

Pharmacological profile of ipragliflozin (ASP1941), a novel selective SGLT2 inhibitor, in vitro and in vivo

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Abstract The pharmacological profile of ipragliflozin (ASP1941; (1*S*)-1,5-anhydro-1-*C*-{3-[(1-benzothiophen-2-yl)methyl]-4-fluorophenyl}-*D*-glucitol compound with *L*-proline (1:1)), a novel SGLT2 selective inhibitor, was investigated. In vitro, the potency of ipragliflozin to inhibit SGLT2 and SGLT1 and stability were assessed. In vivo, the pharmacokinetic and pharmacologic profiles of ipragliflozin were investigated in normal mice, streptozotocin-induced type 1 diabetic rats, and KK-*A*^Y type 2 diabetic mice. Ipragliflozin potently and selectively inhibited human, rat, and mouse SGLT2 at nanomolar ranges and exhibited

stability against intestinal glucosidases. Ipragliflozin showed good pharmacokinetic properties following oral dosing, and dose-dependently increased urinary glucose excretion, which lasted for over 12 h in normal mice. Single administration of ipragliflozin resulted in dose-dependent and sustained antihyperglycemic effects in both diabetic models. In addition, once-daily ipragliflozin treatment over 4 weeks improved hyperglycemia with a concomitant increase in urinary glucose excretion in both diabetic models. In contrast, ipragliflozin at pharmacological doses did not affect normoglycemia, as was the case with glibenclamide, and did not influence intestinal glucose absorption and electrolyte balance. These results suggest that ipragliflozin is an orally active SGLT2 selective inhibitor that induces sustained increases in urinary glucose excretion by inhibiting renal glucose reabsorption, with subsequent antihyperglycemic effect and a low risk of hypoglycemia. Ipragliflozin has, therefore, the therapeutic potential to treat hyperglycemia in diabetes by increasing glucose excretion into urine.

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Introduction

Diabetes is a metabolic disorder that results in hyperglycemia caused by a deficiency in the secretion and/or action of insulin. According to its pathophysiology, diabetes is classified into two categories: type 1 and type 2. Impaired insulin secretion is the major cause of type 1 diabetes, while both impaired insulin secretion and insulin resistance play important roles in the pathophysiology of type 2 diabetes. Irrespective of the type of diabetes, chronic hyperglycemia

leads to glucose toxicity, which further worsens diabetic symptoms (Andrews et al. 1984; Prentki and Nolan 2006). In addition, high incidences of cardiovascular diseases and complications (e.g., nephropathy, neuropathy, and retinopathy) are associated with chronic hyperglycemia (Crofford 1995). Therefore, therapeutic strategies for diabetes are currently focused on controlling blood glucose levels.

To date, several oral drugs that enhance insulin secretion and/or improve insulin sensitivity have been developed for the treatment of diabetes. However, due to limited efficacy and adverse side effects, it is difficult to maintain good glycemic control in most diabetic patients. Therefore, additional agents, especially those that work independently of insulin secretion, are needed for the successful management of diabetes. Among these, the inhibitors of sodium–glucose cotransporter (SGLT) are an attractive option because their modes of action are connected to blood glucose reduction independently from insulin secretion (Idris and Donnelly 2009).

SGLTs exist in at least six subtypes. Among these, physiological and pathophysiological roles of two major SGLT isoforms, SGLT1 and SGLT2, are well investigated, while the roles of an additional four isoforms (SGLT3–6) are presently poorly identified (Wright and Turk 2004; Wright et al. 2007). SGLT2 is a low-affinity, high-capacity transporter that is specifically expressed in the kidneys (Wells et al. 1992; Wright 2001). Mutations in the SGLT2 gene are associated with familial renal glucosuria, a benign syndrome in which glucose excretion occurs in the absence of hyperglycemia (van den Heuvel et al. 2002); thus, SGLT2 plays a critical role in renal glucose reabsorption. In contrast, SGLT1 is a high-affinity, low-capacity transporter that is highly expressed in the small intestine (Pajor and Wright 1992). Mutations in the SGLT1 gene cause glucose/galactose malabsorption; therefore, SGLT1 plays an important role in dietary glucose absorption in the small intestine (Turk et al. 1991).

Phlorizin, a naturally occurring phenol glucoside first isolated from the bark of an apple tree, has a competitive inhibitory activity for SGLT1 and SGLT2. Early animal studies showed that administration of phlorizin induces glucosuria by inhibiting renal glucose reabsorption and ameliorates hyperglycemia in diabetic models without a risk of hypoglycemia (Rossetti et al. 1987a; Ehrenkranz et al. 2005). Phlorizin has also been found to decrease insulin resistance at the peripheral tissue level due to the improvement in hyperglycemia (Abdul-Ghani and DeFronzo 2008). Therefore, the renal glucose reabsorption system could be an important target for improving hyperglycemia in diabetes. However, phlorizin was not developed as a drug for the treatment of diabetes due to its nonselectivity and low bioavailability (Oku et al. 2000). Nonetheless, various orally active and selective SGLT2 inhibitors have been subsequently

developed (Oku et al. 1999; Han et al. 2008). One of the approaches to improve bioavailability is to replace the unstable anomeric oxygen atom of the exo-glycosidic bond by a stable carbon atom (C-glycoside), as reported by two independent institutes including us in 2001 (Washburn 2009). In recent years, it has been demonstrated that some SGLT2 inhibitors, such as dapagliflozin, improve hyperglycemia in type 2 diabetic patients (List et al. 2009).

We have identified a novel orally active and selective SGLT2 inhibitor ipragliflozin (ASP1941; (1*S*)-1,5-anhydro-1-*C*-{3-[(1-benzothiophen-2-yl)methyl]-4-fluorophenyl}-*D*-glucitol compound with *L*-proline (1:1); Fig. 1a), which is a derivative of phlorizin (Fig. 1b). In this report, we describe the *in vitro* and *in vivo* pharmacology of ipragliflozin currently under clinical investigation for use as an antidiabetic agent.

Materials and methods

Materials

Ipragliflozin, dapagliflozin (Meng et al. 2008), and YM471 ([*Z*-4'-[4,4-difluoro-5-{2-(4-dimethylaminopiperidino)-2-oxoethylidene}-2,3,4,5-tetrahydro-1*H*-1-benzazepine-1-carbonyl]-2-phenylbenzanilide monohydrochloride) (Tsukada et al. 2001) were synthesized by Astellas Pharma Inc. (Ibaraki, Japan). Phlorizin, phloretin, methyl- α -*D*-glucopyranoside (AMG), streptozotocin (STZ), glibenclamide, and furosemide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Voglibose (BASEN[®]) was purchased from Takeda Pharmaceutical Company, Ltd. (Osaka, Japan). These drugs were dissolved or suspended in 0.5% methylcellulose solution and administered orally. The doses of ipragliflozin are expressed as the free base form. ¹⁴C-AMG (11.7 GBq/mmol) was obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK).

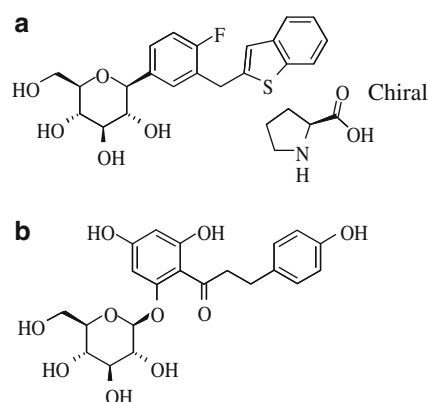


Fig. 1 Chemical structures of **a** ipragliflozin ((1*S*)-1,5-anhydro-1-*C*-{3-[(1-benzothiophen-2-yl)methyl]-4-fluorophenyl}-*D*-glucitol compound with *L*-proline (1:1)) and **b** phlorizin

Animals

Male Sprague–Dawley rats were purchased from Charles River Laboratories Japan (Kanagawa, Japan) at age 5–7 weeks. STZ was dissolved in 50 mM citric acid buffer before intravenous administration at 50 mg/kg. Normal control rats were given physiological saline. Blood glucose levels were measured in the diabetic rats 1 week later, after which the rats were grouped such that the blood glucose levels were similar in each group. Male Institute of Cancer Research normal and KK-A^y type 2 diabetic mice, which exhibit hyperglycemia, insulin resistance, hyperinsulinemia, hyperlipidemia, and obesity, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and CLEA Japan (Kanagawa, Japan), respectively, at the age of 5–6 weeks. The diabetic mice were also grouped such that each group had similar blood glucose levels. All animals were housed under conventional conditions with controlled temperature, humidity, and light (12-h light–dark cycle) and were provided with a standard commercial diet and water (*ad libitum*). Animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, and all procedures were approved by the Animal Ethical Committee of Astellas Pharma Inc.

SGLT2 and SGLT1 inhibition assay

Human, rat, and mouse SGLT2 and SGLT1 full-length complementary deoxyribonucleic acid sequences were cloned and stably transfected into Chinese hamster ovary (CHO) cells using standard techniques as described previously (Katsuno et al. 2007). Cells were seeded into 96-well plates at a density of 3×10^4 cells/well in Ham's F12 medium containing 10% fetal bovine serum. The cells were used 1 day after plating. Test compounds were initially dissolved in dimethyl sulfoxide and diluted to the desired concentration with sodium assay buffer (140 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 5 mM Tris–HCl, pH 7.4). After the medium was removed, the cells were preincubated in 100 μ l choline assay buffer (NaCl in sodium assay buffer was replaced with the same concentration of choline chloride) at 37°C for 20 min. They were then incubated in the test compound solution (25 μ l) containing ¹⁴C-AMG (2.2 μ Ci/ml) and nonlabeled AMG (final concentration 55 μ M) at 37°C for 2 h. Cells were washed twice with 200 μ l ice-cold wash buffer (choline assay buffer containing 10 mM AMG) and then solubilized in 0.5% sodium dodecyl sulfate (SDS) solution (25 μ l). The cell lysate was mixed with 75 μ l MicroScint MS-40 (Packard Instrument Co., Meriden, CT, USA), and radioactivity was measured using a Top Count Microplate Scintillation Counter (Packard Instrument Co.).

Specificity assay

Pharmacological screening assays for various receptors, ion channels, and transporters were performed according to the standard assay procedure of Sekisui Medical Co., Ltd. (Ibaraki, Japan).

Stability against mouse intestinal glucosidases

Under ether anesthesia, the small intestine was removed from overnight fasted normal mice, washed with cold saline, excised, and rinsed with phosphate buffer (48 mM NaCl, 5.4 mM KCl, 28 mM Na₂HPO₄, 43 mM NaH₂PO₄, 35 mM mannitol, 10 mM glucose, pH 6.5). The mucosal tissue was scraped off gently using a slide glass, homogenized with phosphate buffer, and used for the stability study. Test compounds were initially dissolved in acetonitrile at a concentration of 5 mM and then diluted to 100 μ M with the phosphate buffer. Mucosal homogenates (5 mg/ml, 100 μ l) were preincubated at 37°C in microtubes. Thereafter, each compound solution (100 μ l, final concentration 50 μ M) was added and incubated at 37°C for varying time periods. The reaction was stopped by the addition of ice-cold acetonitrile (200 μ l), then 200 μ l of methyl tert-butyl ether was added, and the mixture was centrifuged (15,000 rpm, 10 min). The supernatant was transferred into a tube and evaporated in a vacuum centrifugal concentrator. The residue was dissolved in mobile phase for use as the assay sample. Concentrations of compound in the assay sample were analyzed using a high-performance liquid chromatography (HPLC) with an ultraviolet detector (265 nm for ipragliflozin and 280 nm for phlorizin and phloretin) and a 4.6 \times 250-mm reversed-phase ODS-80Ts column (Tosoh, Tokyo, Japan). The column temperature was maintained at 60°C, 20 mM ammonium acetate/acetonitrile [20:80 (*v/v*)] was used as the mobile phase, and the flow rate was 1.5 ml/min.

Pharmacokinetics

After oral administration of ipragliflozin (3 mg/kg) or phlorizin (100 mg/kg) to non-fasted normal mice, blood was withdrawn from the abdominal vena cava under ether anesthesia. The plasma concentrations of ipragliflozin or phlorizin were measured using HPLC. Acetonitrile (100 μ l) and methyl tert-butyl ether (100 μ l) were added to the plasma samples (100 μ l), mixed, and then centrifuged (15,000 rpm, 10 min). The supernatant was transferred into a tube and evaporated in a vacuum centrifugal concentrator. The residue was dissolved in HPLC mobile phase, 0.1% formic acid solution/acetonitrile [55:45 (*v/v*)] for use as the assay sample. Concentrations of ipragliflozin or phlorizin in the assay samples were measured as described above.

Effect of ipragliflozin on urinary glucose excretion in normal mice

Ipragliflozin (0.01–10 mg/kg) was administered to non-fasted normal mice, and spontaneously voided urine was collected for 24 h after administration while the animals were kept in metabolic cages. After the urine volume had been measured, the glucose concentration in the urine was measured using the Glucose CII test reagent (Wako, Osaka, Japan).

Effect of single administration of ipragliflozin in diabetic animals

To investigate its antihyperglycemic effect, ipragliflozin (0.1–1 mg/kg) was administered to STZ-induced type 1 diabetic rats and KK-*A^y* type 2 diabetic mice in the fed condition. Blood glucose levels were then measured for 8 h under fasting conditions, in order to eliminate the influence of feeding during the experiment. To evaluate sustainability, ipragliflozin (0.1–1 mg/kg) was administered to both types of diabetic animals, which were then fasted for 12 h (overnight). A glucose solution (2 g/kg) was subsequently administered orally, and blood glucose levels were measured as described above.

Effect of repeated administration of ipragliflozin in STZ-induced type 1 diabetic rats

Ipragliflozin (0.3 and 1 mg/kg) was administered to STZ-induced type 1 diabetic rats once daily (at night) for 4 weeks. Body weight and food intake were measured every week. After drug administration on day 26, rats were transferred to metabolic cages and spontaneously voided urine was collected for 24 h. On the morning after the final drug administration on day 28, blood samples were collected under non-fasting conditions, and the pancreas was isolated under ether anesthesia. Blood and urinary glucose concentrations were measured as described above. The pancreas was homogenized by adding acid–ethanol solution (75% ethanol, 23.5% purified water, and 1.5% concentrated hydrochloric acid) and incubating at 4°C for 1 h to extract the insulin. Subsequently, the culture was centrifuged and the supernatant was used as a measurement sample. Plasma and pancreatic insulin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa, Japan). Hemoglobin A_{1c} (HbA_{1c}) levels were measured using a DCA2000 System (Bayer Medical, Tokyo, Japan).

Effect of repeated administration of ipragliflozin in KK-*A^y* type 2 diabetic mice

Ipragliflozin (0.3 and 1 mg/kg) was administered to KK-*A^y* type 2 diabetic mice once daily (at night) for 4 weeks. Body

weight and food intake were measured every week. On the morning after the drug administration on day 28, blood samples were collected under non-fasting conditions. After the drug administration on day 30, mice were transferred to metabolic cages and spontaneously voided urine was collected for 24 h. Blood and urinary glucose concentrations, HbA_{1c}, and plasma insulin levels were measured as described above. Urinary albumin concentration was measured using a mouse albumin ELISA.

Effect of ipragliflozin on fasting blood glucose levels in normal mice

In order to investigate their effects on postprandial hyperglycemia, ipragliflozin (0.03–100 mg/kg) or glibenclamide (0.3–300 mg/kg) was administered to normal mice that had been fasted overnight. After 30 min, glucose solution (2 g/kg) was administered orally, and blood glucose levels were measured. To investigate their effects on hypoglycemia, ipragliflozin (0.03–100 mg/kg) or glibenclamide (0.3–300 mg/kg) was administered to normal mice fasted overnight, and blood glucose levels were measured.

Effect of ipragliflozin on gastrointestinal carbohydrate contents in normal mice

After fasting for 24 h, mice received ipragliflozin (0.3–30 mg/kg) or voglibose (1 mg/kg). After 15 min, a liquid meal (ENSURE-H: carbohydrates 206 mg/ml, fats 53 mg/ml, proteins 53 mg/ml; Abbott, Osaka, Japan) was given orally at 20 ml/kg. Control mice were given purified water instead of a liquid meal. At 1 h after the liquid meal or water administration, blood glucose levels were measured, and gastrointestinal tracts (stomach, upper and lower small intestine, cecum, and lower large intestine) were isolated under ether anesthesia. Isolated gastrointestinal tracts were homogenized with purified water (5 ml) and centrifuged (3,000 rpm, 10 min) to retrieve the supernatant. Glucose concentration was measured as described above. Sucrose and maltose concentrations were measured by a previously described method with minor modifications (Dörner 1977). Fructose concentration was measured using a fructose assay kit (Sigma-Aldrich Co.).

Effect of ipragliflozin on plasma and urinary parameters in KK-*A^y* type 2 diabetic mice

Ipragliflozin (1 mg/kg), furosemide (10 mg/kg), or YM471 (3 mg/kg) was administered to non-fasted diabetic mice, which were then transferred to metabolic cages. The mice were allowed free access to food and water, and spontaneously voided urine samples were collected for 8 h. Thereafter, blood samples were collected from the tail vein

for the determination of blood glucose level. Under ether anesthesia, urine was collected from the bladder, and blood samples were collected from the abdominal vena cava. The volume of spontaneously voided urine combined with the urine in the bladder was measured. Blood and urine samples were centrifuged (15,000 rpm, 10 min), after which the supernatants were used for the determination of several parameters. Plasma and urine osmolalities were measured using a freezing point depression osmometer (Advanced Instruments Inc., Norwood, MA, USA). Plasma and urine electrolyte (Na^+ , K^+ , and Cl^-) concentrations were determined using a flame photometer (Hitachi, Tokyo, Japan), and the urinary electrolyte excretion was calculated as the product of the urine electrolyte concentration and the urine volume.

Statistical analysis

Experimental results are expressed as mean \pm standard error of the mean (SEM), standard deviation (SD), or with 95% confidence limits. The median inhibition concentration (IC_{50}) values were calculated using regression analysis. The areas under the blood glucose concentration–time curves were calculated from blood glucose concentrations measured over time. Student's *t* test was used to analyze the differences between two groups; Dunnett's multiple range test was used for comparisons among multiple groups. A value of $p < 0.05$ was taken to be significant. Statistical and data analyses were conducted using the SAS 8.2 software package (SAS Institute Japan, Ltd., Tokyo, Japan).

Results

SGLT2 and SGLT1 inhibition assay

Ipragliflozin concentration-dependently inhibited mouse, rat, and human SGLT2 activity at nanomolar concentrations (Table 1). For human SGLT2, the inhibitory effect of ipragliflozin was approximately five times greater than that of phlorizin, but for human SGLT1, it was only one ninth of that of phlorizin. Phloretin had very little potency to inhibit either SGLT. The ratios of selectivity (IC_{50} of human SGLT1/SGLT2) of ipragliflozin, phlorizin, and phloretin were 254, 6, and 3, respectively. In addition, ipragliflozin exhibited high selectivity for mouse and rat SGLT2. Dapagliflozin also potently and selectively inhibited mouse and human SGLT2 activity.

Specificity assay

To confirm the specificity, effects of ipragliflozin on several representative receptors, channels, and transporters were

Table 1 Inhibition of SGLT2 and SGLT1 activity by ipragliflozin, dapagliflozin, phlorizin, and phloretin

	Human			Rat			Mouse		
	SGLT2 IC_{50} (nM)	SGLT1 IC_{50} (nM)	Selectivity ^a	SGLT2 IC_{50} (nM)	SGLT1 IC_{50} (nM)	Selectivity ^a	SGLT2 IC_{50} (nM)	SGLT1 IC_{50} (nM)	Selectivity ^a
Ipragliflozin	7.38 (6.75–8.07)	1,876 (1,570–2,242)	254	6.73 (4.07–11.1)	1,166 (1,000–1,359)	173	5.64 (3.76–8.47)	1,380 (1,122–1,698)	245
Dapagliflozin	1.30 (1.17–1.44)	611 (455–822)	470				5.12 (3.28–7.99)	244 (226–262)	48
Phlorizin	34.6 (24.1–49.7)	210 (176–251)	6	49.7 (41.7–59.2)	179 (143–225)	4	72.6 (54.4–96.8)	299 (240–372)	4
Phloretin	23,700 (22,100–25,400)	69,300 (62,400–77,000)	3	30,900 (11,100–86,200)	75,700 (66,700–85,800)	2	47,500 (38,800–58,200)	86,000 (59,500–124,000)	2

IC_{50} values are expressed as mean with 95% confidence limits of three to five independent experiments performed in duplicate

^aSelectivity is the IC_{50} ratio of SGLT1/SGLT2

examined using radioligand binding and enzyme assays. Ipragliflozin did not interact with various receptors, ion channels, and transporters such as adrenergic (α_1 , α_2 , and β), muscarinic (M_1 , M_2 , and non-selective), angiotensin (AT_1 and AT_2), calcium channel (L-type and N-type), potassium channel (K_{ATP} and SK_{Ca}), sodium channel (site 2), cholecystokinin (CCK_A and CCK_B), dopamine (D_1 , D_2 , and transporter), endothelin (ET_A and ET_B), gamma-aminobutyric acid ($GABA_A$ and $GABA_B$), glutamate (AMPA, kainate, and NMDA), serotonin ($5-HT_1$, $5HT_{2B}$, and transporter), histamine (H_1 , H_2 , and H_3), and neurokinin (NK_1 , NK_2 , and NK_3), exhibiting IC_{50} values $>3,000$ nM.

Stability of ipragliflozin against mouse intestinal glucosidases

The *in vitro* biological stability of ipragliflozin and phlorizin against glucosidases was examined using mucosal homogenates of mouse small intestine. While ipragliflozin was not degraded at all (Fig. 2a), phlorizin was quickly degraded in mouse mucosal homogenates into its aglycon, phloretin (Fig. 2b).

Pharmacokinetics

After oral administration of ipragliflozin (3 mg/kg) to normal mice, plasma concentrations of ipragliflozin reached a maximum at 1 h and then gradually decreased (Fig. 3). Obvious plasma concentrations were detected even 8 h after administration. In contrast, when phlorizin (100 mg/kg) was administered orally, plasma drug concentrations were low and rapidly eliminated.

Effect of ipragliflozin on urinary glucose excretion in normal mice

In normal mice, ipragliflozin (0.01–10 mg/kg) dose-dependently and significantly increased urinary glucose excretion, and this effect was still apparent 12–18 h after administration at doses of ≥ 0.3 mg/kg (Fig. 4a). Urine

volume was also significantly increased at doses of 3 and 10 mg/kg (Fig. 4b).

Effect of single administration of ipragliflozin in diabetic animals

In STZ-induced type 1 diabetic rats and KK- A^y type 2 diabetic mice, ipragliflozin (0.1–1 mg/kg) dose-dependently lowered blood glucose levels, and this effect was significant at all tested doses (Figs. 5a and 6a). During the oral glucose tolerance test (OGTT) 12 h after dosing, ipragliflozin (0.1–1 mg/kg) dose-dependently inhibited increases in blood glucose levels. In STZ-induced type 1 diabetic rats, this effect was significant at doses of 0.3 and 1 mg/kg (Fig. 5b), and in KK- A^y type 2 diabetic mice, the effect was significant at all tested doses (Fig. 6b).

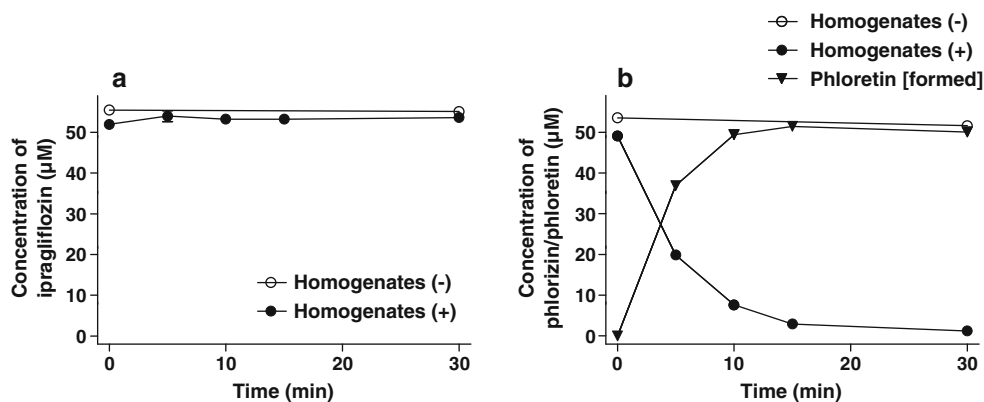
Effects of repeated administration of ipragliflozin in STZ-induced type 1 diabetic rats

Compared to the normal control rats, STZ-induced type 1 diabetic rats had significantly higher mean levels of HbA_{1c} , blood glucose, and urinary glucose excretion and significantly lower plasma insulin levels and pancreatic insulin content under non-fasting conditions (Table 2). Repeated administration of ipragliflozin (0.3 and 1 mg/kg) for 4 weeks significantly reduced the levels of HbA_{1c} and blood glucose. Plasma insulin level was not significantly changed, but pancreatic insulin content was significantly increased at a dose of 1 mg/kg. Urinary glucose excretion was increased dose-dependently, and this was significant at the 1 mg/kg dose. Ipragliflozin did not affect body weight or food intake (data not shown) throughout the study.

Effects of repeated administration of ipragliflozin in KK- A^y type 2 diabetic mice

Repeated administration of ipragliflozin (0.3 and 1 mg/kg) for 4 weeks reduced HbA_{1c} and blood glucose levels, with concomitant increases in urinary glucose excretion

Fig. 2 Stability of **a** ipragliflozin and **b** phlorizin in mouse intestinal mucosal homogenates. Ipragliflozin (50 μ M) or phlorizin (50 μ M) was incubated with mouse intestinal mucosal homogenates (5 mg protein). Data are expressed as the mean \pm SEM of three independent experiments



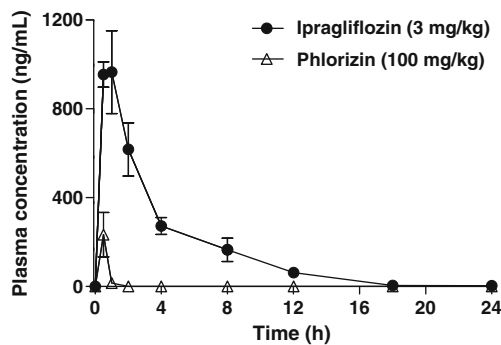


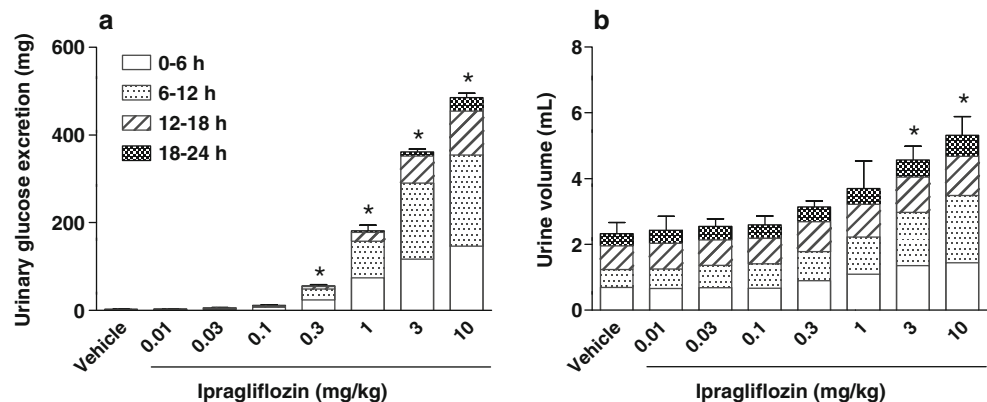
Fig. 3 Pharmacokinetics of ipragliflozin and phlorizin in normal mice. Ipragliflozin (3 mg/kg) or phlorizin (100 mg/kg) was administered orally to non-fasted normal mice. Data are expressed as the mean \pm SD for three animals in each sampling time point

(Table 3). In addition, urinary albumin excretion was significantly decreased. Ipragliflozin treatment did not affect body weight or food intake (data not shown) throughout the study.

Effect of ipragliflozin on fasting blood glucose levels in normal mice

In normal mice, ipragliflozin (0.03–100 mg/kg) dose-dependently inhibited the increase in blood glucose level after glucose loading, and this effect was significant at doses ≥ 0.1 mg/kg (Fig. 7a). Glibenclamide (0.3–300 mg/kg) also dose-dependently inhibited the increase in blood glucose levels; this effect was significant at doses ≥ 3 mg/kg (Fig. 7c). In overnight-fasted mice, ipragliflozin (0.03–100 mg/kg) dose-dependently reduced blood glucose levels, but this effect was only significant at doses ≥ 10 mg/kg, which are 100-fold higher than that in the OGTT (Fig. 7b). Glibenclamide (0.3–300 mg/kg) also dose-dependently reduced fasting blood glucose levels at the same doses as in the OGTT (Fig. 7d). Ipragliflozin did not alter plasma insulin levels under fasting conditions but significantly reduced the increase in plasma insulin levels under glucose loading conditions. In contrast, glibenclamide significantly increased plasma insulin levels under both conditions (data not shown).

Fig. 4 Effects of ipragliflozin on **a** urinary glucose excretion and **b** urine volume in normal mice. The values are the mean or mean \pm SEM for four animals in each group. Each parameter was analyzed statistically over 24 h. * $p < 0.05$ vs. vehicle using Dunnett's multiple range test



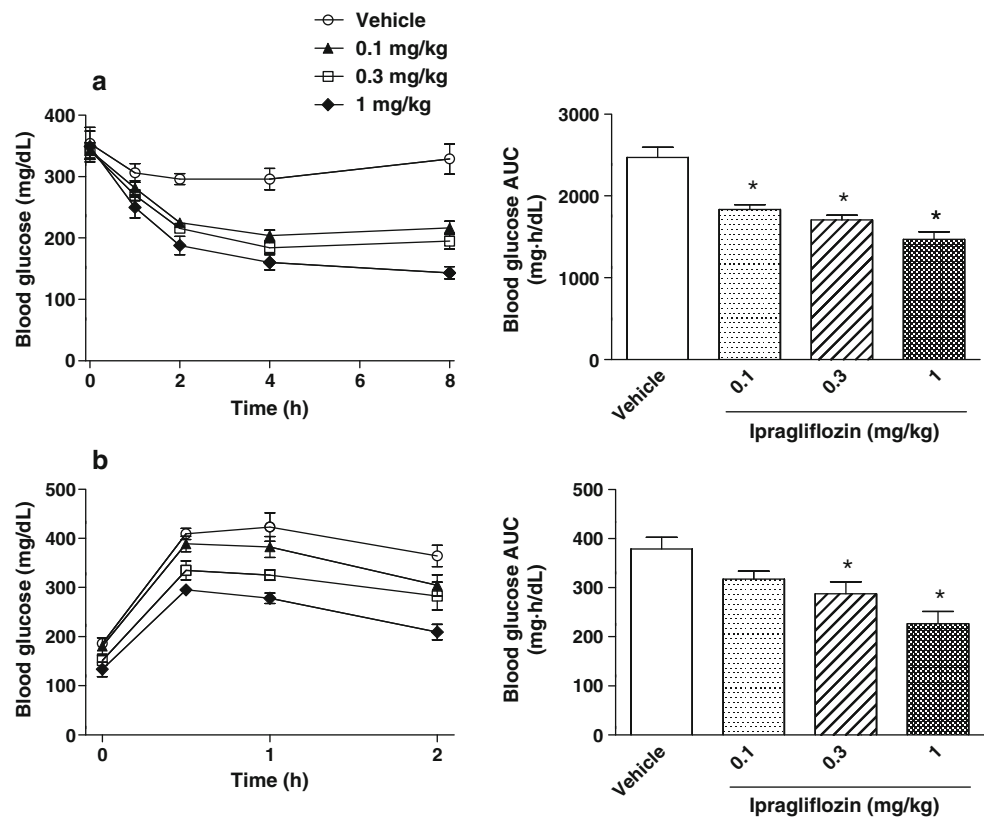
Effect of ipragliflozin on gastrointestinal carbohydrate contents in normal mice

Following liquid-meal loading in normal mice, gastrointestinal disaccharide (sucrose and maltose) and monosaccharide (glucose and fructose) contents increased significantly (Fig. 8). Voglibose (1 mg/kg) significantly increased gastrointestinal disaccharide content (Fig. 8a, b), decreased monosaccharide content (Fig. 8c, d), and significantly inhibited the increase in blood glucose levels (data not shown). In contrast, ipragliflozin (0.3–30 mg/kg) did not significantly affect gastrointestinal disaccharide content, even at the highest dose. In addition, ipragliflozin did not significantly affect gastrointestinal fructose content, and although it did dose-dependently increase glucose content, this effect was only significant at the maximum dose of 30 mg/kg. Ipragliflozin dose-dependently inhibited the increase in blood glucose levels, and this effect was significant at all tested doses (data not shown).

Effect of ipragliflozin on plasma and urinary parameters in KK- A^y type 2 diabetic mice

In type 2 diabetic mice, the loop diuretic, furosemide (10 mg/kg), significantly increased urinary electrolyte (Na^+ , K^+ , and Cl^-) excretion and urine volume, with a concomitant decrease in urinary osmolality (Table 4). This marked diuresis induced by electrolyte excretion also induced a significant decrease in plasma electrolyte concentrations and a significant increase in plasma osmolality. The vasopressin V_{1A}/V_2 receptor antagonist, YM471 (3 mg/kg), significantly increased urine volume without increasing electrolyte excretion and significantly decreased urinary osmolality. This marked water diuresis induced significant increases in plasma electrolyte concentrations and osmolality. In contrast, ipragliflozin (1 mg/kg) markedly increased urinary glucose excretion, with a concomitant slight increase in urine volume, and significantly decreased blood glucose levels, but did not significantly affect plasma or urinary electrolyte balance.

Fig. 5 Effects of ipragliflozin on blood glucose levels in streptozotocin-induced type 1 diabetic rats. **a** Ipragliflozin was administered to non-fasted diabetic rats, and blood glucose levels were measured for 8 h in a fasted state. **b** Ipragliflozin was administered, the rats were fasted for 12 h, then glucose solution was orally administered, and blood glucose levels were measured for 2 h. The values are expressed as mean \pm SEM for four animals in each group. * p <0.05 vs. vehicle using Dunnett's multiple range test



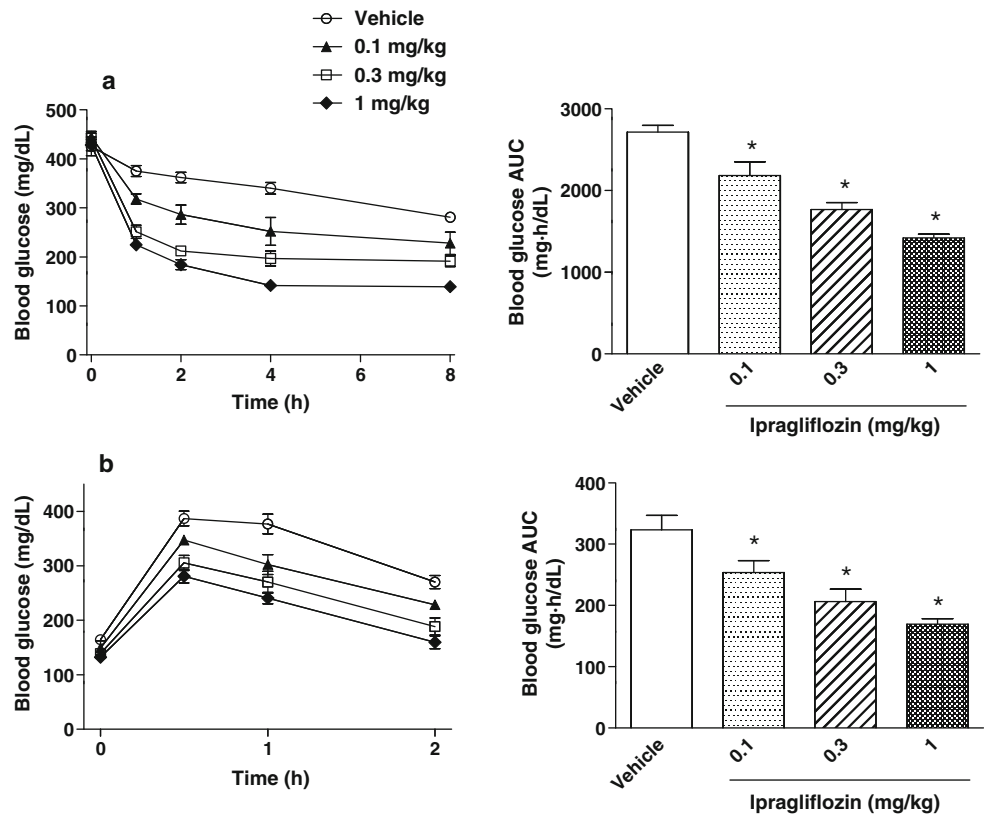
Discussion

Therapeutic strategies for diabetes currently focus on controlling elevated blood glucose levels. Chronic hyperglycemia has been shown to reduce insulin sensitivity and impair β -cell function in animal models (Harmon et al. 2001; Donath et al. 1999); therefore, the correction of hyperglycemia is predicted to improve these important physiological defects in patients with type 2 diabetes (Rossetti et al. 1987a, b). In recent studies, stimulating the excretion of excess glucose in the urine has been proposed as a new approach for the treatment of type 2 diabetes (Isaji 2007; Komoroski et al. 2009). SGLT2 is responsible for most of the glucose reabsorption in the kidneys and has been highlighted as a novel therapeutic target for diabetes. Ipragliflozin is a novel SGLT2 inhibitor, and we have explored its in vitro and in vivo characteristics in this study.

In CHO cells stably expressing either SGLT2 or SGLT1, ipragliflozin potently inhibited human, rat, and mouse SGLT2 with IC_{50} values in the low nanomolar range. However, its potency for SGLT1 inhibition was much weaker among all species studied, with IC_{50} values in the micromolar range. The inhibitory activities and selectivities of ipragliflozin were almost identical to those of dapagliflozin, and there was no appreciable species difference. Furthermore, ipragliflozin did not potently inhibit human SGLT4 and SGLT5

isoforms (IC_{50} >1,000 nM) (unpublished data). In the specificity assays, ipragliflozin did not interact with various receptors, ion channels, and transporters. In addition, ipragliflozin did not inhibit several glucose transporter (GLUT) isoforms, including GLUT1 and GLUT4, in mouse 3T3-L1, rat L6, human Caco-2, and HepG2 cells (IC_{50} >1,000 nM) (unpublished data). These in vitro studies suggest that ipragliflozin is a potent selective SGLT2 inhibitor. In the pharmacokinetic studies in mice, ipragliflozin showed good oral bioavailability and exhibited high drug concentrations for long periods. The absolute bioavailabilities of ipragliflozin were 71.7–90.7% and 74.5–75.3% in rats and monkeys, respectively (unpublished data). In contrast, phlorizin exhibited very low oral bioavailability. In the stability experiment using mucosal homogenates of mouse small intestine, phlorizin was quickly hydrolyzed to phloretin and glucose by intestinal glucosidases such as lactase-phlorizin hydrolase (Leese and Semenza 1973). This suggests that phlorizin administered orally would be easily degraded and poorly absorbed in the small intestine. The low oral bioavailability of phlorizin may be explained by its rapid enzymatic degradation in the gastrointestinal tract. In contrast, ipragliflozin exhibited stable properties against mouse intestinal glucosidases. In the same experiments using intestinal mucosal homogenates or microsomes of another species (rat, canine, monkey, and human), ipragliflozin also

Fig. 6 Effects of ipragliflozin on blood glucose levels in KK-A^y type 2 diabetic mice. **a** Ipragliflozin was administered to non-fasted diabetic mice, and blood glucose levels were measured for 8 h in a fasted state. **b** Ipragliflozin was administered, the mice were fasted for 12 h, then glucose solution was orally administered, and blood glucose levels were measured for 2 h. The values are expressed as mean ± SEM for four animals in each group. **p*<0.05 vs. vehicle using Dunnett's multiple range test



showed stable properties (data not shown). This intestinal stability of ipragliflozin is thought to contribute to its good oral bioavailability.

In normal mice, single administration of ipragliflozin induced marked and sustained increases in urinary glucose excretion which lasted beyond 12 h after administration, with concomitant increase in urine volume. The pharmacokinetic properties of ipragliflozin support these long-lasting glucosuric effects. In STZ-induced type 1 diabetic rats and KK-A^y type 2 diabetic mice, single oral doses ranging from 0.1 to 1 mg/kg of ipragliflozin produced dose-dependent and sustained reductions in hyperglycemia and improvements in glucose tolerance. These results suggest that ipragliflozin exhibits antihyperglycemic effects through the

sustained stimulation of urinary glucose excretion via the inhibition of renal SGLT2. In addition, in both insulin-deficient and obese insulin-resistant diabetic models, once-daily ipragliflozin treatment at doses of 0.3 and 1 mg/kg for 4 weeks significantly lowered blood glucose and HbA_{1c} levels. The concomitant increase in urinary glucose excretion indicates that the antihyperglycemic action of ipragliflozin is derived from the enhancement of urinary glucose disposal. Thus, ipragliflozin exhibits an antihyperglycemic effect, irrespective of the presence of impaired insulin secretion or insulin resistance. In STZ-induced type 1 diabetic rats, ipragliflozin increased pancreatic insulin content, suggesting that amelioration of hyperglycemia with ipragliflozin could induce the economization of pancreatic insulin

Table 2 Antidiabetic effects of repeated administration of ipragliflozin in streptozotocin-induced type 1 diabetic rats

Rats	Ipragliflozin (mg/kg)	Body weight (g)	HbA _{1c} (%)	Blood glucose (mg/dl)	Plasma insulin (ng/ml)	Pancreatic insulin content (ng/mg tissue)	Urinary glucose excretion (mg/day)	Urine volume (ml/day)
Normal	Vehicle	317±11	2.9±0.2	144±10	1.88±0.20	90.5±6.3	0.2±0.1	23±3
Diabetic	Vehicle	238±5**	10.6±0.6**	517±27**	0.30±0.05**	15.8±2.5**	2,220±180**	96±10**
Diabetic	0.3	232±5	8.5±0.3*	425±27*	0.27±0.06	22.4±3.9	2,830±380	100±8
Diabetic	1	227±5	7.6±0.3*	318±19*	0.32±0.07	29.9±4.2*	3,630±49*	114±8

Ipragliflozin was orally administered to streptozotocin-induced type 1 diabetic rats once daily for 4 weeks. Mean ± SEM for six animals in each group

p*<0.05 vs. diabetic vehicle group using Dunnett's multiple range test; *p*<0.05 vs. normal group using Student *t* test

Table 3 Antidiabetic effects of repeated administration of ipragliflozin in KK- A^y type 2 diabetic mice

Ipragliflozin (mg/kg)	Body weight (g)	HbA _{1c} (%)	Blood glucose (mg/dl)	Plasma insulin (ng/ml)	Urinary glucose excretion (mg/day)	Urine volume (ml/day)	Urinary albumin excretion (μ g/day)
Vehicle	43.6 \pm 0.7	8.57 \pm 0.28	470 \pm 32	48.9 \pm 9.8	464 \pm 86	4.73 \pm 0.50	1,250 \pm 70
0.3	44.5 \pm 1.1	6.86 \pm 0.23*	419 \pm 14	52.3 \pm 6.0	699 \pm 50	6.75 \pm 0.96	739 \pm 183*
1	42.0 \pm 0.8	5.94 \pm 0.36*	346 \pm 12*	56.4 \pm 7.8	792 \pm 129*	5.83 \pm 0.75	559 \pm 148*

Ipragliflozin was orally administered to KK- A^y type 2 diabetic mice once daily for 4 weeks. Mean \pm SEM for six animals in each group

* p <0.05 vs. vehicle group using Dunnett's multiple range test

content. In KK- A^y type 2 diabetic mice, ipragliflozin decreased urinary albumin excretion, the established parameter of early stage of diabetic nephropathy. Hyperglycemia is the principal factor for diabetic microvascular complications including nephropathy (UK Prospective Diabetes Study Group 1998); therefore, this would be mainly due to its antihyperglycemic effect. In addition, it has been reported the important role of enhanced glucose reabsorption in the proximal tubule in altering renal hemodynamics and the development of diabetic nephropathy (Vallon et al. 1999). Inhibition of renal glucose reabsorption induced by SGLT2 inhibitor might be expected to have an additional renoprotective effect; further studies are warranted to elucidate its mechanism of action in detail. In this study, chronic

treatment of ipragliflozin slightly reduced body weights of obese diabetic mice, but this was not statistically significant. It has been reported that dapagliflozin reduced body weight in type 2 diabetes (List et al. 2009). This weight reduction was considered to be mainly due to steady caloric loss but might account at least in part for its water loss, through glucosuria. In the another experiment, we have confirmed that ipragliflozin reduced epididymal adipose tissue weights in KK- A^y type 2 diabetic mice (unpublished data), which suggests that SGLT2 inhibitors including ipragliflozin could improve obesity and further studies are warranted to elucidate their effects.

In this study, glibenclamide inhibited postprandial hyperglycemia and decreased fasting blood glucose levels. Both

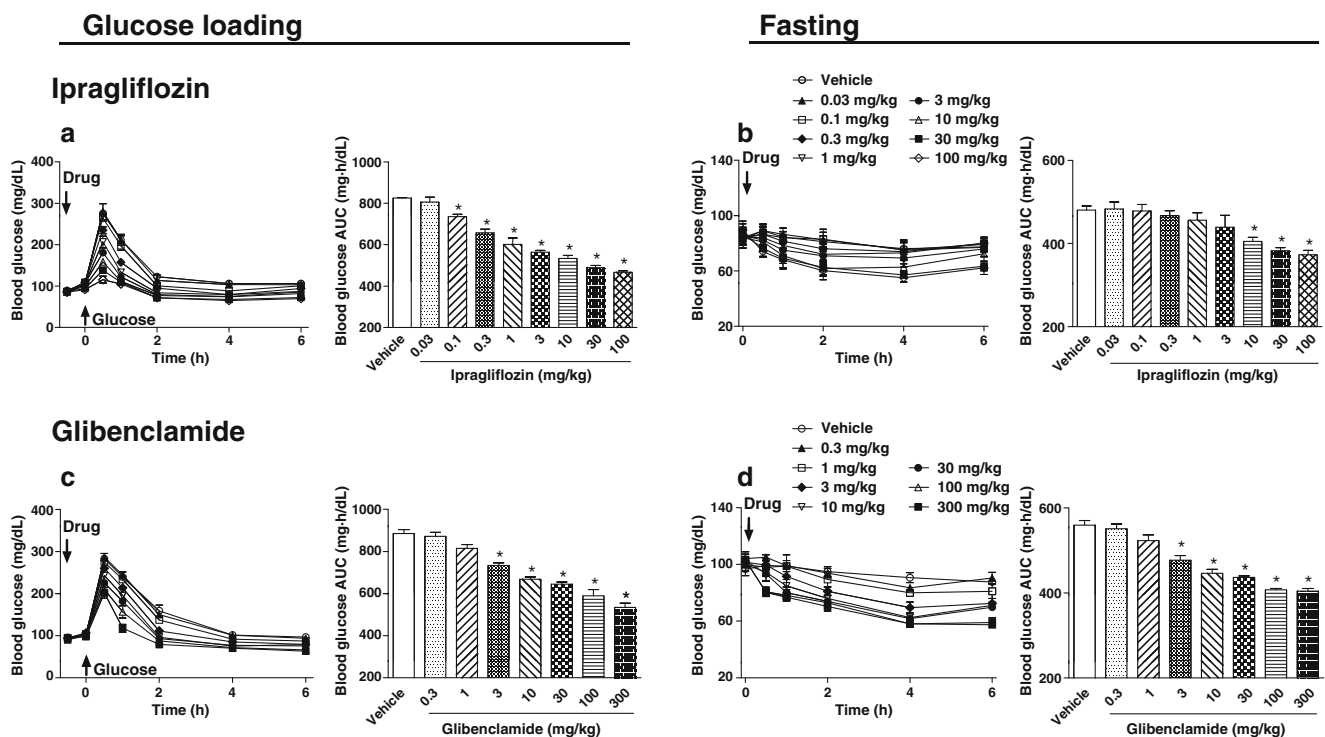
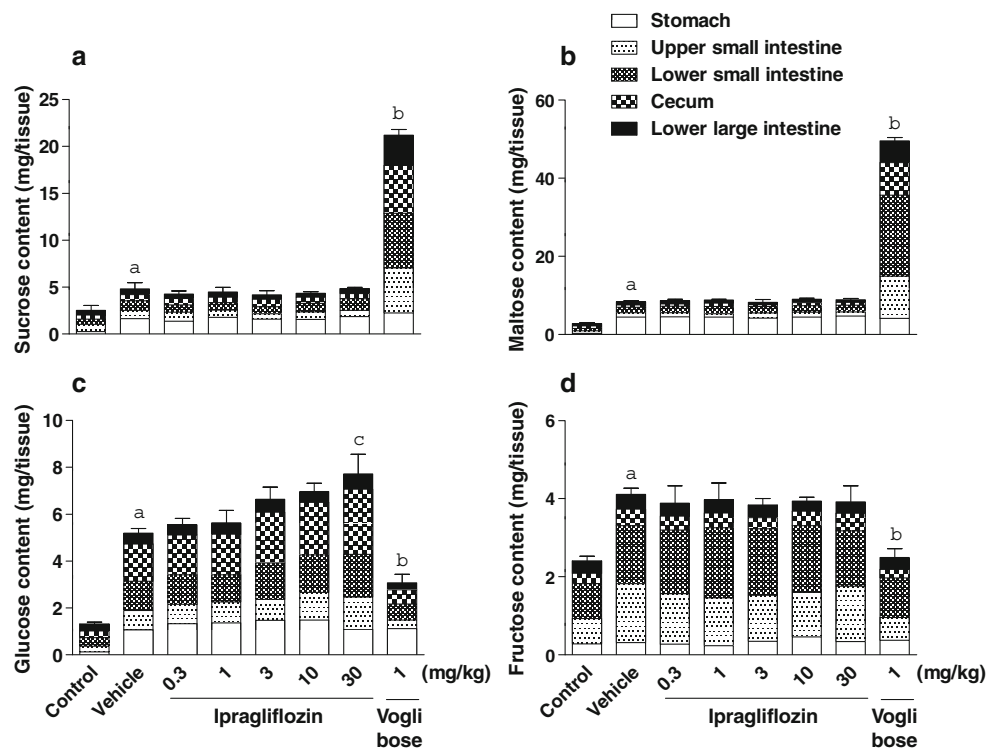


Fig. 7 Effects of ipragliflozin and glibenclamide on fasting blood glucose levels in normal mice. **a** Ipragliflozin or **c** glibenclamide was administered to mice fasted overnight, glucose solution was orally administered 30 min after dosing, and blood glucose levels were

measured for 6 h. **b** Ipragliflozin or **d** glibenclamide was administered to mice fasted overnight and blood glucose levels were measured for 6 h. The values are expressed as mean \pm SEM for four animals in each group. * p <0.05 vs. vehicle using Dunnett's multiple range test

Fig. 8 Effects of ipragliflozin and voglibose on gastrointestinal carbohydrate contents during the liquid meal tolerance test in normal mice. Effect of ipragliflozin and voglibose on gastrointestinal: **a** sucrose, **b** maltose, **c** glucose, and **d** fructose contents 1 h after liquid meal loading. The values are the mean or mean \pm SEM for four animals in each group. ^a $p < 0.05$ vs. control group, ^b $p < 0.05$ vs. vehicle group using Student's *t* test; ^c $p < 0.05$ vs. vehicle group using Dunnett's multiple range test



effects were induced by increases in plasma insulin levels at the same pharmacological doses. In clinical practice, when using sulfonylureas, the optimum dose should have a risk of hypoglycemia while maintaining sufficient therapeutic efficacy (Stahl and Berger 1999). The reduction in the fasting blood glucose level that occurred at the effective doses for glibenclamide is thought to be related to the risk of hypoglycemia. In contrast, by increasing urinary glucose excretion, ipragliflozin inhibited the increase in blood glucose levels after glucose loading in the absence of insulin secretion. Furthermore, as ipragliflozin had no effect on fasting blood glucose levels at pharmacological doses, there should be a low risk of hypoglycemia, making it safe for use as an antihyperglycemic agent. Furthermore, during the chronic treatment studies, significant correction of ambient hyperglycemia was achieved by ipragliflozin in type 1 and type 2 diabetic models, but no evidence of hypoglycemia was observed. The ability to induce an antihyperglycemic effect independently of insulin secretion is a property of ipragliflozin that represents a considerable advantage as a potential antidiabetic medicine for clinical use, and it could be efficacious in a wide variety of diabetic patients.

SGLT2, which is specifically expressed in the kidneys, plays an important role in renal glucose reabsorption (Jabbour and Goldstein 2008). In contrast, SGLT1 is highly expressed in the small intestine and mediates dietary glucose absorption (Pajor and Wright 1992). In order to elucidate the *in vivo* effect of ipragliflozin against intestinal SGLT1, we

examined its effect on gastrointestinal carbohydrate absorption. α -Glucosidase inhibitors, such as voglibose, inhibit the small intestinal disaccharidases, sucrase and maltase (Matsuo et al. 1992). This slows the absorption of carbohydrates from the small intestine, thereby lowering postprandial hyperglycemia (Baron 1998). In this study, voglibose induced a significant increase in intestinal disaccharide content (by delaying disaccharide digestion) that inhibited the increase in blood glucose levels. Thus, α -glucosidase inhibitors are effective at preventing postprandial hyperglycemia by this mechanism. However, osmotic water retention induced by accumulation of intestinal disaccharide content can cause gastrointestinal symptoms such as soft feces or diarrhea (Vichayanrat et al. 2002). Ipragliflozin, however, did not affect intestinal disaccharide or fructose content, but at the maximum dose of 30 mg/kg, it did significantly increase intestinal glucose content. It is known that gastrointestinal expression of SGLTs and absorption of glucose depend mainly on SGLT1 (Turk et al. 1991) and that gastrointestinal drug concentrations immediately after oral administration are at a very high level (Masaoka et al. 2006). Thus, although ipragliflozin shows a 245-fold higher selectivity for mouse SGLT2 versus SGLT1, the significant increase in gastrointestinal glucose content with the highest dose of ipragliflozin is thought to be due to the inhibition of glucose absorption via SGLT1 in the small intestine. Nevertheless, gastrointestinal glucose elevation was only significant at a dose which is 100 times higher than that

Table 4 Effects of ipragliflozin on plasma and urinary parameters in KK-A^y type 2 diabetic mice

	Blood glucose (mg/dl)	Plasma glucose (mg/dl)	Plasma osmolality (mOsm/kg)	Plasma electrolytes (mEq/l)			Urine volume (ml)	Urinary glucose excretion (mg)	Urine osmolality (mOsm/kg)	Urinary electrolyte excretion (μEq)		
				Na ⁺	K ⁺	Cl ⁻				Na ⁺	K ⁺	Cl ⁻
Vehicle	402±7	291±6	291±6	165±3	6.3±0.1	103±2	1.88±0.35	110±18	1,450±60	210±41	379±77	217±53
Ipragliflozin	210±10*	292±7	292±7	166±3	6.2±0.1	109±2	2.35±0.28	283±20*	1,530±50	167±19	321±33	159±20
Furosemide	395±7	319±4*	319±4*	142±2*	4.9±0.1*	88±2*	6.00±0.31*	137±23	684±27*	547±49*	585±22*	513±40*
YM471	405±8	315±8*	315±8*	178±1*	6.7±0.1*	120±3*	4.62±0.25*	126±17	764±34*	248±27	372±35	212±10

Ipragliflozin (1 mg/kg), furosemide (10 mg/kg), or YM471 (3 mg/kg) was orally administered to KK-A^y type 2 diabetic mice and parameters were measured 8 h later. Mean ± SEM for five animals in each group

* $p < 0.05$ vs. vehicle group using Dunnett's multiple range test

which significantly decreased postprandial hyperglycemia. It is therefore considered that therapeutic doses of ipragliflozin would not inhibit intestinal SGLT1, nor affect intestinal carbohydrate digestion and absorption. As such, the risk of gastrointestinal symptoms should be low. During the course of both acute and chronic experiments, no gastrointestinal side effects indicative of significant intestinal SGLT1 inhibition were noted at any dose.

Higher doses of ipragliflozin slightly increased urine volume along with a significant increase in urinary glucose excretion. Since sodium-ion transportation accompanies the glucose transport promoted by SGLT2, we evaluated the effect of ipragliflozin on plasma and urinary electrolyte balance. In this experiment, the loop diuretic, furosemide, induced a marked diuresis with concomitant increase in urinary electrolyte excretion, and decreased plasma concentrations of electrolytes including sodium. This furosemide-induced hyponatremia may be a severe adverse reaction (Sonnenblick et al. 1993). The vasopressin V_{1A}/V₂ receptor antagonist, YM471, also exerted a potent aquaretic effect and increased plasma electrolyte concentrations. In contrast, ipragliflozin (1 mg/kg) markedly increased urinary glucose excretion with a concomitant slight increase in urine volume, but did not affect plasma or urinary electrolyte balance. These results suggest that ipragliflozin would increase urinary glucose excretion without inducing either a marked osmotic diuresis or an electrolyte imbalance at pharmacological doses.

Mutations in the SGLT2 gene lead to the rare disorder, familial renal glucosuria, where glucose reabsorption in the kidneys is severely impaired and large amounts of glucose are excreted in the urine (Francis et al. 2004; Magen et al. 2005). Despite the severe glucosuria, patients appear to have a benign condition, with no serious adverse events or problems in kidney function. Familial renal glucosuria is also not associated with hypoglycemia and generally has no significant clinical manifestations (Santer et al. 2003). In this study, chronic administration of pharmacological doses of ipragliflozin did not induce significant adverse effects in diabetic models. Based on these findings, ipragliflozin seems to be an effective and safe drug for the treatment of hyperglycemia.

In conclusion, the present study shows that ipragliflozin is a potent selective SGLT2 inhibitor, possesses good pharmacokinetic characteristics, and exhibits a sustained antihyperglycemic effect by increasing urinary glucose excretion, without inducing hypoglycemia or insulin secretion. These results indicate the potential usefulness of ipragliflozin for further development as a therapeutic agent for hyperglycemia in patients with diabetes. Clinical trials with ipragliflozin are currently in progress, and proof-of-concept studies should clarify the suitability of ipragliflozin for the treatment of diabetes (Kashiwagi et al. 2010).

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Conflict of interest A. Tahara, E. Kurosaki, M. Yokono, D. Yamajuku, R. Kihara, Y. Hayashizaki, T. Takasu, M. Imamura, L. Qun, M. Sasamata, and M. Shibasaki are employees of Astellas Pharma Inc. H. Tomiyama, Y. Kobayashi, and A. Noda are employees of Kotobuki Pharmaceutical Co. Ltd. Ipragliflozin is in clinical development by Astellas Pharma Inc. and Kotobuki Pharmaceutical Co. Ltd.

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