Tofogliflozin, a Potent and Highly Specific Sodium/Glucose Cotransporter 2 Inhibitor, Improves Glycemic Control in Diabetic Rats and Mice

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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 overexpressing SGLT2, and K 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 sodium/myo-inositol transporter 1 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 the blood glucose level in Zucker diabetic fatty rats. Tofogliflozin also improved postprandial glucose excursion in a meal tolerance test with GK rats. In db/db mice, 4-week tofogliflozin treatment reduced glyced 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 (nephropathy, retinopathy, and neuropathy) complications, 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 disease. The incremental benefit of intensive glycemic control was shown in the Group UK Trial (UKPDS), which compared intensive with conventional treatment in type 2 diabetes mellitus. The intensive treatment group had a 15% lower rate of death due to any cause, a 31% lower rate of death due to cardiovascular disease, and a 15% lower rate of death due to any cause among patients who were free of heart disease at the start of the trial. This benefit was associated with a significant reduction in the progression of nephropathy, retinopathy, and neuropathy. The reduction in the progression of these complications was associated with an overall reduction in the risk of death due to any cause.

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ABBREVIATIONS: T2D, type 2 diabetes mellitus; 2DG, 2-deoxy-glucose; AMG, methyl-α-β-glucopyranoside; ATCC, American Type Culture Collection; CM, carboxymethyl cellulose; Glu AUC, blood glucose area under the curve; GSIS, glucose-stimulated insulin secretion; IRI, immunoreactive insulin; MI, myo-inositol; SGLT, sodium/glucose cotransporter; SMIT, sodium/myo-inositol transporter; UGE, urinary glucose excretion; CHO, Chinese hamster ovary; ZDF, Zucker diabetic fatty; h, human; r, rat; m, mouse; OGTT, oral glucose tolerance test; PF-04971729, (S)-[4-Chloro-3-(4-ethoxybenzyl)phenyl]-1-(hydroxymethyl)-6,8-dioxo-bicyclo[3.2.1]octane-2,3,4-triol. 

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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. Therefore, there is 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 chlorpropamide, a SGLT inhibitor (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 (Santer et al., 2003; Kleta et al., 2004; Magen et al., 2005). 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) because of 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). Because a high safety profile is required for any drug used for lifelong 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 that 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.

**Materials and 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′H]-2′H]-pyran]-3′,4′-triol] (Sato et al., 2010; Fig. 1), dapagliflozin (Meng et al., 2008), canagliflozin (Nomura et al., 2010), ipragliflozin (Tahara et al., 2011), empagliflozin (Grempeler et al., 2012b), luseogliflozin (Kakinuma et al., 2010), and (1S,2S,3′S,4′S)-5-4-chloro-3-(4-ethoxyben-zyloxy)methyl-1-(hydroxymethyl)-6,8-dioxo-bicyclo[3.2.1]octane-2,3,4-triol (PF-04971729) (Mascitti et al., 2011) were all synthesized in our laboratories at Chugai Pharmaceutical Co.

Phlorizin, methyl-α-D-glucopyranoside (AMG), myo-inositol (MI), and fructose were purchased from Sigma-Aldrich (St. Louis, MO); methyl-α-D-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 Electric Company (Tokyo, Japan). Voglibose was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada).

**Animals.** Male SD rats (CD/Crl) and Zucker diabetic fatty (ZDF) rats (ZDF/Crl-Leprdb/+ or cDNA fragments prepared from the kidney or small intestine of human Kidney Marathon-Ready cDNA (Clontech, Mountain View, CA), 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, AJ123622, AK131200, NM001042450, NM052944, AK092248,

Fig. 1. Structure of tofogliflozin (CSG452).
NM001107229, NM022590, NM023845, NM025380) into the multi-cloning site of pcDNA3.1(-) (Invitrogen, Carlsbad, CA). The expression plasmids containing hSGLT1, hSGLT2, hSGLT4, hSGLT6, hSMIT1, mSGLT1, or mSGLT2 cDNA fragment were transfected into Chinese hamster ovary-K1 (CHO) cells [American Type Culture Collection (ATCC), Manassas, VA], and the expression plasmid containing the hSGLT3 cDNA fragment was transfected into human embryonic kidney cells (HEK293H; Invitrogen). Cells 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 VSVG-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, pH 7.4). 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, pH 7.4), each containing 1 mM AMG (mixture of nonradiolabeled AMG and [3H]-AMG), 0.1 mM MI (mixture of nonradiolabeled MI and [3H]-MI), and 0.1 mM fructose (mixture of nonradiolabeled 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. IC₅₀ values of inhibitors were calculated with the empirical four-parameter model fitting of XLfit (IDBS, Guildford, UK) and are indicated as mean ± S.D. of 3 to 22 independent experiments. For measurement of Kᵢ values, the AMG uptake assays were performed in sodium buffer or sodium-free buffer containing various concentrations of AMG. Kᵢ 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 (Coivisto et al., 1991; Bosch et al., 2004), in the presence or absence of tofogliflozin with or without stimulation with 100 or 1000 nM insulin (Sigma-Aldrich) using [3H]-2DG as a substrate.

Glycogen synthesis was evaluated in XM13A1 cells according to the method of Anand et al. (2010). In brief, after overnight serum starvation in starvation medium [Ham’s F-10 medium containing 0.2% bovine serum albumin (Sigma-Aldrich) and 10 mM HEPES, pH 7.2-7.5], cells were incubated in starvation medium plus 175 kBq/ml [14C]-glucose with or without 1 nM 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 noninsulin-stimulated cells from that in insulin-stimulated cells. Glycogen oxidation was evaluated in XM13A1 cells. After overnight serum starvation, cells were incubated with [14C]-glucose with or without tofogliflozin at 37°C for 4 h. [14C]CO₂ content in air exhaled from the culture medium was trapped on a LumaPlate-96 (PerkinElmer Life and Analytical Sciences, Waltham, MA), 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% bovine serum albumin, 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 Inc., 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 medium 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 Chemicals, Osaka, Japan). The reaction mixture containing α-amylase (Biodies International, Kennewick, ME) 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 β-α-mannopyranoside (Sigma-Aldrich), and 4-methylumbelliferyl β-α-mannopyranoside (Sigma-Aldrich) was used as a substrate for the reaction of α-glucosidase, β-glucosidase, β-galactosidase, α-mannosidase, and β-mannosidase, respectively (Hösl, 1977). A reaction mixture containing partially purified glucosidases, 250 mM sodium citrate, pH4.2, and 40 to 160 µM substrate, with or without tofogliflozin, was incubated at room temperature for 1 to 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 µM. 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 nonfasted 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 glucose blood area under the curve (Glu AUC) for each respective 4-h period (Glu AUC₀–₄ₕ, Glu AUC₄–₈ₕ, or Glu AUC₈–₁₂ₕ). These Glu AUCs were determined by using the trapezoidal rule. Blood and urinary glucose concentrations were measured by the hexokinase method (AutoSys S GLU; Sekisui Medical Co., Tokyo, Japan). Plasma tofogliflozin concentrations were measured with an liquid chromatography-tandem mass spectrometry system [high-performance liquid chromatography (Agilent 1100 series; Agilent Technologies, Santa Clara, CA); tandem quadrupole mass spectrometer (API 4000; Applied Biosystems, Foster City, CA)]. Pharmacokinetic parameters (Cmax, Tmax, AUC₀–₂₄ₕ, f₂(4)) were obtained from the quantitative values of each rat by using WinNonlin version 4.1 (Pharsight, Mountain View, CA).
**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 nonfasted 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 /100 g diet) was as follows: 53.8 g of sugar mixture, 0.2 g of L-cysteine, 0.1 g of DL-methionine, equal volumes of maltose and dextrin, 19.2 g of casein, 13.2 g of olive oil, 4.2 g of corn oil, 1.0 g of safflower oil, 4.6 g of mineral mixture, 2.3 g of vitamin mixture, 0.2 g of L-cysteine, 0.1 g of DL-methionine, and 1.4 g of 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 5:00 and 7:00 PM 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 8:00 AM and 12:00 PM 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 immunosay (AutoWako HbA1c; Wako Pure Chemicals). Plasma IRI levels were measured with an insulin ELISA kit (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 nonfasted SD rats (male, 8 weeks of age). Urine samples were collected from the rats' metabolic cages at 0 to 6, 6 to 12, and 12 to 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 the 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, HEK293, or COS-7) by measuring sodium-dependent sugar (AMG, fructose, or MI) uptake. Analysis using Lineweaver-Burk plots showed that both compounds inhibited AMG uptake in a substrate competitive inhibition manner (Fig. 2), and plots showed that both compounds inhibited AMG uptake in a substrate competitive inhibition manner (Fig. 2), and

![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 three times independently.](http://example.com/fig2)

**TABLE 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC_{50} (nM)</th>
<th>SGLT2 Selectivity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IC_{50} hSGLT2</td>
<td>IC_{50} 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>
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\textsubscript{50} values of tofogliflozin against rat and mouse SGLT2 were 14.5 ± 1.9 and 5.0 ± 1.2 nM, respectively, and those of phlorizin were 48.2 ± 11.4 and 16.8 ± 5.6 nM, respectively. Moreover, tofogliflozin was more selective than phlorizin: the selectivity of tofogliflozin toward SGLT2 was 560 times that toward rSGLT1 and 19 times that toward mSGLT1 (IC\textsubscript{50} for SGLT1, 8200 ± 1900 nM; IC\textsubscript{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\textsubscript{50} for rSGLT1, 970 ± 180 nM; IC\textsubscript{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).

In addition, 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 tofogliflozin, no obvious effect was detected in any of these assays. Therefore, the IC\textsubscript{50} values of tofogliflozin against these reactions were over 600 times the IC\textsubscript{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 to 12 h. Simultaneously, tofogliflozin reduced blood glucose levels transiently from 1 to 12 h after administration in a 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\textsubscript{max} = 1.39 μg/ml, 3600 nM) at 2 h after administration (T\textsubscript{max} = 1.42 h), and the concentration decreased (t\textsubscript{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 nonfasted 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: $T_{\text{max}} = 0.25 \text{ h}$; $C_{\text{max}} = 4.38 \mu\text{g/ml}$; $t_{1/2} = 2.7 \text{ h}$ (Supplemental Table 4).

Suppression of Postprandial Hyperglycemia in GK Rats. In addition to the acute hypoglycemic effect in diabetic animals under nonfasted conditions, we examined the suppressive effect of tofogliflozin on postprandial glucose increase using GK rats, a nonobese animal model of type 2 diabetes with glucose intolerance. In these animals, voglibose (0.1, 0.3, or 1 mg/kg), an $\alpha$-glucosidase inhibitor used for the improvement of postprandial hyperglycemia in the clinic, suppressed blood glucose increase dose dependently from 15 to 30 min to 4 h after meal loading (Supplemental Fig. 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 6.7 ± 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 tofogliflozin, blood glucose levels at 4 h after glucose loading were reduced compared with the vehicle group (Fig. 6C); moreover, blood glucose AUC0–6h was 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 nonfasted SD rats, UGE increased dose dependently; the significant increase in urinary glucose in the 10-mg/kg group continued to the period of 12 to 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. In addition, 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 event profiles can differ because of 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). 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-myoo-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 α-glucosidase inhibitors, increases gastrointestinal events (Van de Laar et al., 2005). Because tofogliflozin did not inhibit intestinal glucosidases 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 (Michalski and Klein, 1999; Brady, 2006). The results of comprehensive 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.
blood glucose level. 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. 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 to 6, 6 to 12, and 12 to 24 h after drug administration under nonfasting conditions. A, total urine glucose. B, urine glucose concentration at 0 to 6, 6 to 12, and 12 to 24 h after drug gavage. The amounts of UGE were calculated from urine volume and tofogliflozin (1, 3, or 10 mg/kg) or vehicle was administered to SD rats by oral administration. The relationship between 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. In addition, 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 IC$_{50}$ 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 to 4 h after administration, and the calculated maximum difference in UGE between the vehicle and tofogliflozin groups was approximately 8 mg · kg$^{-1}$ · min$^{-1}$. It is reported that endogenous glucose production is approximately 14 mg · kg$^{-1}$ · min$^{-1}$ in ZDF rats compared with approximately 5 to 6 mg · kg$^{-1}$ · min$^{-1}$ in nondiabetic 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. Because glucotoxicity contributes to the deterioration of β-cell function through oxidative stress under hyperglycemic conditions (Poitout and Robertson, 2008), and because 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. In general, the risks of hypoglycemia are believed to be low with SGLT2 inhibitors for the following reasons. First, 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. Second, 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.

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 IC$_{50}$ value and one-quarter of the rSGLT1 IC$_{50}$ 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.

In addition, 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 SGLT2 IC50 value and one-seventh the SGLT1 IC50 value (data not shown).

These results suggest that the absence of hyperglycemia at the maximal tofogliflozin dose in rats is related to the residual SGLT1 activity that is preserved with tofogliflozin’s high selectivity toward SGLT2. Because 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 hyperglycemia 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 hyperglycemia 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: Suzuki, Honda, Sato, Kawabe, and Ikeda.

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

Conducted reagents or analytic tools: Kobuyashi and Sato.

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

References


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Supplemental data

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

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Suzuki et al.
Tofogliflozin, a potent and highly selective sodium/glucose cotransporter 2 inhibitor, improves glycemic control in diabetic rats and mice

Supplemental Table 1  Inhibition activity of tofogliflozin on the specific ligand binding to receptors, channels, and transporters.

<table>
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<th>Target</th>
<th>Origin</th>
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Inhibition (%) is expressed as percentage of inhibition of control-specific binding in the presence of tofogliflozin (10 μmoL/L).
Suzuki et al.

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

Supplemental Table 2  Inhibition activity of tofogliflozin against glucose-related cellular functions.

<table>
<thead>
<tr>
<th>Action</th>
<th>Source</th>
<th>Detection</th>
<th>Tofogliflozin (μmol/L)</th>
<th>Inhibition (% Mean±SD)</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Glucose uptake</td>
<td>XM13A1 cells (human myoblast)</td>
<td>[3H] labeled 2-deoxy-glucose uptake</td>
<td>100</td>
<td>3.3, 0.8</td>
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<td></td>
<td>L6 cells (rat myoblast)</td>
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<td>100</td>
<td>13.0 ± 2.1</td>
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<td></td>
<td>3T3-L1 cells (mouse adipocyte)</td>
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<td>100</td>
<td>24.3 ± 6.6</td>
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<td>Glucose oxidation</td>
<td>XM13A1 cells</td>
<td>[14C]-CO₂ production from D-[14C] glucose</td>
<td>100</td>
<td>19.3, 20.2</td>
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<td>Glycogen synthesis</td>
<td>XM13A1 cells</td>
<td>Incorporation of [13C] into glycogen from D-[13C] glucose</td>
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<td>Primary rat hepatocytes</td>
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<td>Glucose stimulated</td>
<td>Isolated rat pancreatic islets</td>
<td>Insulin secretion to culture medium</td>
<td>10</td>
<td>-4.4 ± 16.0</td>
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Insulin secretion
Suzuki et al.

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

**Supplemental Table 3** Inhibition activity of tofogliflozin against glucosidase reactions.

<table>
<thead>
<tr>
<th>Glucosidase reaction</th>
<th>Source</th>
<th>Detection</th>
<th>Tofogliflozin (μmol/L)</th>
<th>Inhibition (% Mean±SD)</th>
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<tr>
<td>Human α-amylase</td>
<td>Purified amylase from human pancreas</td>
<td>Starch</td>
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<tr>
<td>Rat glucoamylase</td>
<td>Glucosidase</td>
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<td>100</td>
<td>4.7 ± 0.4</td>
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<td>Rat sucrase</td>
<td>Partially purified glucosidases from rat intestinal acetone powder</td>
<td>Sucrose</td>
<td>100</td>
<td>-0.2 ± 3.1</td>
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<tr>
<td>Rat trehalose</td>
<td>Partially purified glucosidases from rat intestinal acetone powder</td>
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<td>Rat isomaltase</td>
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<td>Partially purified glucosidases from human skin fibroblast (XPEMB1 cells)</td>
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<td>Human β-galactosidase</td>
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<td>Human α-mannosidase</td>
<td>Partially purified glucosidases from human skin fibroblast</td>
<td>4MU-α-D-mannopyranoside</td>
<td>100</td>
<td>-5.6 ± 12.6</td>
<td>3</td>
</tr>
<tr>
<td>Human β-mannosidase</td>
<td>Partially purified glucosidases from human skin fibroblast</td>
<td>4MU-β-D-mannopyranoside</td>
<td>100</td>
<td>-9.0 ± 2.7</td>
<td>3</td>
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</table>
Tofogliflozin, a potent and highly selective sodium/glucose cotransporter 2 inhibitor, improves glycemic control in diabetic rats and mice

Supplemental Table 4  Tofogliflozin pharmacokinetic parameters in ZDF rats and db/db mice.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>C_{max}  (μg/mL)</th>
<th>T_{max} (h)</th>
<th>AUC_{0-24} (μg·h/mL)</th>
<th>t_{1/2} (h)</th>
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<td>0.03</td>
<td>2.50</td>
<td>0.20</td>
<td>NC</td>
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<td>2.00</td>
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<tr>
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<td>6</td>
<td>1.39</td>
<td>1.42</td>
<td>6.49</td>
<td>2.9</td>
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<td>6</td>
<td>4.63</td>
<td>2.33</td>
<td>22.97</td>
<td>3.0</td>
</tr>
<tr>
<td>db/db mice</td>
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<td>0.03</td>
<td>0.25</td>
<td>0.07</td>
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<td>0.08</td>
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<td>0.25</td>
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<tr>
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<td>3</td>
<td>4.38</td>
<td>0.25</td>
<td>10.10</td>
<td>2.7</td>
</tr>
</tbody>
</table>

NC: Not calculated
Suzuki et al.

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**Supplemental Figure 1** Effects of single oral administration of voglibose on postprandial blood glucose levels during meal tolerance test in GK rats.

GK rats were fasted overnight, and voglibose (0.1, 0.3, or 1 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.