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RESEARCH PAPER

TS-071 is a novel, potent and selective renal sodium-glucose cotransporter 2 (SGLT2) inhibitor with anti-hyperglycaemic activity

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BACKGROUND AND PURPOSE

The renal sodium-glucose cotransporter 2 (SGLT2) plays an important role in the reuptake of filtered glucose in the proximal tubule and therefore may be an attractive target for the treatment of diabetes mellitus. This study characterizes the pharmacological profile of TS-071 ((1*S*)-1,5-anhydro-1-[5-(4-ethoxybenzyl)-2-methoxy-4-methylphenyl]-1-thio-D-glucitol hydrate), a novel SGLT2 inhibitor *in vitro* and *in vivo*.

EXPERIMENTAL APPROACH

Inhibition of glucose uptake by TS-071 was studied in CHO-K1 cells stably expressing either human SGLT1 or SGLT2. Single oral dosing studies were performed in rats, mice and dogs to assess the abilities of TS-071 to increase urinary glucose excretion and to lower plasma glucose levels.

KEY RESULTS

TS-071 inhibited SGLT2 activity in a concentration-dependent manner and was a potent and highly selective inhibitor of SGLT2. Orally administered TS-071 increased urinary glucose excretion in Zucker fatty rats and beagle dogs at doses of 0.3 and 0.03 mg·kg^{-1} respectively. TS-071 improved glucose tolerance in Zucker fatty rats without stimulating insulin secretion and reduced hyperglycaemia in streptozotocin (STZ)-induced diabetic rats and *db/db* mice at a dose of 0.3 mg·kg^{-1}.

CONCLUSION AND IMPLICATIONS

These data indicate that TS-071 is a potent and selective SGLT2 inhibitor that improves glucose levels in rodent models of type 1 and 2 diabetes and may be useful for the treatment for diabetes mellitus.

Abbreviations

α-MG, methyl-α-D-glucopyranoside; AUC, area under the curve; GLUT, glucose transporter; CHO-K1, Chinese hamster ovary-K1; CMC-Na, carboxymethyl cellulose sodium salt; 2-DG, 2-deoxy-D-glucose; IBMX, 3-isobutyl-1-methylxanthine; SD, Sprague-Dawley; SGLT, sodium-glucose cotransporter; STZ, streptozotocin; TS-071, (1*S*)-1,5-anhydro-1-[5-(4-ethoxybenzyl)-2-methoxy-4-methylphenyl]-1-thio-D-glucitol hydrate

Introduction

The kidney plays an important role in the regulation of plasma glucose levels by reabsorbing nearly all of the filtered load of glucose in the proximal tubule (Silverman and Turner, 1992). Glucose reuptake in the proximal tubule is mediated by sodium-glucose cotransporters (SGLTs) (Wright, 2001). The low-capacity, high-affinity sodium-glucose cotransporter,

SGLT1, is highly expressed in the gastrointestinal tract, moderately expressed in the heart and kidneys and has an important function in the intestinal absorption of glucose (Hediger and Rhoads, 1994; Zhou *et al.*, 2003). The high-capacity, low-affinity sodium-glucose cotransporter, SGLT2, is expressed exclusively in the kidney and is located primarily in the brush-border membrane of epithelial cells of the S1 segment of proximal renal tubules (Kanai *et al.*, 1994; You *et al.*, 1995). More than 90% of renal glucose reabsorption is mediated by SGLT2 in the S1 segment, whereas SGLT1 is found distally in the S3 segment and is responsible for less than 10% of renal glucose reabsorption (Hediger and Rhoads, 1994). Recently, SGLT2 deficient mice were generated and characterized. This mouse model confirmed that SGLT2 mediated glucose reabsorption in the early proximal tubule and almost all of the glucose reabsorption in the kidney (Vallon *et al.*, 2011).

Mutations in the human SGLT2 gene are associated with cases of familial renal glucosuria, a disease characterized by glucosuria in the absence of hyperglycaemia and generalized signs of proximal tubular dysfunction. Except for glucose excretion into urine, no other complications are associated with this disease (Santer *et al.*, 2003). Mutations in the SGLT1 gene are associated with glucose–galactose malabsorption syndrome in the gut with little or no glucosuria (Wright *et al.*, 2002). These findings suggest that SGLT2 is the major transporter responsible for glucose reuptake in the kidney, while SGLT1 plays only a limited role in this response. First, a pharmacological approach was taken to explore the role of SGLT in glucose reabsorption using phlorizin. Previous studies have indicated that administering a non-selective SGLT inhibitor increases the excretion of glucose and lowers plasma glucose levels in diabetic animals (Ehrenkranz *et al.*, 2005). However, phlorizin is not orally active due to hydrolysis by β-glucosidase in the intestine (Oku *et al.*, 1999). These results suggest that inhibition of SGLT2 may be a therapeutic approach to treating diabetes mellitus by increasing the loss of filtered glucose and could also potentially improve insulin resistance through weight loss, consequent on the loss of excess calories.

Several analogues of phlorizin have been reported to be SGLT2 inhibitors. In an earlier study, we reported a novel compound, TS-071, (1*S*)-1,5-anhydro-1-[5-(4-ethoxybenzyl)-2-methoxy-4-methylphenyl]-1-thio-D-glucitol hydrate, to be a SGLT2 inhibitor (Kakinuma *et al.*, 2010). In the present study, we have investigated the potency and selectivity of TS-071 *in vitro*. We have also explored the effects of TS-071 on the excretion of glucose and on plasma glucose levels in rodents and dogs and in animal models of type 1 and 2 diabetes.

Methods

Preparation of SGLT1 and SGLT2 expressing cell lines

The cDNAs encoding for human SGLT1 (hSGLT1) and human SGLT2 (hSGLT2) were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from the poly $(A)^+$ RNA isolated from human small intestine and kidney, respectively, using primers designed from published sequences (GenBank accession numbers NM_000343 and NM_003041). The cDNA fragments for hSGLT1 and hSGLT2 were cloned into the *Hin*dIII/*Xho*I site of the pCMV-tag5A vector (Stratagene, La Jolla, CA) and into the *Eco*RI/*Xho*I site of the pCAG-Neo^{r}2 vector respectively. The pCAG-Neo^{r}2 mammalian cell stable expression vector was created from the pCAGGS vector for the promoter (Niwa *et al.*, 1991), and from the pCMV-Script vector (Stratagene) for the backbone carrying the neomycin resistance gene. Briefly, the CAG promoter and the expression unit were excised from the *Sal*I/*Hin*dIII site of the pCAGGS vector; and the region including the neomycin/kanamycin resistance gene of pCMV-Script (location 1195–4278 in GenBank accession number AF028239) was amplified by PCR. The resulting two DNA fragments were ligated. To this vector, designated pCAG-Neo^r, a multicloning site containing *Eco*RI, *Nhe*I, *Xho*I, *Kpn*I, *Mlu*I and *Not*I sites was added to the *Eco*RI sites, and the 3′-terminal *Eco*RI site was changed to a *Not*I site. This yielded the pCAG-Neo^{r}2 vector.

Each plasmid vector was transfected into Chinese hamster ovary-K1 (CHO-K1) cells using Lipofectamine 2000 or Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. hSGLT1 or hSGLT2 expressing clones were selected on the basis of neomycin resistance by Geneticin (Invitrogen), and stable cell lines expressing hSGLT1 or hSGLT2 were established. The highest mRNA expression of hSGLT1 or hSGLT2 in each stable cell lines was detected using RT-PCR with sequence-specific primers. The functional expression of hSGLTs was confirmed by measuring SGLT activity in a [^{14}C]methyl-α-D-glucopyranoside (α-MG) uptake assay (see below for details). Established stable cell lines expressing hSGLT1 or hSGLT2 showed transport activity of 3.80 or 5.26 pmol min^{-1} per well respectively, as compared with vector-transfected cell lines (0.00044 pmol min^{-1} per well). The hSGLT1 or hSGLT2-overexpressing cell lines were maintained in F-10 Nutrient Mixture (Invitrogen) containing 10% fetal bovine serum (FBS) and 500 or 250 $\mu g \cdot mL^{-1}$ Geneticin in a humidified 5% CO_2 and 95% air atmosphere at 37°C.

SGLT1 and SGLT2 assays

For SGLT2, the cells were seeded in a 96-well plate at 1.5×10^4 cells per well in F-10 Nutrient Mixture containing 10% FBS. A [^{14}C]α-MG uptake assay was carried out using the cells 2 days after seeding. The cells were incubated in a buffer (0.14 $mol \cdot L^{-1}$ choline chloride, 2 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ $CaCl_2$, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ HEPES, 5 $mmol \cdot L^{-1}$ Tris, pH 7.2–7.4) at 37°C for 20 min. Then, the cells were incubated in uptake buffer [Na+] (composition; 0.14 $mol \cdot L^{-1}$ NaCl, 2 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ $CaCl_2$, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ HEPES, 5 $mmol \cdot L^{-1}$ Tris, pH 7.2–7.4) containing 1 $mmol \cdot L^{-1}$ α-MG substrate (120 $KBq \cdot mL^{-1}$ [^{14}C]α-MG) and various concentrations of the test agent, or vehicle (DMSO) at 37°C for 60 min in triplicate. The uptake reaction was terminated by washing the cells twice with the pre-treatment buffer containing 10 $mmol \cdot L^{-1}$ α-MG, and then the cells were then lysed in 0.25 $mol \cdot L^{-1}$ NaOH. Radioactivity was measured using a liquid scintillation counter. Sodium-independent uptake was measured in a sodium-free uptake buffer [Na^-] (composition; 0.14 $mol \cdot L^{-1}$ choline chloride, 2 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ $CaCl_2$, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ HEPES, 5 $mmol \cdot L^{-1}$ Tris, pH 7.2–7.4) containing 1 $mmol \cdot L^{-1}$ α-MG

substrate (120 KBq·mL^{-1} [^{14}C]α-MG) and DMSO as the vehicle. The sodium-dependent α-MG uptake was calculated by subtraction of the sodium-independent uptake count from each count measured in the presence of sodium in the buffer. The amount of α-MG uptake was calculated from a standard curve of radioactivities in [^{14}C]α-MG diluents. For SGLT1, the cells were seeded in a 96-well plate at 2×10^4 cells per well, 2 days before the [^{14}C]α-MG uptake assay. The concentration of the α-MG substrate was 100 µmol·L^{-1} (59.6 KBq·mL^{-1} [^{14}C]α-MG and α-MG). The uptake assay was performed for 30 min in triplicate.

2-deoxy-D-glucose (2-DG) uptake assays in 3T3-L1 adipocytes

3T3-L1 fibroblast cells (mouse embryo, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (D-MEM) containing 10% FBS in humidified 5% CO_2 and 95% air at 37°C. The cells were seeded in a 24-well collagen-coated plate at 1×10^5 cells per well in the growth medium (D-MEM containing 10% FBS) for 5 days. For the differentiation of 3T3-L1 cells to adipocytes, the cells were stimulated with D-MEM containing 10% FBS, 0.5 mmol·L^{-1} 3-isobutyl-1-methylxanthine (IBMX, Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 µmol·L^{-1} dexamethasone and 10 µg·mL^{-1} insulin for 2 or 3 days, and the medium was then replaced with the growth medium. The culture was continued for 8–9 days after the start of stimulation. The [^{14}C]2-DG uptake assay was carried out using the cells that had differentiated into adipocytes. The 3T3-L1 adipocytes were pre-incubated in treatment buffer (composition; 0.14 mol·L^{-1} NaCl, 5 mmol·L^{-1} KCl, 1 mmol·L^{-1} $CaCl_2$, 2.5 mmol·L^{-1} $MgCl_2$, 20 mmol·L^{-1} HEPES, pH 7.5) at 37°C for 4 h and further incubated in the treatment buffer with 100 nmol·L^{-1} insulin or without insulin for 30 min. Glucose uptake was initiated by the addition of 50 µmol·L^{-1} 2-DG substrate containing 37.4 KBq·mL^{-1} [^{14}C]2-DG and test compound or DMSO as the vehicle into each well. The cells were incubated at 37°C for 30 min, washed four times with ice-cold PBS and lysed in 0.5 mol·L^{-1} NaOH. The radioactivity was measured using a liquid scintillation counter. The uptake assays were conducted in triplicate. Percent inhibition in the presence or absence of insulin was calculated using the counts measured at each dose of the test compound and the vehicle control.

Animals

All animal care and study protocols were approved by the Ethics Review Committee for Animal Experimentation of the Taisho Pharmaceutical Animal Care Committee. Male Sprague-Dawley (SD), Zucker-*fa/fa* (Zucker fatty) and fa/+ or +/+ (Zucker lean) rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). Male BKS.Cg-+Lepr^{db}/ + Lepr^{db}/Jcl (*db/db*, former name: C57BL/KSJ-lepr^{db}/lepr^{db}) and BKS.Cg-m + / + Lepr^{db}/Jcl (*db/m*, former name: C57BL/KSJ-lepr^{db}/ + m) mice were purchased from CLEA Japan (Tokyo, Japan). Male beagle dogs were purchased from LSG Corporation (Tokyo, Japan). The rats and mice were allowed *ad libitum* access to food (laboratory chow diet, MF pellet; Oriental Yeast Co., Ltd., Tokyo, Japan) and water unless otherwise stated. The dogs were housed in individual cages and allowed once daily food (laboratory chow diet, DS-A; Oriental Yeast Co., Ltd.) and *ad libitum* access to water unless otherwise stated. Animal rooms were maintained at 23 ± 5°C, 50 ± 20% humidity and a 12 h light/dark cycle with lights on at 7:15 a.m.

Urinary glucose excretion, volume and electrolytes in rats

Male Zucker fatty rats (11 weeks of age) were fasted overnight for 17 h. The test compound was suspended in an aqueous 0.5% solution of carboxymethyl cellulose sodium salt (CMC-Na). The rats (mean body weight, approximately 440 g) were given vehicle or test compounds orally at doses of 0.1–3 mg·kg^{-1} (5 mL·kg^{-1} dosing volume) and placed in metabolic cages. Thirty minutes after administration, the rats were orally dosed with an aqueous 40% solution of glucose (2 g/5 mL·kg^{-1}). Urine was collected for 24 h after the test compound dosing, and urinary volume was then measured. The urinary glucose concentration was measured using a commercial assay kit, Glucose C2 Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and urinary glucose excretion were calculated (0–8 h, 8–24 h and 0–24 h) over 8 h intervals. Urinary electrolytes were measured using Model 7060 Automatic Analyzer (Hitachi, Ltd, Tokyo, Japan). Urine osmolality was measured using Model 3900 Multi-Sample Osmometer (Advanced Instruments, Inc., Norwood, MA, USA).

Urinary glucose excretion and volume in dogs

Male beagle dogs (7–12 months of age) were fasted for about 21 h. The dogs (body weight, 8.1–9.6 kg) were then given various doses of vehicle or test compounds orally at doses of 0.003–1 mg·kg^{-1} (5 mL·kg^{-1} dosing volume) and placed in metabolic cages. One hour after treatment, the dogs were orally dosed with an aqueous 50% solution of glucose (2 g/4 mL·kg^{-1}). Urine was collected for 24 h after dosing of the test compound, and urinary volume was then measured. The amounts of urinary glucose excretion (0–24 h) were measured.

Oral glucose tolerance test (OGTT) in rats

Male Zucker fatty rats and Zucker lean rats (10 weeks of age) were fasted overnight (17 h). The rats were given vehicle or test compounds orally at doses of 0.1–3 mg·kg^{-1}. Thirty minutes after administration, the rats were orally dosed with an aqueous 40% solution of glucose (2 g/5 mL·kg^{-1}). Blood was collected in heparinized tubes from the orbital venous sinus under ether anaesthesia at 15, 30, 60, 90 and 120 min after glucose administration. Plasma glucose concentration was measured using a commercial assay kit, as described above, and the difference from baseline plasma glucose levels over the study time was calculated for the delta area under the curve (ΔAUC). Plasma insulin level was measured using a commercial enzyme-linked immunosorbent assay kit (Shibayagi Co., Ltd., Gunma, Japan).

Glucose lowering effect in diabetic rats and mice

Male SD rats (7 week old) that were fasted overnight (17 h) were injected with streptozotocin (STZ; 50 mg·mL^{-1}·kg^{-1}, i.v.) via the tail vein to induce hyperglycaemia. The diabetic rats were used to examine glucose lowering effects 1 week after

STZ injection. Non-fasted rats were orally administered vehicle or test compounds at doses of 0.1–3 mg·kg^{-1}. Blood was collected in heparinized tubes from the orbital venous sinus under ether anaesthesia at 0.5, 1, 2, 4, 6, 8 and 24 h after test compound administration. Non-fasted male *db/db* mice (7 weeks of age) were orally administered vehicle or test compounds at doses of 0.1–3 mg·kg^{-1}. Blood was collected in heparinized tubes from the orbital venous sinus under ether anaesthesia at 0.5, 1, 2, 4, 6, 8 and 24 h after test compound administration. Plasma glucose concentration was measured, and the area under the curve (AUC) was calculated.

Effects on normoglycaemia in rats

Male SD rats (8 weeks of age) that were fasted overnight (17 h) or non-fasted rats were orally administered vehicle or test compounds at doses of 0.3–3 mg·kg^{-1}. Blood was collected in heparinized tubes from the orbital venous sinus under ether anaesthesia at 0.5, 1, 2, 4, 8 and 24 h after test compound administration. Plasma glucose concentration was measured at each time point.

Caco-2 monolayer permeability

Caco-2 cells were obtained from the American Type Culture Collection. The cells (passage number 50 to 60) were seeded on 24-well cell culture inserts (Transwell™, 0.3 μm pores, 6.5 mm id) and then cultured for 21 days. Hank's balanced Salt Solution (HBSS, KAC, Kyoto, Japan) was used in all experiments after adjusting the pH to 6.5 for the apical side and the pH 7.4 for the basolateral side. TS-071 was dissolved in HBSS (pH 6.5) at a final concentration of 10 μmol·L^{-1}. Samples (50 μL) were taken from the basolateral side at 30, 60, 90 and 120 min after dose administration. The volume of basolateral side was maintained by adding HBSS (pH 7.4). All experiments were performed in triplicate at 37°C. Concentrations of TS-071 were determined by LC-MS/MS methods (API3000, AB SCIEX, Foster City, CA, USA).

Metabolism of TS-071 by hepatocytes

TS-071 (5 μmol·L^{-1}) was incubated with 1 × 10^6 human cryopreserved hepatocytes (Product No. HMHP-F, In Vitro Technologies, Baltimore, MD, USA) in HBSS at 37°C for 240 min. The reactions were quenched by the addition of DMSO, and TS-071 concentration in the supernatant was analysed by LC-MS/MS (API3000, AB SCIEX).

Pharmacokinetics

Male SD rats (8 week old) and male beagle dogs (7–8 month old, Japan Laboratory Animals, Tokyo, Japan) were used in the experiments. The animals were fasted overnight (about 17 h). The animals were allowed free access to the drinking water. The plasma samples obtained after i.v. or p.o. administration of TS-071 were extracted using an SPE column (OASIS HLB, Waters, Milford, MA, USA) cartridge. Concentration of TS-071 was determined by LC-MS/MS (API4000, AB SCIEX).

Statistical analysis

Data are presented as mean ± standard error. The statistical analysis was performed using Microsoft Excel 2000 and 2003 (Microsoft Japan Co. Ltd., Tokyo, Japan) and SAS System Version 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). The statistical significance of difference was evaluated by *t*-test when comparing two groups and by Dunnett's test for multiple-group comparison. To evaluate effects on normoglycaemia in rats, two-way repeated measures ANOVA was used. A value of $P < 0.05$ was considered to be significant.

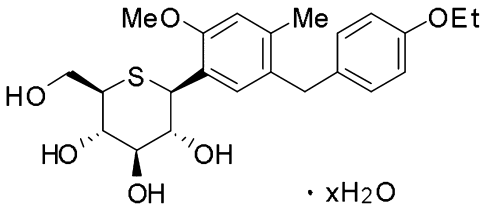

Figure 1
Chemical structure of TS-071.

Materials

TS-071, (1*S*)-1,5-anhydro-1-[5-(4-ethoxybenzyl)-2-methoxy-4-methylphenyl]-1-thio-D-glucitol hydrate (Figure 1), was synthesized by Medicinal Chemistry Laboratories, Taisho Pharmaceutical Co., Ltd. (Saitama, Japan) (Kakinuma *et al.*, 2010). Methyl-α-D-[U-^{14}C] glucopyranoside ([^{14}C]α-MG) and 2-deoxy-D-[U-^{14}C]glucose ([^{14}C]2-DG) were purchased from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, UK). Methyl-α-D-glucopyranoside (α-MG), 2-deoxy-D-glucose (2-DG), phlorizin, cytochalasin B, streptozotocin (STZ) and glibenclamide were purchased from Sigma-Aldrich Co. (St. Louis, MO). The TS-071 used in these studies was a hydrate that contained 3.18–3.96% water. A correction was made for the water content and the doses reported for anhydrous TS-071. Drug and molecular target nomenclature follows Alexander *et al.*, (2009)

Results

Inhibitory effects on human SGLTs

The inhibitory effects of TS-071 and phlorizin, a non-selective SGLT inhibitor on SGLT activity in CHO cells expressing hSGLT1 or hSGLT2 are shown in Figure 2. These compounds inhibited α-MG uptake in a concentration-dependent manner.

Effects on 2-DG uptake

The effects of TS-071 on insulin-dependent and insulin-independent 2-DG uptake in 3T3-L1 adipocytes are shown in Table 1. TS-071 at a concentration of 100 μmol·L^{-1} reduced 2-DG uptake in the presence and absence of insulin with 43.3% and 41.1% inhibition respectively. Very little inhibition was seen at a concentration of 10 μmol·L^{-1}. Cytochalasin B at 10 μmol·L^{-1} as a positive control strongly inhibited 2-DG uptake in 3T3-L1 adipocytes.

Effects of TS-071 on urinary glucose excretion, volume, electrolytes and urine osmolality in rats

The effects of TS-071 on urinary glucose excretion (mg/24 h) are shown in Table 2. The urinary glucose output after oral

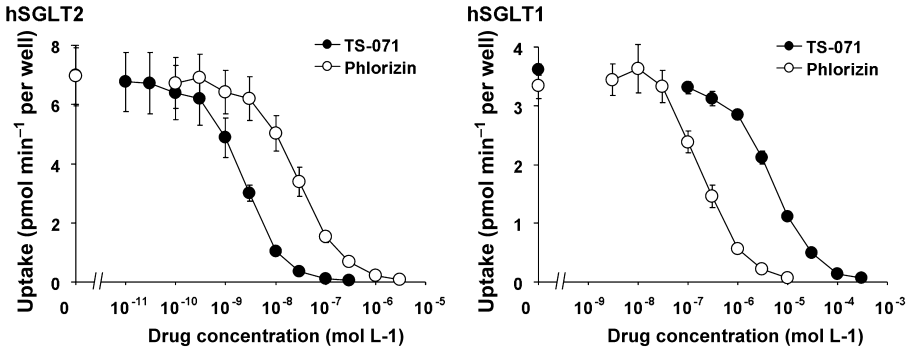

Figure 2

Inhibitory effects of TS-071 and phlorizin on hSGLT1 and hSGLT2. Each value shown represents the mean ± SEM of the sodium-dependent α-MG uptake (pmol min^{-1} per well) from 3-4 experiments.

Table 1

Effects of TS-071 on 2-DG uptake in 3T3-L1 adipocytes

	Concentration ($\mu mol \cdot L^{-1}$)	Inhibition (%) Insulin (+)	Insulin (–)
TS-071	1	2.66 ± 0.52	3.25 ± 0.84
	10	10.4 ± 1.55	11.1 ± 0.70
	100	43.3 ± 0.26	41.1 ± 1.44
Cytochalasin B	10	96.3 ± 0.14	93.3 ± 0.32

Data represents the mean ± SEM ($n = 4$).

Table 2

Effects of TS-071 on urinary glucose excretion after oral glucose loading in Zucker fatty rats

	Dose (mg kg^{-1})	Urinary glucose output (mg) 0–8 h	8–24 h	0–24 h
Vehicle	–	0.68 ± 0.10	0.52 ± 0.08	1.20 ± 0.11
TS-071	0.1	0.95 ± 0.23	0.89 ± 0.22	1.84 ± 0.26
	0.3	94.26 ± 23.05	1.81 ± 0.38	96.07 ± 23.07***
	1	272.72 ± 47.23	95.06 ± 44.31	367.79 ± 25.63***
	3	580.69 ± 27.42	493.10 ± 75.54	1073.79 ± 80.00***

Data represents the mean ± SEM ($n = 8$). The statistical analysis was performed on the natural logarithm of urinary glucose output (mg per 24 h). Significance level was set with Bonferroni's correction.
***$P < 0.001/4$ significantly different from vehicle group (Welch's t-test).

administration of TS-071 increased remarkably in Zucker fatty rats, a model of type 2 diabetes. Most of the glucose was excreted during the first 8 h after administration of TS-071. TS-071 (0.3 – 3.0 $mg \cdot kg^{-1}$) significantly increased the urinary glucose excretion in Zucker fatty rats. In these experiments, slight increases in urinary volume and potassium excretion (Table 3) were observed at the highest dose (3 $mg \cdot kg^{-1}$); however, no significant changes were observed at either 0.3 or 1 $mg \cdot kg^{-1}$ of TS-071. Neither urinary excretion of sodium and chloride nor urine osmolality was affected by TS-071 treatment.

Effects of TS-071 on urinary glucose excretion and volume in dogs

The urinary glucose output in dogs is shown in Table 4. A small amount of urinary glucose output was observed after oral glucose loading in dogs treated with vehicle. TS-071 at

Table 3

Effects of TS-071 on urinary volume, electrolytes and urine osmolality after oral glucose loading in Zucker fatty rats

	Dose (mg kg^{-1})	Urinary volume (mL per 24 h)	Urinary sodium (mmol per 24 h)	Urinary potassium (mmol per 24 h)	Urinary chloride (mmol per 24 h)	Urine osmolality (mOsm kg^{-1}·H_2O)
Vehicle	–	6.7 ± 0.6	0.21 ± 0.03	0.49 ± 0.04	0.12 ± 0.02	972 ± 64
TS-071	0.1	7.8 ± 0.5	0.29 ± 0.07	0.50 ± 0.06	0.16 ± 0.03	873 ± 74
	0.3	7.5 ± 0.5	0.28 ± 0.04	0.51 ± 0.03	0.17 ± 0.02	990 ± 50
	1	7.5 ± 0.9	0.24 ± 0.05	0.47 ± 0.05	0.12 ± 0.02	1130 ± 82
	3	12.2 ± 0.4***	0.38 ± 0.07	0.68 ± 0.05*	0.31 ± 0.08	1168 ± 63

Data represents the mean ± SEM (n = 8).
*P < 0.05, ***P < 0.001 significantly different from vehicle group (Dunnett's test).

Table 4

Effects of TS-071 on urinary glucose excretion and volume after oral glucose loading in dogs

	Dose (mg kg^{-1})	Urinary glucose output (mg per 24 h)	Urinary volume (mL per 24 h)
(A)			
Vehicle	–	3.05 ± 0.49	138.8 ± 17.0
TS-071	0.003	3.28 ± 0.62	154.1 ± 22.6
	0.01	5.07 ± 1.69	123.2 ± 14.2
	0.03	325.29 ± 72.46***	140.3 ± 12.9
(B)			
Vehicle	–	7.64 ± 1.95	191.4 ± 26.9
TS-071	0.1	5599.12 ± 478.94***	187.7 ± 14.3
	0.3	13287.10 ± 691.19***	238.4 ± 20.5
	1	20129.68 ± 1870.83***	374.3 ± 33.0***

Data represents the mean ± SEM (A: n = 9, B: n = 10). The statistical analysis was performed on the natural logarithm of urinary glucose output (mg per 24 h), or urinary volume (mL per 24 h).
***P < 0.0001 significantly different from vehicle group (two-way Dunnett-type test).

0.03 mg·kg^{-1} or more significantly increased urinary glucose excretion. The urinary glucose output (mg/24 h) after oral administration of TS-071 increased markedly at 1 mg·kg^{-1}, showing a 2635-fold increase. Urinary volume was increased only at the highest dose (1 mg·kg^{-1}).

Effects of TS-071 on oral glucose tolerance test in type 2 diabetic rats

In Zucker fatty rats, impaired glucose tolerance was ameliorated by oral administration of TS-071 (Figure 3A, B). The Δglucose $AUC_{0-120\,min}$ values were significantly lower in the TS-071 group at a dose of 0.3, 1 or 3 mg·kg^{-1} than in the vehicle group. At the same time, the increases in plasma insulin levels after glucose loading were suppressed by TS-071 treatment (Figure 3C, D).

Glucose lowering effects in STZ-induced diabetic rats and type 2 diabetic db/db mice

The intravenous injection of 50 mg·kg^{-1} STZ in SD rats induced hyperglycaemia, which averaged approximately 22 mmol·L^{-1}. Oral administration of TS-071 reduced plasma glucose levels (Figure 4). TS-071 significantly reduced plasma glucose levels in STZ-induced diabetic rats at a dose of 0.3, 1 or 3 mg·kg^{-1}. In *db/db* mice, a model of type 2 diabetes, TS-071 significantly reduced plasma glucose levels at a dose of 0.3, 1 and 3 mg·kg^{-1} (Figure 5).

Effects of TS-071 on plasma glucose levels in non-diabetic rats

These results are presented in Figure 6. Oral administration of TS-071 reduced plasma glucose levels in non-fasted SD rats, but the changes were slight, transient and within the normal range of plasma glucose levels. In rats fasted for about 17 h, the oral administration of TS-071 reduced plasma glucose levels from 1 to 4 h after an oral dose of 3 mg·kg^{-1}. In these studies, glibenclamide at 10 mg·kg^{-1} also reduced non-fasting and fasting plasma glucose levels. These data suggested that TS-071 might affect normoglycaemia under severe fasting conditions at high doses within the pharmacological dosing range.

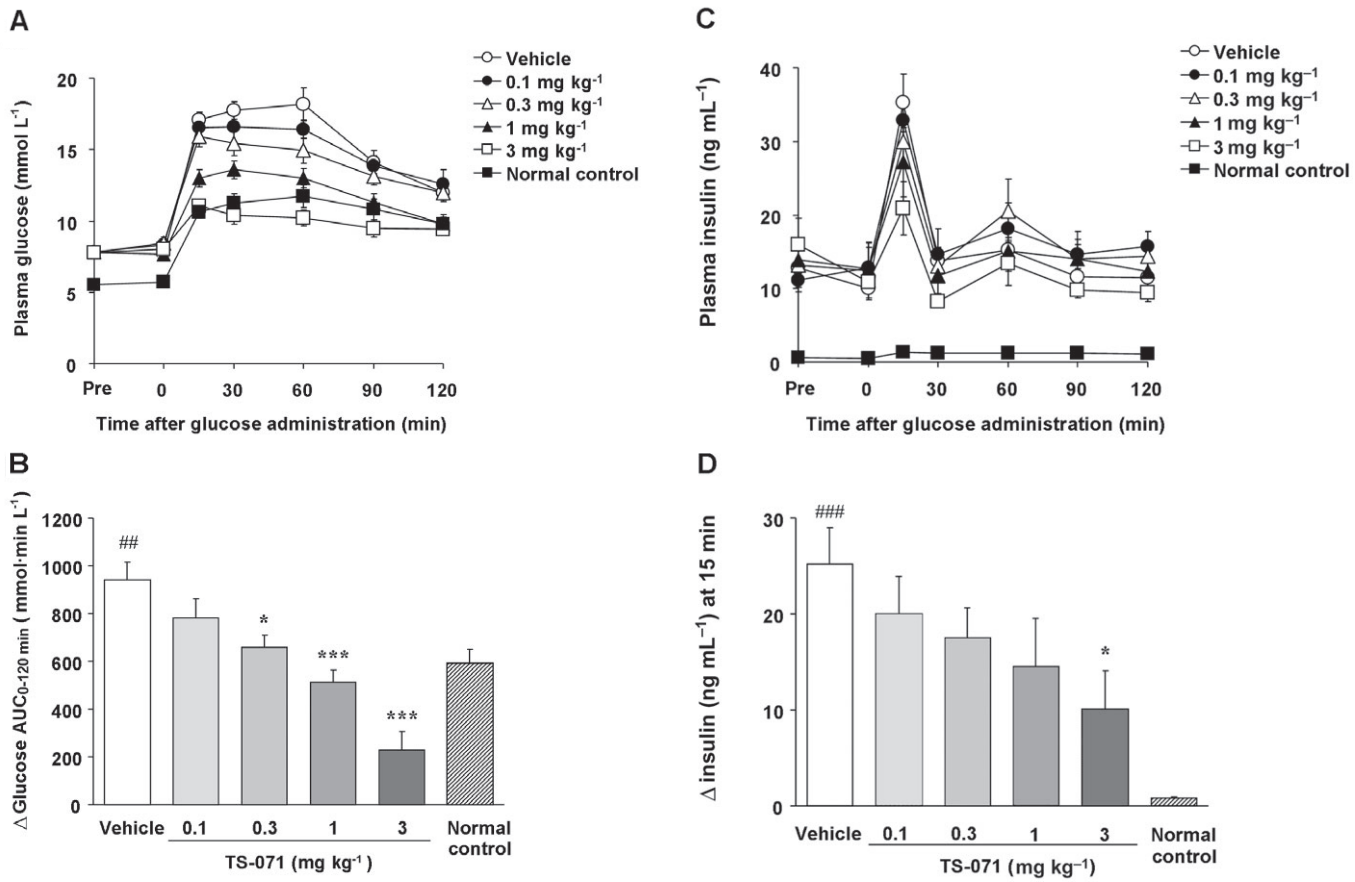

Figure 3

Effects of TS-071 on plasma glucose and insulin levels after oral glucose loading in Zucker fatty rats. (A) Each value shown represents the mean ± SEM of plasma glucose levels (mmol·L^{-1}) (n = 8). (B) Each value shown represents the mean ± SEM of Δglucose $AUC_{0-120\ min}$ (mmol·min L^{-1}). $^{\#\#}P < 0.01$ significantly different from normal control (Student's *t*-test). $^{*}P < 0.05$, $^{***}P < 0.001$ significantly different from vehicle group (Dunnett's test). (C) Each value shown represents the mean ± SEM of plasma insulin levels (ng·mL^{-1}) ($n = 8$). (D) Each value shown represents the mean ± SEM of changes in insulin levels (ng·mL^{-1}) from 0 to 15 min. $^{\#\#\#}P < 0.001$ significantly different from normal control (Welch's *t*-test). $^{*}P < 0.05$ significantly different from vehicle group (Dunnett's test). Zucker lean rats were used as normal control group.

Pharmacokinetics

The permeability of TS-071, i.e., passage through the cell monolayer of Caco-2 cells, was assessed and the intrinsic clearance (CL_{int}) was evaluated by an assay using human cryo-preserved hepatocytes. Results are shown in Table 5.

The pharmacokinetic profiles of TS-071 were estimated after a single intravenous or an oral administration at 1 mg·kg^{-1} to rats and dogs (Table 5). After oral administration, the C_{max} and the $AUC_{0-\infty}$ were measured in dogs and in rats and are shown in Table 5. After intravenous administration, the volume of distribution (*V*dss) in dogs and rats were also measured (Table 5).

Discussion and conclusions

Recently, SGLT2 has been recognized as an attractive target for a new class of anti-diabetic agents. Inhibition of SGLT2 promotes glucose excretion by inhibiting reuptake of filtered glucose in the proximal tubules and lowers plasma glucose levels. According to this mechanism, SGLT2 inhibitors would be expected to have advantages over current therapies. The sulphonylureas are associated with hypoglycaemia, weight gain and β-cell exhaustion resulting from excessive insulin secretion. The use of thiazolidinediones has been associated with weight gain and oedema (Inzucchi, 2002). The reduction of plasma glucose by SGLT2 inhibition is not dependent on insulin secretion from the pancreas. Therefore, SGLT2 inhibitors may carry a lower risk of hypoglycaemia as they may lead to better preservation of β-cell function. Furthermore, total calorie loss through urinary glucose excretion may not cause weight gain and may even achieve weight loss.

Phlorizin was found to be a SGLT inhibitor and has been used to evaluate the role of SGLT in glucose metabolism (Ehrenkranz *et al.*, 2005). However, phlorizin was unsuitable as an oral anti-diabetic drug because it is a non-selective inhibitor of both SGLT1 and SGLT2. It also has low bioavailability following oral administration (Oku *et al.*, 1999). Subsequently, selective SGLT2 inhibitors, T-1095, sergliflozin and remogliflozin, which were orally active pro-drugs, were developed (Oku *et al.*, 1999; Katsuno *et al.*, 2007; Fujimori *et al.*,

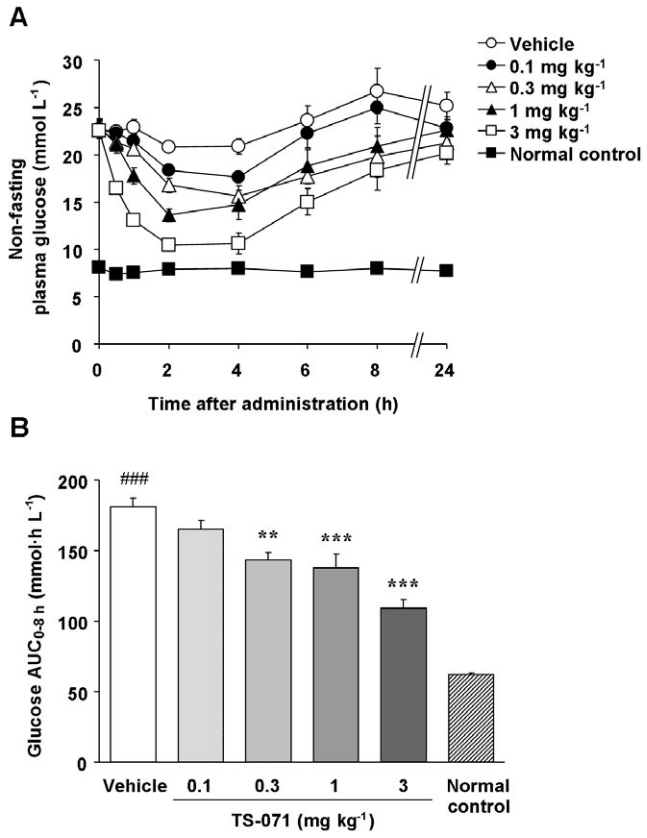

Figure 4

Effects of TS-071 on non-fasting plasma glucose levels in STZ-induced diabetic rats. (A) Each value shown represents the mean ± SEM of plasma glucose levels (mmol·L^{-1}) (n = 8). (B) Each value shown represents the mean ± SEM of glucose $AUC_{0-8\ h}$ (mmol·h L^{-1}). $^{###}P < 0.001$ significantly different from normal control (Welch's t-test). $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different from vehicle group (Dunnett's test).

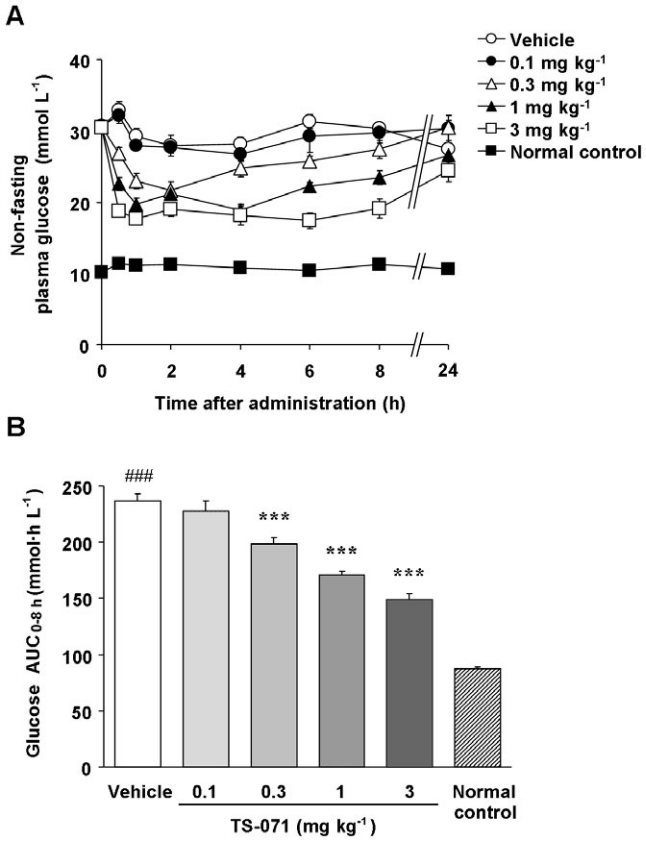

Figure 5

Effects of TS-071 on non-fasting plasma glucose levels in *db/db* mice. (A) Each value shown represents the mean ± SEM of plasma glucose levels (mmol·L^{-1}) (n = 8). (B) Each value shown represents the mean ± SEM of glucose $AUC_{0-8\ h}$ (mmol·h L^{-1}). $^{###}P < 0.001$ significantly different from normal control (Welch's t-test). $^{***}P < 0.001$ significantly different from vehicle group (Dunnett's test). The *db/m* mice were used as normal control group.

2008). Dapagliflozin, a form active by itself, was reported to be an orally active and selective SGLT2 inhibitor (Han *et al.*, 2008).

We focused on developing a novel orally active and selective inhibitor for the SGLT2 molecule. As a consequence of screening a large number of compounds using an hSGLT2-expressing cell system, we discovered that TS-071 was a potent SGLT2 inhibitor with an IC_{50} value of 2.26 nmol L^{-1} and exhibiting a 1765-fold selectivity for inhibition of hSGLT2 over hSGLT1 (IC_{50} value: 3990 nmol L^{-1} for SGLT1; Kakinuma *et al.*, 2010). Also, TS-071 was 12-fold more potent than phlorizin (IC_{50} value: 27.8 nmol L^{-1}; Kakinuma *et al.*, 2010) in blocking hSGLT2 activity. In comparison, dapagliflozin has been reported to be a potent and highly selective SGLT2 inhibitor with 1242-fold selectivity among several hSGLT2 inhibitors examined by pharmaceutical companies (Han *et al.*, 2008). These results indicate that TS-071 has higher selectivity for SGLT2 than dapagliflozin. To assess the selectivity for other glucose transporters, effects on glucose transport activity using 3T3-L1 adipocytes were examined. Glucose uptake is dependent on the activity of glucose transporters (GLUTs) and regulates the homeostasis of blood glucose levels and cellular glucose metabolism. GLUT1 is ubiquitously expressed and provides basal glucose uptake. Furthermore, GLUT4 is expressed in insulin-sensitive tissues in which glucose uptake is dependent on insulin stimulation (Olson and Pessin, 1996). In 3T3-L1 adipocytes, insulin-stimulated glucose uptake is mediated by GLUT4 activity and basal glucose uptake is mainly mediated by GLUT1 (Calderhead *et al.*, 1990). TS-071 inhibited both insulin-dependent and -independent glucose uptake by about 40% inhibition at a very high concentration (100 μmol·L^{-1}). It had no effect at concentration less than 10 μmol·L^{-1}. These results indicate that TS-071 is a selective SGLT2 inhibitor with a 50 000-fold greater selectivity for SGLT2 over the GLUTs. Furthermore, in the *in vitro* radioligand binding assays to various receptors, ion channels and transporters (in our previous studies), TS-071 at 100 μmol·L^{-1} exhibited 67% inhibition against only Na^+ channel site 2 (reference compound; Ref., dibucaine), but less than 35% inhibition for various other ligands, and had less than 18% inhibition at 10 μmol·L^{-1} [adenosine A_1 receptor (Ref., DPCPX; 8-cyclopentyl-1,3-dipropylxanthine),

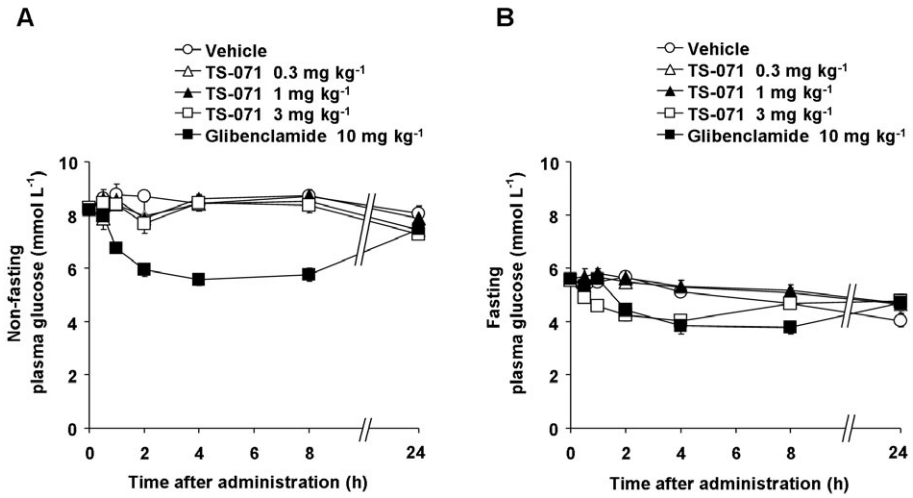

Figure 6

Effects of TS-071 on plasma glucose levels in normal rats. (A) Each value shown represents the mean ± SEM of non-fasting plasma glucose levels (mmol·L^{-1}) (n = 8). Vehicle group compared with TS-071 groups: Significant effect in two-way repeated measures ANOVA (main effect of dose: P = 0.0238, main effect of time: P < 0.0001, interaction effect: P = 0.8442). Vehicle group compared with glibenclamide group: Significant effect in two-way repeated measures ANOVA (main effect of dose: P < 0.0001, main effect of time: P < 0.0001, interaction effect: P < 0.0001). (B) Each value shown represents the mean ± SEM of fasting plasma glucose levels (mmol·L^{-1}) (n = 8). Vehicle group compared with TS-071 groups: Significant effect in two-way repeated measures ANOVA (main effect of dose: P = 0.0021, main effect of time: P < 0.0001, interaction effect: P < 0.0001). Vehicle group compared with glibenclamide group: Significant effect in two-way repeated measures ANOVA (main effect of dose: P = 0.0766, main effect of time: P < 0.0001, interaction effect: P < 0.0001).

Table 5

Pharmacokinetic parameters of TS-071

In vitro	
Permeability[1]	14×10^6 cm·s^{-1}
Intrinsic clearance (CL_{int})[2]	0.64 mL·min^{-1}·kg^{-1}
In vivo	
Rats (1 mg·kg^{-1})	
T_{max}	0.33 ± 0.14 h
C_{max}	87.8 ± 31.5 ng·mL^{-1}
$AUC_{0-\infty}$	180 ± 34.3 ng·h·mL^{-1}
Vdss	2.6 ± 0.6 L·kg^{-1}
Dogs (1 mg·kg^{-1})	
T_{max}	0.67 ± 0.29 h
C_{max}	914 ± 73.4 ng·mL^{-1}
$AUC_{0-\infty}$	4880 ± 654 ng·h·mL^{-1}
Vdss	0.8 ± 0.06 L·kg^{-1}

Data shown are mean± SEM (n = 3 in rats, n = 3 in dogs).
[1]Permeability was evaluated by Caco-2 cell assay.
[2]CL_{int} was evaluated by human cryo-preserved hepatocyte assay.

angiotensin AT_1 receptor (Ref., angiotensin II), bradykinin B_2 receptor (Ref., HOE140), mineralcorticoid receptor (Ref., aldosterone), vasopressin V_2 receptor (Ref., Arg^8-vasopressin), Ca^{2+} channel type L dihydropyridine (Ref., nitrendipine), Cl^- channel (Ref., picrotoxin), K^+ channel K_A (Ref., dendrotoxin), K^+ channel K_{ATP} (Ref., glibenclamide), adenosine transporter (Ref., nitrobenzylthioinosine), dopamine transporter (Ref., GBR12909), noradrenaline transporter (Ref., desipramine)]. As a result of these studies, TS-071 was confirmed as a highly selective SGLT2 inhibitor.

To examine the efficacy of TS-071 on type 2 diabetes, we gave it orally to Zucker fatty rats and *db/db* mice. Doses of TS-071 of 0.3 mg·kg^{-1} or more enhanced urinary glucose excretion and suppressed postprandial hyperglycaemia in Zucker fatty rats. It also reduced non-fasting high glucose levels in *db/db* mice. These data confirmed the glucose lowering effects of TS-071 to be dependent on urinary glucose excretion mediated through SGLT2 inhibition in proximal renal tubules and its potential effects in type 2 diabetes. The urinary glucose excretion increased markedly at 1 mg·kg^{-1}, showing approximately 300-fold increase in Zucker fatty rats. Although slight increases in urinary volume and urinary potassium excretion were observed at the highest dose, urinary sodium and chloride excretion were not affected. Likewise, urine osmolality was not affected by TS-071. These results revealed that only the highest dose of TS-071 might induce a slight osmotic diuresis resulting from the excessive urinary glucose excretion. Similarly, sergliflozin, a SGLT2 inhibitor, had no almost effect on urinary electrolyte excretion (Katsuno *et al.*, 2007). In addition, SGLT2-deficient mice exhibited glucosuria and polyuria but no other changes in urinary excretion of electrolytes (Vallon *et al.*, 2011). Therefore, a marked electrolyte imbalance was not provoked by treatment with TS-071. In the OGTT, TS-071 did not stimulate plasma insulin increase but, rather, suppressed the plasma insulin secretion, corresponding to the reduction in plasma glucose levels. Thus, the action of TS-071 was independent of insulin secretion from the pancreas.

To induce hyperglycaemia, STZ (50 mg·kg^{-1}) was intravenously injected into rats (Rakieten *et al.*, 1963) and one week after STZ treatment, the mean plasma glucose level was over 22 mmol·L^{-1}. TS-071 reduced plasma glucose levels in these STZ-induced diabetic rats. These results suggested that this class of drugs may be useful in type 1 diabetes. In type 1 diabetes, there is an absolute lack of insulin secretion and insulin is, and will remain, the major therapy. The predictable benefit of SGLT2 blockade would be weight loss associated with the caloric wasting, in contrast to the weight gain observed with insulin therapy. This feature may also provide an advantage over the sulphonylureas. Thus, SGLT2 inhibitors should be useful for treating both type 1 and type 2 diabetes mellitus.

To assess the risk of hypoglycaemia with TS-071, glucose-lowering activities on both fasting and non-fasting condition in normal rats were evaluated. TS-071 caused a slight and transient reduction in plasma glucose levels in non-fasting rats and a high dose of TS-071 decreased plasma glucose levels to 4.0 mmol·L^{-1} in fasting animals. These data indicate that the risk of hypoglycaemia with TS-071 is low when plasma glucose concentration is in the normal range.

The tissue distribution of SGLT2 is limited to the kidney, and , therefore, a high selectivity for SGLT2 would clearly restrict mechanism-based side effects of SGLT2 inhibitors. This novel and unique mechanism means that SGLT2 inhibitors have potential for use in combination with other anti-diabetic agents. Combination therapy with distinct mechanisms of action will not only improve glycaemic control but should also result in lower overall dose of all agents and consequently reduced adverse effects.

To examine the efficacy of TS-071 in dogs, we gave it orally to dogs that had been orally loaded with glucose. TS-071 at 0.03 mg·kg^{-1} or more enhanced urinary glucose excretion and this increase was considerable (107–2635-fold). When dogs and rats received the same dose (1 mg·kg^{-1}) of TS-071 orally, the peak plasma level in dogs was approximately 10 times higher than that in rats. Observed minimum efficacious doses for urinary glucose excretion in dogs and rats were 0.03 and 0.3 mg·kg^{-1} respectively. These findings suggested that the efficacy of TS-071 in preclinical species depended on plasma exposure or the pharmacokinetics of TS-071. TS-071 showed very low intrinsic clearance (CL_{int}, 0.64 mL·min^{-1}·kg^{-1}), a moderate volume of distribution ($Vdss$, 0.8 L kg^{-1} in dogs and 2.6 L kg^{-1} in rats). Furthermore, TS-071 showed good permeability in Caco-2 epithelial cell monolayers greater than propranolol (used a positive high permeability control), indicating that TS-071 should have good absorption. From these results, the pharmacokinetic profile of TS-071 in humans is expected to be excellent. Therefore, TS-071 could exhibit efficacy at a relatively low dose and have a prolonged duration of action in humans. TS-071 is currently in phase II of clinical development.

In conclusion, the present study indicates that TS-071 is a potent and selective SGLT2 inhibitor and provides anti-hyperglycaemic activity that is independent of insulin secretion from the pancreas *in vivo*. The results suggest that TS-071 has some advantages over current anti-diabetic drugs with the lower risk of pancreatic β-cells exhaustion and hypoglycaemia, and has the clinical potential for the treatment of both type 1 and type 2 diabetes.

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Conflict of interest

All authors are employees of Taisho Pharmaceutical Co., Ltd.

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