weight and food consumption were measured at intervals of 1 to 4 days. Blood samples were collected from the tail vein between 09:00 and 12:00 on day −1 and day 28, and the blood glucose, glycated hemoglobin, and plasma immunoreactive insulin (IRI) levels were determined. Glycated hemoglobin levels were measured by turbidimetric inhibition immunoassay (AutoWako HbA1c; Wako Pure Chemical Industries). Plasma IRI levels were measured with an insulin ELISA kit (no. 200718; Morinaga Institute of Biological Science, Kanagawa, Japan). Four days after final administration (day 31), an OGTT (oral glucose load: 3 g/kg) was performed with overnight-fasted mice. Blood samples were collected from the tail vein 1 min before and 0.5, 1, 2, and 4 h after the glucose administration and blood glucose levels were determined as described above.

Urinary glucose excretion and plasma glucose levels in normal rats (SD rats)

Tofogliflozin (1, 3, or 10 mg/kg) or vehicle (0.5% CMC; 5 mL/kg) was orally administered to non-fasted SD rats (male, 8 weeks of age). Urine samples were collected from the rats’ metabolic cages at 0–6, 6–12, and 12–24 h after administration, and the urine volume and the urinary glucose concentration was measured as described above. The blood glucose levels were measured by a blood glucose monitoring system (Accu-check Aviva; Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± S.D. in in vitro experiments and mean ± S.E.M. in in vivo experiments. Statistical analysis was performed by using SAS System for Windows, Release 8.02 (SAS Institute.
Japan, Tokyo, Japan). Statistical significance was determined by the parametric Dunnett’s multiple comparison.
Results

In vitro SGLT2 inhibition and SGLT2 selectivity

The inhibitory activities of tofogliflozin and phlorizin against human, rat, and mouse SGLT2 were examined in cells (CHO, COS-7) over-expressing each SGLT2 by evaluating sodium-dependent AMG uptake. Analysis using Lineweaver-Burk plots showed that both compounds inhibited AMG uptake in a substrate competitive inhibition manner (Fig. 2), and $K_i$ values of phlorizin for human, rat, and mouse SGLT2 inhibition were $13.6 \pm 1.4$, $39.4 \pm 0.8$, and $13.8 \pm 0.7$ nM, respectively. Tofogliflozin inhibited each SGLT2 more strongly than did phlorizin: $K_i$ values of tofogliflozin for human, rat, and mouse SGLT2 inhibition were $2.9 \pm 0.2$, $14.9 \pm 5.9$, and $6.4 \pm 0.8$ nM, respectively.

Next, the inhibitory activities of tofogliflozin and other SGLT inhibitors against the 7 human SGLTs (hSGLT1, hSGLT2, hSGLT3, hSGLT4, hSGLT5, hSGLT6, and hSMIT1) were compared in SGLT-overexpressing cells (CHO, HEK293, or COS-7) by measuring sodium-dependent sugar (AMG, fructose, or MI) uptake (Table 1). Tofogliflozin showed the highest selectivity toward hSGLT2 versus hSGLT1, hSGLT6, and hSMIT1. The compounds with the highest selectivity toward hSGLT2 versus hSGLT3, hSGLT4 and hSGLT5 were dapagliflozin, luseogliflozin and PF-04971729, respectively, indicating that among the SGLT inhibitors tested tofogliflozin was the most selective SGLT2 inhibitor.

In addition to the selectivity of tofogliflozin toward hSGLT2 versus that toward other human SGLTs, the inhibitory activities of tofogliflozin against rat and mouse SGLT1 and SGLT2 were compared. Tofogliflozin inhibited both rat and mouse SGLT2 at lower concentrations than phlorizin: $IC_{50}$ values of tofogliflozin against rat and mouse SGLT2 were $14.5 \pm 1.9$ and $5.0 \pm 1.2$ nM, and those of phlorizin were $48.2 \pm 11.4$ and $16.8 \pm 5.6$ nM, respectively. Moreover, tofogliflozin was more selective than phlorizin: the selectivity of tofogliflozin toward SGLT2 was 560 times that toward rSGLT1 and 360 times that toward mSGLT1 ($IC_{50}$ for rSGLT1, 8200 ± 1900 nM; $IC_{50}$ for mSGLT1, 1800 ± 870 nM), whereas the selectivity of phlorizin toward SGLT2 was 20 times that
toward rSGLT1 and 19 times that toward mSGLT1 (IC$_{50}$ for rSGLT1, 970 ± 180 nM; IC$_{50}$ for mSGLT1, 310 ± 110 nM).

**Effects on non-SGLT-related reactions and functions**

Because tofogliflozin has a glucose moiety in its structure, we evaluated the effects of this compound on glucose metabolism (such as basal or insulin-induced glucose uptake) *in vitro* with human myoblasts (XM13A1 cells), rat myoblasts (L6 cells), and mouse adipocytes (3T3-L1 cells). We also evaluated the effects on glucose oxidation and insulin-induced glycogen synthesis in XM13A1 cells, hepatic glucose production induced by glucagon in rat primary hepatocytes, and GSIS in rat pancreatic islets. In these experiments, no marked effect was observed with tofogliflozin concentrations of 10 or 100 μM (Supplemental Table 2).

Glucosidases also play critical roles in glucose metabolism; therefore, the effects of tofogliflozin on several glucosidase activities were also evaluated by using purified human pancreatic α-amylase, partially purified rat intestinal glucosidases, or partially purified human lysosomal glucosidases from XPMB1 cells. Tofogliflozin (100 μM) exhibited no marked inhibition against these enzymes (Supplemental Table 3).

Additionally, selectivity of tofogliflozin to various molecular targets was also examined with *in vitro* radioligand binding assays to 71 receptors, 5 ion channels, and 3 transporters, and it was shown that tofogliflozin (10 μM) did not markedly inhibit specific ligand binding to these targets (Supplemental Table 1).

At 10 μM of tofogliflozin no obvious effect was detected in any of these assays. Therefore, the IC$_{50}$ values of tofogliflozin against these reactions were over 600 times the IC$_{50}$ value of tofogliflozin against SGLT2.
Increase of renal glucose clearance and reduction of blood glucose levels

in ZDF rats

The acute effects of tofogliflozin on renal glucose clearance and blood glucose levels were examined in ZDF rats, an obese diabetic model. Tofogliflozin increased renal glucose clearance dose-dependently (Fig. 3a), and at dosages of 3 and 10 mg/kg, significant increases in renal glucose clearance continued until the period of 8–12 h. Simultaneously, tofogliflozin reduced blood glucose levels transiently from 1 to 12 h after administration in dose-dependent manner (Fig. 3b). The maximum glucose lowering effect was observed at 4 to 6 h after administration, with the plasma glucose levels falling to within the normal range but not below 100 mg/dL at the highest dose at this time point.

Changes in plasma tofogliflozin concentrations in these rats were examined over time (Fig. 3c). After administration, plasma tofogliflozin concentration rapidly increased in a dose-dependent manner. At 3 mg/kg, plasma tofogliflozin concentration peaked ($C_{\text{max}}$: 1.39 μg/mL, 3600 nM) at 2 h after administration ($T_{\text{max}}$: 1.42 h), then the concentration decreased ($t_{1/2}$: 2.9 h) to 0.069 μg/mL (180 nM) at 12 h (Supplemental Table 4).

Improvement of hyperglycemia in db/db mice

The acute effect of tofogliflozin on blood glucose levels in non-fasted db/db mice was then tested to confirm the glucose-lowering effect also in mice. Tofogliflozin rapidly reduced blood glucose levels in a dose-dependent manner, and the reduction of blood glucose levels was maintained until 12 h after drug administration at dosages of 1 mg/kg or higher (Fig. 4). The maximum glucose lowering effects were observed at 4 h after administration in the 0.1 to 1 mg/kg groups and 6 h after administration in the 3 and 10 mg/kg groups. As was the case with the ZDF rats, the plasma glucose levels of db/db mice also fell within the normal range at the highest dose but were not lowered to below 100 mg/dL.
The plasma tofogliflozin concentration in db/db mice increased rapidly just after the administration, and the increment was dose-dependent. Pharmacokinetic parameters at 10 mg/kg tofogliflozin are as follows: $T_{max}$ 0.25 h, $C_{max}$ 4.38 μg/mL and $t_{1/2}$ 2.7 h (Supplemental Table 4).

**Suppression of postprandial hyperglycemia in GK rats**

In addition to the acute hypoglycemic effect in diabetic animals under non-fasted conditions, we examined the suppressive effect of tofogliflozin on postprandial glucose increase using GK rats, a non-obese animal model of type 2 diabetes with glucose intolerance. In these animals, voglibose (0.1, 0.3, or 1 mg/kg), an α-glucosidase inhibitor used for the improvement of postprandial hyperglycemia in the clinic, suppressed blood glucose increase dose-dependently from 15–30 min to 4 h after meal loading (Supplemental Figure 1). The suppressive effects of tofogliflozin (1, 3, or 10 mg/kg) on glucose increase after meal loading (Fig. 5) were comparable to those of voglibose, indicating that tofogliflozin improves postprandial hyperglycemia.

**Improvement of hyperglycemia and glucose intolerance by long-term administration of tofogliflozin in db/db mice**

To examine the long-term effects of tofogliflozin on hyperglycemia and glucose tolerance in db/db mice, tofogliflozin was orally administered once daily for 4 weeks at the doses at which acute blood glucose reduction was observed (0.1–10 mg/kg). Tofogliflozin dose-dependently reduced glycated hemoglobin after 4-week administration, and the glycated hemoglobin levels at doses of 0.3 mg/kg and over were significantly lower than in the group treated with vehicle only (Fig. 6a). During the 4-week administration, no difference in food intake or body weight due to the tofogliflozin treatment was observed. Although the plasma IRI levels of the vehicle group decreased from 23.0 ± 2.0 ng/mL on day −1 to 6.6 ± 0.7 ng/mL on day 28, tofogliflozin significantly prevented the decrease of IRI levels at doses of 3 and 10 mg/kg (Fig. 6b).
Four days after the final administration, an OGTT was performed to test whether long-term glucose control by tofogliflozin improves glucose tolerance. In the groups treated with 0.3 mg/kg or more of tofogliflozin, blood glucose levels at 4 h after glucose loading were reduced compared with the vehicle group (Fig. 6c); moreover, blood glucose AUC0–4h of the 3 and 10 mg/kg groups were lower than that of the vehicle group (Fig. 6d). Therefore, long-term administration of tofogliflozin improved hyperglycemia and thereby ameliorated glucose intolerance of the obese diabetic mice.

Effects on urinary glucose excretion and blood glucose levels in normal rats

Effects of tofogliflozin on urinary glucose and blood glucose levels in normal rats were examined to explore the hypoglycemic potential of tofogliflozin under normoglycemic conditions. After the oral administration of tofogliflozin (1, 3, or 10 mg/kg) to non-fasted SD rats, UGE increased dose-dependently; the significant increase in urinary glucose in the 10 mg/kg group continued to the period of 12–24 h (Fig. 7a). However, no changes in blood glucose levels in any of the tofogliflozin treatment groups were observed (Fig. 7b).
Discussion

In the present study, we demonstrated that tofogliflozin had potent SGLT2 inhibitory activity and the highest selectivity toward SGLT2 versus other SGLT family members among SGLT2 inhibitors without any direct effect on other aspects of glucose metabolism. Tofogliflozin improved T2D pathological conditions by lowering blood glucose levels through the inhibition of renal glucose reabsorption. Additionally, tofogliflozin did not change plasma glucose levels in normal rats despite the increased levels of UGE.

Pharmaceutical companies have been trying to find potent and highly bioavailable SGLT2 inhibitors, and several SGLT2 inhibitors are currently being developed for diabetes treatment. Dapagliflozin, one of such compounds under clinical development, induced UGE and reduced blood glucose and HbA1c levels in T2D patients (Musso et al., 2011), indicating that SGLT2 inhibition is a practical drug target for the treatment of diabetes.

However, even among drugs belonging to the same class, adverse events profiles can differ owing to differences in their off-target effects. Therefore, to examine whether this type of emerging therapeutic class can safely fulfill the unmet medical needs in T2D treatment, we believe that case-by-case evaluations of the efficacy and safety of highly selective SGLT2 inhibitors are needed, both in clinical trials and with long-term clinical experience.

As shown in this study, tofogliflozin is a potent SGLT2 inhibitor and the most selective among the compounds in this class. The highly selective inhibition of SGLT2 by tofogliflozin is an important characteristic because safe and efficacious antidiabetics are needed and SGLT family members are thought to play a number of important physiological roles. SGLT1 plays a role in the absorption of several carbohydrates, and a dysfunction of SGLT1 results in glucose–galactose malabsorption (Martín et al., 1996). In addition, SGLT1 is reported to be expressed in various organs such as the lungs, heart, and liver (Wright et al., 2011). SGLT6 (Roll et al., 2002) and SMIT1 (Berry et al., 1995) are sodium-dependent MI transporters expressed in various organs including the kidneys. Inhibition of MI transport may impair the function of the kidneys, because MI, an organic
osmolyte, mediates osmolarity and maintains cell volume and fluid balance in various cells (Beck et al., 1998). For example, methylene-\textit{myo}-inositol, an MI transporter inhibitor, damages renal tubules and induces acute renal failure in normal rats (Kitamura et al., 1998). SGLT4 and SGLT5 have each been reported as sodium-dependent sugar transporters, with SGLT4 highly expressed in the small intestine and kidneys (Tazawa et al., 2005) and SGLT5 specifically expressed in the kidneys (Grempler et al., 2012a), and SGLT3 has been reported as a glucose sensor, rather than a sugar transporter, highly expressed in the skeletal muscles, small intestine, and kidneys (Diez-Sampedro et al., 2003); however, the precise roles of these three SGLTs remains unclear.

Acarbose, an antidiabetic in the class of $\alpha$-glucosidase inhibitors, increases gastrointestinal events (Van de Laar et al., 2005). Since tofogliflozin did not inhibit intestinal glucosidases \textit{in vitro}, it may have a low-risk of adverse gastrointestinal effects. In addition, the absence of any inhibitory activity of tofogliflozin against lysosomal glucosidases suggests a low-risk of undesirable side effects, because lysosomal glucosidase deficiency is known to be responsible for various lysosomal diseases (Brady, 2006; Michalski and Klein, 1999). The results of comprehensive \textit{in vitro} binding assays to molecular targets (71 receptors, 5 ion channels, 3 transporters; shown in Supplemental Table 1) also suggest no off-target effects of tofogliflozin. Although a low safety concern is suggested from the high selectivity of tofogliflozin to SGLT2, more extensive and comprehensive analyses in long-term clinical studies with a large number of patients will be needed to confirm the safety of tofogliflozin.

Since SGLT1 and SGLT2 both contribute to urinary glucose reabsorption, we evaluated whether the selective inhibition of SGLT2 with tofogliflozin is sufficient to induce UGE to lower blood glucose levels. In ZDF rats, tofogliflozin increased renal glucose clearance until at least 12 h after administration, together with a reduction in plasma glucose. In these animals, the estimated unbound tofogliflozin concentrations in plasma, as estimated from the rat plasma protein binding ratio (83–84\%) and measured plasma tofogliflozin concentrations, were at levels higher than the rat SGLT2 IC$_{50}$ value (14.5 nM) in the 3 and 10 mg/kg dosing groups until at least 12 h after administration. The relationship between the estimated unbound concentration of tofogliflozin and
its duration of efficacy strongly suggests that the blood glucose lowering effects of tofogliflozin are due to the induction of UGE by SGLT2 inhibition. Additionally, when the maximum concentration of tofogliflozin in the plasma of rats in the 10 mg/kg group peaked at 4.15 μg/mL (11,000 nM), the estimated unbound concentration (1700 nM) would not have exceeded the rat SGLT1 IC50 value (8200 ± 1900 nM), indicating that the induction of UGE in ZDF rats was only due to SGLT2 inhibition.

Moreover, we could account for the blood glucose lowering effects of tofogliflozin by the amount of UGE induced in ZDF rats. Tofogliflozin increased UGE in the period 0–4 h after administration, and the calculated maximum difference in UGE between the vehicle and tofogliflozin groups was approximately 8 mg/kg/min. It is reported that endogenous glucose production is approximately 14 mg/kg/min in ZDF rats as compared with approximately 5–6 mg/kg/min in non-diabetic rats (Upton et al., 1998), and the difference was comparable to the UGE induced by tofogliflozin as described above.

On the other hand, blood glucose levels are influenced by various factors such as insulin secretion and the insulin sensitivity of skeletal muscle, liver, and adipose tissue. We observed that tofogliflozin induced acute glucose-lowering effects without an increase in plasma insulin levels in ZDF rats (data not shown). These in vivo observations together with the results of the in vitro experiments discussed above suggest that tofogliflozin’s glucose-lowering effects are independent of pancreatic insulin secretion and insulin sensitivity. These experimental data indicated that by inducing UGE with selective inhibition of SGLT2, tofogliflozin could substantially improve hyperglycemia in diabetic rodent models.

It is believed that daily management of blood glucose levels could improve the pathological condition of diabetes. In our experiments on db/db mice, administration of tofogliflozin for 4 weeks reduced glycated hemoglobin levels and prevented IRI reduction. Furthermore glucose tolerance was improved in mice treated with 3 or 10 mg/kg tofogliflozin. These results suggest that long-term treatment with tofogliflozin may improve the pathological condition of diabetes by better preservation of β-cell function. Since glucotoxicity contributes to the deterioration of β-cell function...
through oxidative stress under hyperglycemic conditions (Poitout and Robertson, 2008), and since tofogliflozin lowers blood glucose but has no direct effect other than SGLT2 inhibition, the preservation of β-cell function with tofogliflozin might be due to the reduction of oxidative stress through the sustained glucose-lowering effects.

Hypoglycemia is a very frequent adverse effect seen in the clinical treatment of diabetes. Generally, the risks of hypoglycemia are believed to be low with SGLT2 inhibitors for the following reasons. Firstly, SGLT inhibition lowers blood glucose independently of insulin which induces hypoglycemia directly through utilization of glucose and also inhibits anti-hypoglycemic actions, such as an increase in endogenous glucose production. Secondly, no hypoglycemia has been reported in patients with familial renal glucosuria whose SGLT2 function is decreased. This can be explained by the fact that both SGLT1 and SGLT2 reabsorb glucose in normal tubules and SGLT1 is able to partially reabsorb glucose in patients with SGLT2 dysfunction.

Actually, in ZDF rats in the 10 mg/kg tofogliflozin group, the maximum unbound tofogliflozin concentrations in plasma were estimated to be more than 100 times the rSGLT2 IC50 value and one-quarter of the rSGLT1 IC50 value, by which renal glucose reabsorption via SGLT2 should be almost completely inhibited and that via SGLT1 should be almost completely maintained. Under these conditions, although the blood glucose level was decreased to within the normal range with the increase of renal glucose clearance, no hypoglycemia was observed.

Additionally in SD rats, although UGE was induced with tofogliflozin dose-dependently, no significant reduction in blood glucose levels was observed with tofogliflozin even at 10 mg/kg. In separate pharmacokinetic experiments using SD rats treated with tofogliflozin at 10 mg/kg, the maximum unbound tofogliflozin concentrations in plasma were estimated to be 85 times the rSGLT2 IC50 value and one-seventh the rSGLT1 IC50 value (data not shown).

These results suggest that the absence of hypoglycemia at the maximum tofogliflozin dose in rats is related to the residual SGLT1 activity that is preserved with tofogliflozin’s high selectivity toward SGLT2. Since the selectivity of tofogliflozin toward SGLT2 versus that toward SGLT1 is
higher for human SGLTs than for rat SGLTs, we consider that the risk of hypoglycemia in humans treated with tofogliflozin will be lower than that in rats.

The present study indicates that tofogliflozin is the most specific SGLT2 inhibitor and achieves potent antidiabetic effects by increasing UGE. It also suggests that tofogliflozin has clinical potential as an antidiabetic drug without the risks of hypoglycemia and unpredicted side effects due to interactions with glucose-related reactions and off-target reactions. Therefore, tofogliflozin is the most attractive candidate among compounds of the SGLT2 inhibitor class.
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Authorship contributions

Participated in research design: Ikeda, Honda, Sato, Suzuki, and Kawabe.

Created new drug: Sato and Kobayashi.

Conducted experiments: Suzuki, Fukazawa, Ozawa, Hagita, T. Kawai, Takeda, Yata, M. Kawai, Fukuzawa, and Honda.

Wrote or contributed to the writing of the manuscript: Suzuki, Fukazawa, Honda, and Kawabe.
References


Footnotes

Results of this study were presented in part at the 71st Scientific Sessions of the American Diabetic Association (San Diego, LA, USA, July 2011): Masayuki Suzuki, Kiyofumi Honda, Masanori Fukazawa, Kazuharu Ozawa, Hitoshi Hagita, Takahiro Kawai, and Sachiya Ikeda. Tofogliflozin, a novel, potent, and highly selective SGLT2 inhibitor, improves glycemic control in diabetic mice and rats. 1136-P

Request for reprints should be addressed to Masanori Fukazawa, Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan.
Phone +81 550 87 8431; Fax +81 550 87 5219; E-mail: fukazawamsn@chugai-pharm.co.jp
Figure legends

**Fig. 1** Structure of tofogliflozin (CSG452)

**Fig. 2** Inhibition of hSGLT2 by tofogliflozin. Sodium-dependent and sodium-independent AMG uptake was measured with CHO cells overexpressing hSGLT2 in the presence or absence of tofogliflozin with various concentrations of AMG. Sodium-dependent AMG uptake velocity (v) was calculated and used for the Lineweaver-Burk plots against 1/[s]. Experiments were performed 3 times independently.

**Fig. 3** Effects of single oral administration on blood glucose level and renal glucose clearance in ZDF rats. Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle was administered to ZDF rats by oral gavage. a) Renal glucose clearance was calculated as described in the text every 4 h for 12 h after drug administration. b) Blood glucose levels were determined before (0 h) and at 1, 2, 4, 6, 8, 12 and 24 h after drug administration under non-fasting conditions. c) Plasma tofogliflozin concentrations were measured at 0.5, 1, 2, 4, 8, 12, and 24 h after drug administration. Data are expressed as mean ± S.E.M. (n=6). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett’s multiple comparison test.

**Fig. 4** Effects of single oral administration on blood glucose level in db/db mice. Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle was administered to db/db mice by oral gavage. Blood glucose levels were determined before (0 h) and at 1, 2, 4, 6, 8, 10, 12, and 24 h after drug administration under non-fasting conditions. Data are expressed as mean ± S.E.M. (n=6). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett’s multiple comparison test.
Fig. 5  Effects of single oral administration on postprandial blood glucose levels during meal tolerance test in GK rats. GK rats were fasted overnight, and tofogliflozin (1, 3, or 10 mg/kg) or vehicle was administered by oral gavage 5 min before meal administration. Blood glucose levels were determined before (0 h) and at 0.25, 0.5, 1, 2, 3, and 4 h after meal administration. Data are expressed as mean ± S.E.M. (n=6). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett’s multiple comparison test.

Fig. 6  Effects of 4-week repetitive administration on glycated hemoglobin (Hb) in db/db mice. Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) was administered once daily between 17:00 and 19:00 by oral gavage for 4 weeks. Blood was collected between 09:00 and 12:00 on day −1 and day 28. a) Blood glycated Hb levels. b) Plasma IRI levels. c) Changes in blood glucose level in the OGTT 4 days after final administration in mice fasted overnight. Blood glucose levels were determined before (0 h) and at 0.5, 1, 2, and 4 h after oral glucose administration (3 g/kg). d) Blood glucose AUC\(_{0-4h}\) (Glu AUC\(_{0-4h}\)) calculated by the trapezoidal rule. Data are expressed as mean ± S.E.M. (n=10). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett’s multiple comparison test.

Fig. 7  Effects of single oral administration on UGE in SD rats. Tofogliflozin (1, 3, or 10 mg/kg) or vehicle was administered to SD rats by oral gavage. The amounts of UGE were calculated from urine volume and urine glucose concentration at 0–6, 6–12, and 12–24 h after drug administration under non-fasting conditions. a) Total urine glucose. b) Blood glucose level. Data are expressed as mean ± S.E.M. (n=6). *p < 0.05, ***p < 0.001 versus vehicle-treatment group by Dunnett’s multiple comparison test.
Table 1  SGLT2 IC\textsubscript{50} values and SGLT2 selectivity of SGLT2 inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC\textsubscript{50} (nM)</th>
<th>SGLT2 selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hSGLT2</td>
<td>hSGLT1</td>
</tr>
<tr>
<td>Tofogliflozin</td>
<td>2.9 ± 0.7</td>
<td>2900</td>
</tr>
<tr>
<td>Dapagliflozin</td>
<td>1.3 ± 0.2</td>
<td>610</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>6.7 ± 2.9</td>
<td>290</td>
</tr>
<tr>
<td>Ipragliflozin</td>
<td>2.8 ± 0.5</td>
<td>860</td>
</tr>
<tr>
<td>Empagliflozin</td>
<td>3.6 ± 1.6</td>
<td>1100</td>
</tr>
<tr>
<td>Luseogliflozin</td>
<td>3.1 ± 0.1</td>
<td>1600</td>
</tr>
<tr>
<td>PF-04971729</td>
<td>1.4 ± 0.1</td>
<td>1300</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>16.4 ± 5.2</td>
<td>11</td>
</tr>
</tbody>
</table>

SGLT2 selectivity is expressed as the ratio of IC\textsubscript{50} of a compound against either hSGLT1, 3, 4, 5, 6, or hSMIT1 to its IC\textsubscript{50} against hSGLT2.
Renal glucose clearance (mL/h/kg)

Time after drug administration (h)

Vehicle
0.1 mg/kg
0.3 mg/kg
1 mg/kg
3 mg/kg
10 mg/kg
Figure 5. Graph showing the effect of different doses of medication (1 mg/kg, 3 mg/kg, 10 mg/kg) on blood glucose levels (mg/dL) over time after meal loading (h).

- Vehicle
- 1 mg/kg
- 3 mg/kg
- 10 mg/kg

Significance indicated by asterisks:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
The figure shows the effect of different dosages of a medication (0.1 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg) on blood glucose levels over time after glucose loading. The y-axis represents blood glucose levels in mg/dL, and the x-axis represents time after glucose loading in hours.

- **Vehicle**
- **0.1 mg/kg**
- **0.3 mg/kg**
- **1 mg/kg**
- **3 mg/kg**
- **10 mg/kg**

Significance levels are indicated with asterisks: *

**Significance levels:**
* p < 0.05
** p < 0.01
*** p < 0.001