Characterization and comparison of sodium-glucose cotransporter 2 inhibitors: Part 2. Antidiabetic effects in type 2 diabetic mice

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Abstract

Previously we investigated the pharmacokinetic, pharmacodynamic, and pharmacologic properties of all six sodium-glucose cotransporter (SGLT) 2 inhibitors commercially available in Japan using normal and diabetic mice. We classified the SGLT2 inhibitors with respect to duration of action as either long-acting (ipragliflozin and dapagliflozin) or intermediate-acting (tofogliflozin, canagliflozin, empagliflozin, and luseogliflozin). In the present study, antidiabetic effects of repeated administration of these SGLT2 inhibitors in type 2 diabetic mice were investigated. When repeatedly administered for 4 weeks, all SGLT2 inhibitors significantly exhibited antihyperglycemic, antihyperinsulinemic, and pancreas-protective effects, as well as insulin resistance-improving effects. When compared at doses producing comparable reduction in hyperglycemia across all drugs, the antidiabetic effects of ipragliflozin and dapagliflozin were more potent than those of the other four drugs, but these differences among the six drugs were not statistically significant. Further, an oral glucose tolerance test performed after repeated administration demonstrated significant improvement in glucose tolerance only with ipragliflozin and dapagliflozin, implying improved insulin resistance and secretion. Taken together, these findings demonstrate that, although all SGLT2 inhibitors exert antidiabetic effects in type 2 diabetic mice, these pharmacologic effects might be slightly superior with the long-acting drugs, which are able to provide favorable blood glucose control throughout the day.

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1. Introduction

Type 2 diabetes is characterized by hyperglycemia and relative insulin deficiency as a result of impaired insulin secretion from pancreatic β-cells or insulin resistance, and its incidence has increased dramatically due to increasing prevalence of obesity and physical inactivity (1,2). In addition, substantial evidence suggests that chronic hyperglycemia alone can directly impair both insulin secretion and sensitivity, a phenomenon known as “glucose toxicity” and which contributes to the progressive worsening of hyperglycemia (3). Effective glycemic control is therefore important to prevent both the onset of diabetes and the progressive deterioration of the diabetic disease state. However, while a number of antidiabetic drugs are available, maintaining good long-term glycemic control remains difficult in most type 2 diabetic patients, even when used in combination (4), highlighting the need for efficient new therapeutic strategies for treating type 2 diabetes.

In recent years, sodium-glucose cotransporter (SGLT) 2 inhibitors, which can inhibit reabsorption of filtered glucose in the kidney and increase urinary glucose excretion, have been developed and proposed as novel antihyperglycemic agents for treating type 2 diabetes (5), with several shown to improve hyperglycemia in this patient population (6). However, while many studies have focused on nonclinical and clinical pharmacologic effects of SGLT2 inhibitors (7,8), most have examined these drugs on an individual basis, with only one study comparing several SGLT2 inhibitors in terms of in vitro inhibitory activity and selectivity for SGLT2 (9) and none comparing their in vivo pharmacologic effects. In our previous experiment, we investigated and compared the pharmacokinetic, pharmacodynamic, and basic pharmacologic properties of all six SGLT2 inhibitors commercially available in Japan using normal and diabetic mice (10). We then classified the SGLT2 inhibitors with

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respect to duration of action as either long-acting (ipragliflozin and dapagliflozin) or intermediate-acting (tofogliflozin, canagliflozin, empagliflozin, and luseogliflozin). That study examined single dosing under free-feeding conditions. In contrast, the present study was planned to investigate the antidiabetic effects of these SGLT2 inhibitors in type 2 diabetic mice after repeated dosing under restricted feeding conditions based on these basic data, as well as parameters including not only hyperglycemia but also pancreatic insulin content and glucose tolerance.

2. Materials and methods

2.1. Materials

Ipragliflozin (11), dapagliflozin (12), tofogliflozin (13), canagliflozin (14), empagliflozin (9), and luseogliflozin (15) were synthesized at Astellas Pharma Inc. (Ibaraki, Japan) and suspended in 0.5% methylcellulose solution for oral administration. Doses of drugs are expressed as the free base form.

2.2. Animals

Male C57BL/6 (normal) and KK/Ay type 2 diabetic mice were purchased from CLEA Japan at age 6 weeks (Kanagawa, Japan) and uniformly grouped by blood glucose levels at age 7 weeks. All animals were housed under conventional conditions with controlled temperature, humidity, and light (12-h light—dark cycle) and were provided with standard commercial diet and water. Starting from the day after grouping, animals received feed only during the active period (i.e. fed for 14-h from 19:00 to 9:00, and fasted for the rest of the 24-h period), and three days after grouping, drug administration was started. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Astellas Pharma Inc., Tsukuba Research Center has been awarded Accreditation Status by the AAALAC International.

2.3. Antidiabetic effects of repeated administration of SGLT2 inhibitors

Vehicle or each SGLT2 inhibitor (ipragliflozin and dapagliflozin: 0.1–1 mg/kg, tofogliflozin, canagliflozin, empagliflozin, and luseogliflozin: 1–10 mg/kg) was orally administered to diabetic mice (and vehicle was orally administered to normal mice) once daily (just before feeding at night) for 4 weeks. Doses of drugs were set to produce comparable increase in 24-h urinary glucose excretions based on the results of our preliminary study. After administration on Day 1, blood samples (approximately 15 μL) for the evaluation of blood glucose and plasma insulin levels were obtained from the tail vein using capillary glass tubes at every 24-h sampling point. Blood sampling was done during the night (dark period) using a spotlight to minimize lighting, taking special care not to affect food consumption or related parameters. After drug administration on Day 26, mice were transferred to metabolic cages, and spontaneously voided urine was collected for 24 h. The morning (5:00–8:00) after the final drug administration (Day 30), blood samples were collected under nonfasting conditions, and tissues including pancreas were isolated under isoflurane anesthesia.

2.4. Effects of repeated administration of SGLT2 inhibitors on glucose tolerance

Vehicle or each SGLT2 inhibitor (ipragliflozin and dapagliflozin: 0.3 mg/kg, tofogliflozin, canagliflozin, empagliflozin, and luseogliflozin: 3 mg/kg) was orally administered to diabetic mice once daily (just before feeding at night) for 4 weeks. The final administration (Day 29) was followed by a 2-day withdrawal period, and on the day after completion of withdrawal, an oral glucose tolerance test (OGTT) was performed. After mice had fasted during the inactive period (from 9:00 to 19:00), blood was sampled from a tail vein for evaluation of fasting blood glucose and plasma insulin levels. The glucose solution (2 g/kg) was then orally administered, followed by blood sampling for 2 h.

2.5. Biochemical determinations

Blood and urinary glucose concentrations were measured using Glucose Cl II test reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma insulin levels were measured using an ultra-high-sensitivity mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan). Hemoglobin A1c (HbA1c) levels were measured using a DCA2000 System (Bayer Medical, Tokyo, Japan). Pancreatic hormone levels (insulin, glucagon, and somatostatin) were measured in accordance with the method previously reported (16). Plasma fibroblast growth factor 21 (FGF-21), leptin, and adiponectin concentrations were measured using commercial ELISA kits (R&D Systems Inc., Minneapolis, MN, USA).

2.6. Statistical analysis

The experimental results are expressed as the mean ± standard error of means (SEM). The areas under the curve (AUCs) and standard deviation (SD) were calculated from blood glucose and plasma insulin concentrations measured over time. The Matsuda-DeFronzo index was calculated using the following formula: 10,000/square root of [(fasting blood glucose × fasting plasma insulin) × (mean blood glucose × mean plasma insulin during OGTT)], and disposition index was calculated using the following formula: (plasma insulin AUC/blood glucose AUC during OGTT) × Matsuda-DeFronzo index (17). Significance of differences between normal and diabetic vehicle groups was assessed using Student’s t-test, while that between the vehicle- and drug-treated groups was assessed using one-way ANOVA followed by post-hoc Tukey’s and Dunnett’s multiple comparison tests. A value of $P < 0.05$ was considered to be significant. Statistical and data analyses were conducted using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

The 24-h blood sampling study revealed fasting hyperglycemia in diabetic mice, with increased blood glucose levels after start of feeding which remained high throughout the 14-h feeding (active) period compared with normal mice. After the feeding period, blood glucose levels gradually decreased but still remained high during the fasting (inactive) period. In association with blood glucose levels, plasma insulin levels also remained extremely high across the active and inactive periods. Administration of SGLT2 inhibitors dose-dependently and significantly reduced blood glucose and plasma insulin levels (Fig. 1 and Fig. 2). The effects of ipragliflozin and dapagliflozin on reduction in blood glucose and plasma insulin levels were potent immediately after administration but were gradually attenuated from 2 h post-dose, leading to obvious hyperglycemia and hyperinsulinemia in the latter half of the active period and throughout the inactive period,
Fig. 1. Antihyperglycemic effects of SGLT2 inhibitors in type 2 diabetic mice. 24-h blood glucose levels were measured on the first day of repeated administration for 4-weeks. (A) Time course of changes in blood glucose levels, (B) the area under the blood glucose concentration–time curve (AUC), and (C) standard deviation (SD) of blood glucose levels for 24 h. The values are the mean ± SEM for six animals per group. *$P<0.05$ vs. normal group, $^{\#}P<0.05$ vs. diabetic vehicle group.
Fig. 2. Antihyperinsulinemic effects of SGLT2 inhibitors in type 2 diabetic mice. 24-h plasma insulin levels were measured on the first day of repeated administration for 4-weeks. (A) Time course of changes in plasma insulin levels, (B) the area under the plasma insulin concentration–time curve (AUC), and (C) standard deviation (SD) of plasma insulin levels for 24 h. The values are the mean ± SEM for six animals per group. *P < 0.05 vs. normal group, #P < 0.05 vs. diabetic vehicle group.
demonstrating their shorter-acting properties compared with ipragli- 
flozin and dapagli- 
flozin. The SD of blood glucose levels, a 
parameter of blood glucose control throughout the day, was also 
significantly decreased with all drugs, but more markedly with 
ipragli- 
flozin and dapagli- 
flozin, compared with the other four drugs, with an even larger difference than that of blood glucose level (Fig. 2C). The 24-h urine sampling 
study revealed dose-dependent, significant increase in urinary 
glucose excretion and associated slight increases in urine volume 
with all drugs (Fig. 3). These increases in urinary glucose excretion 
were comparable for all drugs at 24 h but varied in persistence: the 
effects were significant with all drugs until 14 h after administra-
tion (active period) but only remained significant in the subsequent 
inactive period with ipragli- 
flozin and dapagli- 
flozin.

After 4-week repeated administration, all SGLT2 inhibitors 
dose-dependently and significantly reduced nonfasting blood 
glucose, HbA1c, and insulin levels and increased pancreatic insulin 
content (Fig. 4). No drugs significantly affected pancreatic glucagon 
or somatostatin content (data not shown). In addition, reductions 
in plasma FGF-21 and leptin levels and an increase in plasma adi-
ponectin levels were noted (Fig. 5).

Although there was no marked and statistically significant 
differences in these effects among six drugs, comparison at doses 
inducing comparable increases in urinary glucose excretion and 
reduction in hyperglycemia (ipragli- 
flozin and dapagli- 
flozin, 0.3 mg/kg; tofogli- 
flozin, canagli- 
flozin, empagli- 
flozin, and luseo-
flozin, 3 mg/kg) demonstrated the slight superiority of ipragli-
flozin and dapagli- 
flozin in controlling daily variability of blood 
glucose levels and improvement in diabetic parameters (decrease 
in plasma insulin levels, increase in pancreatic insulin content, and 
decrease in plasma FGF-21 levels) compared with the other four 
drugs (Fig. 6). In addition, decreased plasma leptin levels and 
increased plasma adiponectin levels induced by ipragli- 
flozin and dapagli- 
flozin were also slightly more potent than with the other 
four drugs (Fig. 5).

The OGTT performed after 4-week repeated administration and 
subsequent 2-day withdrawal revealed significant reductions in 
fasting blood glucose and plasma insulin levels and improvement 
in glucose tolerance with ipragli- 
flozin and dapagli- 
flozin, but only a 
non-significant trend toward improvement with the other four 
drugs (Fig. 7). Similarly, Matsuda-DeFronzo and disposition in-
dexes, determined in the test, indicated significant improvement or 
a trend toward improvement only with ipragli- 
flozin and dapagli-
flozin (Fig. 8).

![Fig. 3. Increase in urinary glucose excretion by SGLT2 inhibitors in type 2 diabetic mice. Urine samples were collected for 24 h at Week 4 of repeated administration. Urinary glucose excretion (left) and urine volume (right) for (A) 0–7 h, (B) 7–14 h, (C) 14–24 h, and (D) 0–24 h. The values are the mean ± SEM for six animals per group. *P < 0.05 vs. normal group, #P < 0.05 vs. vehicle group.](image)
4. Discussion

To ensure that the antidiabetic effects observed in our study reflected those in a clinical setting as accurately as possible, daily feeding timing for diabetic mice was set at 14 h from 19:00 to 9:00, in reference to the human active period (14 h from 7:00 to 21:00). This setting allowed diabetic mice to show hyperglycemia due to feeding in the active period and exhibit reduced blood glucose levels due to fasting in the inactive period, as well as demonstrate associated variation in plasma insulin levels, thereby enabling investigation of daily blood glucose control by SGLT2 inhibitors in a setting similar to diabetic patients. The 24-h blood and urine sampling study demonstrated dose-dependent, significant effects of all SGLT2 inhibitors on increase in urinary glucose excretion and reduction in blood glucose and plasma insulin levels. Since drug doses were set to produce comparable increases in 24-h urinary glucose excretion based on results of our preliminary study, although the effective doses differed, all drugs similarly increased urinary glucose excretion and reduced hyperglycemia on a daily basis.

However, duration of action varied; notably, while 24-h persistent effects were noted with ipragliflozin and dapagliflozin, the effects of the other four drugs (tofogliflozin, canagliflozin, empagliflozin, luseogliflozin) were potent immediately after administration but were gradually attenuated subsequently. This drop-off in efficacy led to abrogation of increases in urinary glucose excretion and obvious hyperglycemia and hyperinsulinemia in the latter half of the feeding (active) period through the fasting (inactive) period. Previously, the persistence of pharmacologic effects of these SGLT2 inhibitors was confirmed to be strongly dependent on their pharmacokinetics, particularly on their distribution and retention in the target organ, kidney (10). In addition, the amplitudes of daily blood glucose and insulin excursions were significantly decreased with all drugs, but more markedly with the long-acting ipragliflozin and dapagliflozin. Based on these findings, the six SGLT2 inhibitors studied were classified with respect to duration of action into two categories: long-acting, comprising ipragliflozin and dapagliflozin; and intermediate-acting, comprising tofogliflozin, canagliflozin, empagliflozin, and luseogliflozin. Our findings suggested that the long-acting SGLT2 inhibitors allow favorable blood glucose control throughout the day via persistent effects on increasing urinary glucose excretion and reducing blood glucose and plasma insulin levels.

Following 4-week repeated administration, all SGLT2 inhibitors dose-dependently and significantly improved hyperglycemia and hyperinsulinemia and increased pancreatic insulin content, indicating that the effects on reduction in blood glucose and plasma insulin levels identified in the 24-h blood sampling study on Day 1 were maintained without being attenuated throughout the 4-week repeated administration period. Further, these drugs did not significantly affect pancreatic glucagon or somatostatin content, suggesting that the increase in pancreatic insulin content noted in the present study was attributable not to the growth of pancreatic endocrine cells but to the prevention of pancreatic exhaustion by reduction in plasma insulin levels associated with improvement in hyperglycemia.

In type 2 diabetic patients, elevated plasma leptin (18) and FGF-21 (19) levels and reduced plasma adiponectin (20) levels have been observed and are known to be closely correlated with pathogenesis and progression of insulin resistance (21,22). In the present study as well, type 2 diabetic mice showed variations in these adipocyte-derived hormones similar to observations in diabetic patients, which were reversed with improvement of diabetic conditions by SGLT2 inhibitors, suggesting that SGLT2 inhibitors improve insulin resistance of type 2 diabetes. Comparison of pharmacologic effects of these SGLT2 inhibitors at doses producing comparable increases in urinary glucose excretion and reductions in hyperglycemia demonstrated a slight but not statistically significant superiority of ipragliflozin and dapagliflozin in the amplitude of daily blood glucose excursion and improvement in diabetic parameters (hyperinsulinemia, pancreatic exhaustion, and markers of insulin resistance) compared with the other four drugs. In addition, other diabetic parameters, including body weight, fat weight, plasma lipid levels and hepatic lipid contents also exhibited a similar improvement by drugs without affecting food intake (data not shown).
The OGTT performed after repeated administration also demonstrated significant glucose tolerance improvement only with iragliflozin and dapagliflozin. In the present study, 4-week repeated administration was followed by 2-day withdrawal, and after completion of the withdrawal, the effects of increased urinary glucose excretion by SGLT2 inhibitors were confirmed to have disappeared before performing the OGTT. The glucose tolerance-improving effects noted in the present study were therefore assumed to be attributable not to an acute antihyperglycemic effect via increased urinary glucose excretion but to improvement in glucotoxicity due to long-term reduction in hyperglycemia. The Matsuda-DeFronzo index, a parameter of insulin resistance, and the disposition index, a parameter of insulin secretion, were also improved following administration of long-acting SGLT2 inhibitors, implying improved insulin resistance and secretion. These results were consistent with the above-mentioned variations in adipocyte-derived hormones and pancreatic protection induced by SGLT2 inhibitors.

Many epidemiological and interventional studies have confirmed the association between hyperglycemia and the development of diabetic symptoms, including glucose intolerance, insulin resistance, and pancreatic exhaustion, and various complications such as obesity and nephropathy (23,24). Currently, HbA1c is considered the gold standard of long-term glycemic control and is recommended as a routine test in diabetic patients. However, recent studies have suggested that blood glucose variability throughout the day, irrespective of the magnitude of hyperglycemia, may confer an additional risk for development of diabetic micro- and macrovascular complications (25,26). It has been reported that parameters of blood glucose variability, such as mean amplitude of glucose excursions (MAGEs) and standard deviation of blood glucose (SDBG), are closely correlated with exacerbation of inflammation/oxidative stress and progression of atherosclerosis in type 2 diabetic patients (27–29). Thus, better daily control of blood glucose excursion, which is independent of HbA1c level, is also important for attenuating progression of diabetic symptoms and complications. In the present study, while all SGLT2 inhibitors reduced HbA1c levels comparably, the long-acting SGLT2 inhibitors were associated with smaller amplitude of daily blood glucose excursion (SDBG) and were superior in pancreas protection and

![A. Tahara et al. / Journal of Pharmacological Sciences 131 (2016) 198–208](image-url)
improvement in insulin resistance and glucose tolerance compared with the intermediate-acting drugs. These results suggested that not only long-term blood glucose control, as measured by HbA1c, but also daily favorable blood glucose control, as measured by SDBG, and improvement in hyperinsulinemia are crucial for improving diabetes.

To our knowledge, this is the first report comparing and differentiating multiple SGLT2 inhibitors in antidiabetic effects using mouse model of type 2 diabetes. The present study focused only on the pancreatic protective effects and the principle causes of type 2 diabetes, such as insulin resistance, and did not include investigation of other important components in the pathogenesis of type 2 diabetes such as obesity and inflammation, or of complications including nephropathy. Further comprehensive investigation of these factors is warranted.

The results of the present study provided evidence for superiority of the long-acting SGLT2 inhibitors in antidiabetic effects. However, concerns with these long-acting SGLT2 inhibitors are nocturia and hypoglycemia due to increased urine volume and decreased blood glucose levels associated with persistent increase in urinary glucose excretion. Increased urine volume on administration of SGLT2 inhibitors is principally attributable to osmotic diuresis associated with increased urinary glucose excretion. In the present study as well, increased urine volumes were noted along with increased urinary glucose excretions; however, the increments were negligible. The increases in urine volume during the inactive period (which corresponds to nighttime for humans) induced by long-acting SGLT2 inhibitors were also not significant. The 24-h blood glucose measurement study showed that the long-acting SGLT2 inhibitors reduced blood glucose level during the inactive period but not below the normal blood glucose level, with no excessive reduction noted. Further, no hypoglycemic symptoms—such as excessive weight loss or decreased locomotor activity—were noted throughout the study period, nor were any changes in lipid metabolism including plasma ketone body levels observed (data not shown). These findings suggested that long-

![Graphs](image-url)
Fig. 7. Improvement in glucose tolerance by repeated administration of SGLT2 inhibitors in type 2 diabetic mice. Time course of changes in the (A) blood glucose and (D) plasma insulin levels, fasting (B) blood glucose and (E) plasma insulin levels, and the area under the (C) blood glucose and (F) plasma insulin concentration–time curve (AUC) during the oral glucose tolerance test. The values are the mean ± SEM for six animals per group. *P < 0.05 vs. normal group, #P < 0.05 vs. vehicle group.
acting SGLT2 inhibitors are unlikely to cause nocturia or hypoglycemia.

In conclusion, findings from the present study provide evidence that, although all SGLT2 inhibitors studied exert antidiabetic effects, such as reduction in hyperglycemia through increase in urinary glucose excretion in type 2 diabetic mice, these drugs vary in terms of the duration of action. Inhibitors are classified with respect to duration of action into two categories—long-acting and intermediate-acting—and the long-acting drugs exert favorable blood glucose control throughout the day and might be slightly superior to intermediate-acting drugs in pancreas protection and improvement in insulin resistance and glucose tolerance.

Conflicts of interest

The authors have no conflicts of interest.

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Fig. 8. Improvement in insulin resistance and secretion by repeated administration of SGLT2 inhibitors in type 2 diabetic mice. Indexes of (A) Matsuda-DeFronzo and (B) disposition constant in insulin resistance and glucose tolerance.
