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Pharmacological profile of tofogliflozin, a novel SGLT2 inhibitor, in preclinical studies

（新規 SGLT2 阻害剤トホグリフロジンの前臨床薬効評価）

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I. ABBREVIATIONS

AMG : α-methyl-D-glucopyranoside

ANCOVA : analysis of covariance

ATCC : American Type Culture Collection

BSA : bovine serum albumin

BW : body weight

BWC : body water content

CANA : canagliflozin

CFU : colony-forming units

CMC : carboxymethylcellulose sodium salt

DAPA : dapagliflozin

DBW : dry body weight

DIO : diet-induced obese

2-DG : 2-deoxy-glucose

DTCB : deduced total calorie balance

EE : energy expenditure

EPI : epididymal adipose tissue

FC : food consumption

FFAs : free fatty acids

GK rats : Goto-Kakizaki rats

GLP-1 : glucagon-like peptide 1

Glu AUC : glucose area under the curve

G-PBS : phosphate buffered saline with 0.01% gelatin

GSIS : glucose-stimulated insulin secretion

Hb : hemoglobin

HE : hematoxylin-eosin

HFD : high-fat diet

ING : inguinal adipose tissue

IRI : immunoreactive insulin

MES : mesenteric adipose tissue

MI : *myo*-inositol

NASH : nonalcoholic steatohepatitis

ND : normal diet

OGTT : oral glucose tolerance test

PIO : pioglitozone

PPAR : peroxisome proliferator-activated receptor

RET : retroperitoneal adipose tissue

RQ : respiratory quotient

SDA : Sabouraud dextrose agar

SGLT : sodium/glucose cotransporter

SMIT1 : sodium/myoinositol transporter 1

T2D : type 2 diabetes

TC : total cholesterol

TG : triglycerides

TKB : total ketone bodies

TOFO : tofogliflozin

UGC : urinary glucose concentration

UGE : urinary glucose excretion

UTI : urinary tract infection

VO_2 : consumed oxygen

VCO_2 : produced carbon dioxide

ZDF rats : Zucker diabetic fatty rats

II. ABSTRACT

Sodium/glucose cotransporter 2 (SGLT2) is the predominant mediator of renal glucose reabsorption and is the emerging molecular target for the treatment of diabetes. A novel potent and selective SGLT2 inhibitor, tofogliflozin (CSG452), created by Chugai Pharmaceutical Co., Ltd., induced urinary glucose excretion (UGE), improved hyperglycemia and reduced body weight in clinical trials. Sanofi K. K. and Kowa Company Ltd. received Japan Pharmaceuticals and Medical Devices Agency approval to tofogliflozin for the treatment of type 2 diabetes (T2D) on 24th March 2014. It is expected that tofogliflozin, an oral hypoglycemic agent with body weight reduction, would improve quality of life of patients with T2D.

To clarify the potency of tofogliflozin as a medicine for T2D treatment, we investigated its pharmacological profiles with preclinical experiments focusing on following 3 viewpoints.

1) Fundamental Profile by in Vitro and in Vivo Pharmacological Studies
2) Mechanism of Body Weight Reduction in Obese Animal Models
3) Mechanism of Inducing Urinary Tract Infection in Novel Animal Model

1) Fundamental Profile by in Vitro and in Vivo Pharmacological Studies

Tofogliflozin competitively inhibited SGLT2 in cells overexpressing SGLT2, and *Ki* values for human, rat, and mouse SGLT2 inhibition were 2.9, 14.9, and 6.4 nM, respectively. The selectivity of tofogliflozin toward human SGLT2 versus human SGLT1, SGLT6, and sodium/myoinositol transporter 1 (SMIT1) was the highest among the tested SGLT2 inhibitors under clinical development. Furthermore, no interaction with tofogliflozin was observed in any of a battery of tests examining glucose-related physiological processes, such as glucose uptake, glucose oxidation, glycogen synthesis, hepatic glucose production, glucose-stimulated insulin secretion (GSIS), and

glucosidase reactions.

A single oral gavage of tofogliflozin increased renal glucose clearance and lowered the plasma glucose level in Zucker diabetic fatty rats (ZDF rats). Tofogliflozin also improved postprandial glucose excursion in a meal tolerance test with Goto-Kakizaki rats (GK rats). In db/db mice, 4-week tofogliflozin treatment reduced glycated hemoglobin (Hb) and improved glucose tolerance in the oral glucose tolerance test (OGTT) 4 days after the final administration. No plasma glucose reduction was observed in normoglycemic SD rats treated with tofogliflozin.

These findings demonstrate that tofogliflozin inhibits SGLT2 in a specific manner, lowers plasma glucose levels by increasing renal glucose clearance, and improves pathological conditions of T2D with a low hypoglycemic potential.

2) Mechanism of Body Weight Reduction in Obese Animal Models

The effect of tofogliflozin on body weight was investigated in detail with obese and diabetic animal models. Diet-induced obese (DIO) rats and mouse diabetic model with obesity, KKAy mice, were fed diets containing tofogliflozin, and body weight, body composition, biochemical parameters, and metabolic parameters were evaluated.

In DIO rats with 9-weeks administration of tofogliflozin, UGE was induced and body weight gain was prevented. Food consumption (FC) was increased without change of energy expenditure (EE), and deduced total calorie balance (DTCB = FC – UGE – EE) was decreased. Body fat mass was decreased without significant change in bone and lean body mass. Respiratory quotient (RQ) value and plasma triglycerides (TG) level were decreased, and plasma total ketone bodies (TKB) level was increased. Moreover, plasma leptin level, cell size of adipocytes, and CD68 positive cells in mesenteric adipose tissue were decreased.

In KKAy mice treated with tofogliflozin for 3 or 5 weeks, plasma glucose level and body weight gain were decreased. Liver weight and TG content were decreased without

reducing body water content. By combination therapy with tofogliflozin and pioglitazone, pioglitazone-induced body weight gain was suppressed, and glycated Hb level was reduced more effectively than monotherapy with either pioglitazone or tofogliflozin alone.

These data demonstrate that the body weight reduction with tofogliflozin is mainly due to the calorie loss with the increased UGE. In addition, metabolic shift from carbohydrates oxidation to fatty acids oxidation was also induced with tofogliflozin, which may lead to the prevention of fat accumulation and inflammation in adipose tissue and liver. Tofogliflozin may have a potency to prevent or improve obesity, hepatic steatosis, insulin resistance as well as hyperglycemia.

3) Mechanism of Inducing Urinary Tract Infection in Novel Animal Model

Urinary tract infection (UTI) is a common clinical problem in diabetic patients; however, the relationship between UTI and glucosuria remains uncertain. To investigate the relationship, we examined the effect of glucosuria induced by SGLT2 inhibitors on the progression of UTI in mice.

From 1 day before transurethral inoculation with *Candida albicans*, female mice were treated orally once a day with an SGLT2 inhibitor in different treatment regimens: 1) dapagliflozin at 10 mg/kg for 2, 3, or 6 days, 2) dapagliflozin at 0.1, 1, or 10 mg/kg for 3 days, 3) dapagliflozin, canagliflozin, or tofogliflozin at 10 mg/kg for 3 days. To evaluate ascending UTI, the kidneys were removed 6 days after the inoculation, and the number of viable *C. albicans* cells in kidney was measured as colony-forming units (CFU).

In mice treated with dapagliflozin, the number of *C. albicans* CFU in kidney increased in accordance with both treatment duration and dose. The number of CFU significantly increased when mice were treated with 10 mg/kg dapagliflozin or canagliflozin but not tofogliflozin. With dapagliflozin and canagliflozin, urine glucose

concentration (UGC) was significantly increased up to 24 h after drug administration; with tofogliflozin, UGC was significantly increased only up to 12 h after administration.

Our data indicate that increased susceptibility to UTI is associated with a persistent increase in UGC.

These results indicate that tofogliflozin has a potency to induce UGE and improve obesity, hepatic steatosis, insulin resistance as well as hyperglycemia. With the highly selectivity and the short half-life, tofogliflozin would induce fewer unexpected side-effects or UTI. It is assumed that tofogliflozin would be an oral hypoglycemic agent with wide therapeutic window and improve quality of life of patients with T2D.

III. INTRODUCTION

According to the World Health Organization, 346 million people worldwide have diabetes mellitus, and this number is expected to more than double by the year 2030 (1). Approximately 90% of all patients with diabetes mellitus have T2D, a progressive metabolic disease characterized by chronic hyperglycemia due to insulin resistance and impaired insulin secretion from the pancreatic β-cells (2). T2D is associated with a high incidence of both macrovascular (cardiovascular disease) and microvascular (nephropathy, retinopathy, and neuropathy) complications, and patients with T2D have a higher cardiovascular and all-causes mortality than do people without diabetes. Although the benefit of intensive glycemic control to reduce the increased risk of cardiovascular disease in people with T2D is still controversial, intensive glycemic control is required for the prevention of diabetes-related microvascular complications in these patients. Many drugs are currently available for the clinical treatment of patients with T2D. In most cases, however, the current treatment options are not sufficient to prevent disease progression, resulting in treatment with combinations of drugs or with insulin therapy. In addition, antidiabetic drugs can cause undesirable side effects such as hypoglycemia, body weight gain, gastric symptoms, and fluid retention. Therefore, there is a high unmet medical need for novel and potent drugs to improve glycemic control with a good safety and tolerability profile.

Glucose exists in free form in the plasma and is filtered freely through the glomerulus. In healthy individuals, the kidney retrieves glucose from the filtrate, and the urine is therefore practically glucose free. Glucose reabsorption is mediated by sodium/glucose cotransporters (SGLTs), namely high-affinity sodium/glucose cotransporter 1 (SGLT1) and low-affinity sodium/glucose cotransporter 2 (SGLT2), in the proximal tubules of the kidney (3). Therefore, the induction of UGE by the inhibition of SGLTs is thought to be a strategy to control plasma glucose levels. Indeed, the hyperglycemia in a diabetic animal model is improved with the induction of UGE

by treatment with phlorizin, a SGLT inhibitor (4). SGLT2 is specifically expressed in the proximal tubules of the kidney. In humans, mutations of the SGLT2 gene resulting in defective transport activity cause familial renal glucosuria (5, 6, 7). Despite UGE occurring in individuals with familial renal glucosuria, these people are generally asymptomatic and have normal plasma glucose levels and a normal glucose tolerance. SGLT1, on the other hand, transports glucose and galactose not only in the renal tubules but also in the small intestine (8). In infants, mutations of the SGLT1 gene resulting in defective transport activity cause diarrhea and dehydration (glucose-galactose malabsorption) because of the reduction of both glucose and galactose absorption in the small intestine, and patients with these mutations also show mild renal glucosuria (9, 10). These genetic disorders suggest that SGLT2 plays a dominant role in renal glucose reabsorption and that SGLT1 inhibition could cause gastrointestinal symptoms. In addition, SGLT1 is reported to be expressed in various organs such as the lungs, heart, and liver (8). Moreover other SGLT subtypes expect SGLT1 express in various tissue and might have their own physiological function (Table 1). SGLT6 (11) and SMIT1 (12) are sodium-dependent MI transporters expressed in various organs including the kidneys. Inhibition of MI transport may impair the function of the kidneys, because MI, an organic osmolyte, mediates osmolarity and maintains cell volume and fluid balance in various cells (13). For example, methylene-myo-inositol, an MI transporter inhibitor, damages renal tubules and induces acute renal failure in normal rats (14). SGLT4 and SGLT5 have been reported as sodium-dependent sugar transporters, with SGLT4 highly expressed in the small intestine and kidneys (15) and SGLT5 specifically expressed in the kidneys (16), and SGLT3 has been reported as a glucose sensor, rather than a sugar transporter, highly expressed in the skeletal muscles, small intestine, and kidneys (17); however, the precise roles of these three SGLTs remains unclear. Therefore, selective SGLT2 inhibition, because of its potency to induce UGE and its low safety concerns, is an

attractive target for the development of a next-generation antidiabetic drug.

Table 1 Distributions and deduced physiological functions of SGLT subsypes.

Name		Substrate	Distribution	Function
SGLT1	SLC5A1	Glucose, Galactose	Small intestine, Kidney, Heart, etc.	Intestinal Glucose/Galactose absorption
SGLT2	SLC5A2	Glucose	Kidney	Glucose renalreabsorption
SGLT3	SLC5A4	Glucose	Small intestine, Skeletal muscle	Unknown
SGLT4	SLC5A9	Mannose	Small intestine, Skeletal muscle	Unknown
SGLT5	SLC5A10	Mannose, Fructose	Kidney	Unknown
SGLT6	SLC5A11	Chiro-inositol, Myo-inositol	Ubiquitously	Intestinal inositol absorption, inositol renalreabsorption, and so on
SMIT	SLA5A3	Myo-inositol, Scyllo-inositol	Ubiquitously	Intestinal inositol absorption, inositol renalreabsorption, and so on

Currently, several SGLT2 inhibitors with various degrees of selectivity toward SGLT2 versus SGLT1 are being tested in clinical trials (18) and launched (19). Because a high safety profile is required for any drug used for lifelong treatment of T2D, long-term experience will be needed to examine whether this emerging class of drugs can safely fulfill the unmet medical needs of T2D treatment. Recently, we identified a potent and highly selective SGLT2 inhibitor, tofogliflozin (20). The small number of patients with familial renal glucosuria limits confidence in the lower safety concern that is normally applied to long-term SGLT2 inhibition by virtue of the benign condition of the patient population. Therfore, it is required to make intensive and multidimensional profiling of this emerging class of drugs of value in drug development, especially for T2D.

In the first study “Fundamental Profile by in Vitro and in Vivo Pharmacological Studies” (21), we examined the pharmacological profiles of tofogliflozin (CSG452), both in vitro and in vivo, including evaluation not only of its selectivity toward other SGLTs but also of its effect on glucose-related physiological processes, such as glucose uptake, glucose oxidation, glycogen synthesis, hepatic glucose production, GSIS, and glucosidase reactions. We found that tofogliflozin was highly specific to SGLT2 (its

selectivity toward SGLT2 versus other SGLT members was the highest among the SGLT2 inhibitors we tested) and that it improved T2D pathological conditions by lowering plasma glucose levels through the inhibition of renal glucose reabsorption, with a low hypoglycemic potential.

On the other hand, epidemiological studies identify obesity as a major risk factor for T2D (22, 23), and intra-abdominal adiposity is profoundly associated with the pathogenesis of T2D via inflammation in adipose tissues, insulin resistance, and impaired glucose regulation caused by fat accumulation (24, 25). Therefore, diet and exercise is regarded as an important strategy to prevent and delay progression of T2D (26). However, it is difficult to control body weight and plasma glucose solely by diet and exercise(27,28).

Furthermore, few antidiabetics have any antiobesity effect. Insulin analogues, insulin secretagogues, and peroxisome proliferator-activated receptor γ (PPARγ) agonists inevitably increase body weight (29, 30), and metformin (31) and dipeptidyl peptidase 4 inhibitors (32) do not obviously affect body weight. Although glucagon-like peptide 1 (GLP-1) analogues can reduce body weight (33), they are used via subcutaneous self-injection and also have gastrointestinal side effects. These facts indicate that an orally available antidiabetic drug to control both plasma glucose and body weight is required for patients with T2D.

Recent clinical studies have indicated that oral administration of SGLT2 inhibitors induces UGE, improves hyperglycemia, and reduces body weight of patients with T2D (34, 35, 36). Tofogliflozin, a potent and highly selective SGLT2 inhibitor, induces UGE and improves hyperglycemia in rodents with a low hypoglycemic potential (21,37), and, tofogliflozin improved hyperglycemia and reduced body weight in clinical studies (38, 39). However, the mechanism through which tofogliflozin reduces body weight is unclear.

In the second study “Mechanism of Body Weight Reduction in Obese Animal Models”

(40), we investigated the mechanism of body weight reduction with tofogliflozin by using DIO rats as an obesity model and KKAy mice as an animal model of diabetes with obesity.

From the point of view of side effects or safety, the several clinical trials indicated that SGLT2 inhibitors increased frequency of urinary and genital infections, including asymptomatic candiduria and vulvovaginal candidiasis (18, 41, 42, 43). It has been reported that diabetes mellitus is associated with a high risk of infections (44, 45). UTI is a common clinical problem in diabetic patients (46, 47). Diabetic women, in particular, are considered to be more likely to have asymptomatic bacteriuria and symptomatic UTI, including fungal infections (particularly *Candida* infections) (48, 49).

Although the presence of glucosuria can possibly explain the presence of UTI in patients with diabetes, the actual relationship between UTI and glucosuria remains uncertain. Enhanced bacterial growth was observed in in vitro studies with the high glucose concentrations relevant to the UGC of poorly controlled patients (50); however, it could not be confirmed that glucosuria was a risk factor for UTI in diabetic women (51). Because pathological conditions such as hyperglycaemia, microvascular disease and neuropathy might also be associated with susceptibility to infection (43, 52), there are limitations to clinical evaluations of the relationship between UTI and UGC. For the purpose of evaluating the effects of UGC on the risk of UTI, the use of UTI animal models can be an option.

Published murine models of UTI include hematogenous spread or ascending infection routes, with or without urethral ligature or the insertion of a foreign body to impede urinary flow (53, 54, 55). For analogy with human UTI, the animal model used to evaluate the impact of glucosuria will preferably be one with the ascending rather than the hematogenous infection route and without urinary tract obstruction. However, normal urine flow is a major protective factor that prevents microbial colonization of

the urinary tract, and the spread of apparent ascending infection is not observed in the unobstructed murine UTI models when microbes are inoculated into the bladder.

The susceptibility of streptozotocin-induced diabetic mice to UTI is reported to be higher than that of the normal mice (56). However, the UTI of these diabetic animal models might be influenced by several mechanisms in addition to glucosuria, including immunologic impairment due to their hyperglycaemia (57). Therefore, to accurately examine the impact of glucosuria on UTI, a unique model of UTI is required.

In the clinical trials of several SGLT2 inhibitors, increased frequency of urinary and genital infections, including asymptomatic candiduria and vulvovaginal candidiasis, was reported (18, 41, 42, 43), although the infections were mild and were resolved with routine antifungal therapy (41). From these trials, it can be inferred that increased UGC plays a role in the increased incidence of UTI. In addition, a tendency towards an increased prevalence of *Candida* species in the urine of diabetic patients treated with canagliflozin was reported (42), suggesting that *Candida* species also contribute to the increased rate of UTI as uropathogens.

In the third study “Mechanism of Inducing Urinary Tract Infection in Novel Animal Model” (58), to clarify the relationship between glucosuria and UTI, we constructed a novel mouse model of ascending UTI with normoglycaemic mice and *Candida albicans* as an uropathogen. We evaluated the effect of glucosuria induced by SGLT2 inhibition for various lengths of time on the susceptibility of the mice to the *Candida*-induced UTI. Our data indicate that increased susceptibility to UTI is associated with a persistent increase in UGC.

To evaluate efficacy and side-effects of tofogliflozin, we utilized many animal models in this investigation. In the first study “Fundamental Profile by in Vitro and in Vivo Pharmacological Studies”, leptin-receptor-deficient models such as ZDF rat and db/db mouse were used. In these animal models, one of the most popular T2D animal models, hyperphagia is exhibited, and obesity, insufficient insulin secretion, insulin

resistance, and severe hyperglycemia are accompanied with their ageing. In general, because urine volume is grater in the rat compared to in the mouse, urine glucose could be measured more correctly in the rat. Therefore, we used ZDF rats to evaluate effects on UGE and causal plasma glucose level after single oral gavage of tofogliflozin. On the other hand, the mouse needs smaller volume of drug compounds and narrower space to live, so we selected db/db mice to evaluate long-term effect on plasma glucose level and pathological index (glucose intolerance) after repeated oral administration. GK rat is a non-obese Wistar substrain which develops early in life T2D with mild hyperglycemia, hyperinsulinemia, and insulin resistance. We used this model to evaluate effects on postpradial plasma glucose level. SD rat was used to evaluate hypoglycemia induction via tofogliflozin, one of the most frequent side effects of medicines for diabetes.

In the second study, “Mechanism of Body Weight Reduction in Obese Animal Models” DIO rat and KKAy mouse were used to analysis the mechanism of body weight reduction. DIO rat is obese animal model induced by eating high-calorie diet containing fats, generally accompanied with mild insulin resistance and hyperglycemia in the long-term (more than 6 months) experiment. KKAy mouse is a T2D animal model maintaining insulin secretion, accompanied with obesity, severe insulin resistant, and sever hyperglycemia.

In the third study, “Mechanism of Inducing Urinary Tract Infection in Novel Animal Model” because we wanted to analysis the direct effect of high UGC on infection, normal mouse strain, C57B6J, was used for UTI model. In the mouse without hyperglycemia, vascular insufficiency, neuropathy, and immunologic impairment, impact of glucosuria on UTI could be evaluated.

IV. Fundamental Profile by in Vitro and in Vivo Pharmacological Studies

1. Materials and Methods

Chemicals

Tofogliflozin ((1*S*, 3′*R*, 4′*S*, 5′*S*, 6′*R*) -6-[(4-ethylphenyl)methyl]-3′,4′,5′,6′-tetrahydro-6′-(hydroxymethyl)-spiro[isobenzofuran-1(3*H*),2′-[2*H*] pyran]-3′,4′,5′-triol) (59; Fig. I-1), dapagliflozin (60), canagliflozin (61), ipragliflozin (62), empagliflozin (63), luseogliflozin (64), PF-04971729 (65) were all synthesized in our laboratories at Chugai Pharmaceutical Co.

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

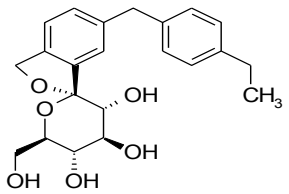


Fig. I-1 Structure of tofogliflozin (CSG452).

Animals

Male SD rats (CD/Crl) and ZDF rats (ZDF/Crl-Lepr^{fa}; fa/fa) were purchased from Charles River Laboratories Japan (Yokohama, Japan). Male db/db mice (BKS.Cg-+ Lepr^{db}/+ Lepr^{db}/Jcl) and GK rats (GK/Jcl) were purchased from Clea Japan (Tokyo,

Japan). Male Wistar rats (Slc:Wistar) were purchased from Japan SLC (Shizuoka, Japan). These animals were housed under a 12-h/12-h light/dark cycle (lights on 7:00 AM–7:00 PM) with controlled room temperature (20–26°C) and humidity (35–75%), and were allowed *ad libitum* access to a diet of laboratory chow (Purina5008 pellets [PMI Nutrition International LLC, St. Louis, MO, USA] for ZDF rats; CE-2 pellets [Clea Japan] for other animals) and water. All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical Co., Ltd. The protocols were approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical.

SGLT inhibition

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

kidney fibroblast cells (COS-7; ATCC), and the cells transiently expressing each SGLT were used for the AMG or fructose uptake assay.

For the AMG, MI, or fructose uptake assay, the cells expressing each SGLT were cultured in 96-well plates for 2 or 3 days and washed twice with sodium-free buffer (140 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris pH7.4). The cells were then incubated in sodium-free buffer or sodium buffer (140 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris pH7.4) each containing 1mM AMG (mixture of non-radiolabeled AMG and [^{14}C]-AMG), 0.1 mM MI (mixture of non-radiolabeled MI and [^{3}H]-MI), 0.1 mM fructose (mixture of non-radiolabeled fructose and [^{14}C]-fructose) at 37°C for 45 min. Sodium-dependent AMG, MI, or fructose uptake was calculated by subtracting the radioactivity detected in cells incubated in the sodium-free buffer from the radioactivity detected in the cells incubated in the sodium buffer. IC_{50} values of inhibitors were calculated with the empirical 4-parameter model fitting of XLfit (IDBS, Guildford, UK) and are indicated as mean ± S.D. of 3 to 12 independent experiments. For measurement of K_i values, the AMG uptake assays were performed in sodium buffer or sodium-free buffer containing various concentrations of AMG. K_i values of inhibitors were calculated from Lineweaver-Burk plots.

Effects on cellular functions relating to glucose metabolism

Glucose uptake was evaluated by 2DG uptake assay in XM13A1 cells, a temperature-sensitive SV40 large T-antigen immortalized cell line derived from human skeletal muscle (Lonza, Basel, Switzerland), differentiated L6 rat skeletal muscle cells (L6 cells; ATCC), and mouse adipocytes (3T3-L1 cells; ATCC) according to previously described methods (66, 67), in the presence or absence of tofogliflozin with or without stimulation with 100 or 1000 nM insulin (Sigma-Aldrich) using [^{3}H]-2DG as a substrate.

Glycogen synthesis was evaluated in XM13A1 cells according to the method of Anand et al. (68). Briefly, after overnight serum starvation in starvation medium (Ham's F-10 Medium containing 0.2% bovine serum albumin [BSA; Sigma-Aldrich] and 10 mM HEPES pH7.2–7.5) cells were incubated in starvation medium plus 175 kBq/mL [^{14}C]-glucose with or without 1 μM insulin in the presence or absence of tofogliflozin at 37°C for 2 h. Glycogen in the lysate of the cells was precipitated by ethanol, and insulin-stimulated glycogen synthesis was calculated by subtracting the radioactivity detected in glycogen of non-insulin-stimulated cells from that in insulin-stimulated cells.

Glucose oxidation was evaluated in XM13A1 cells. After overnight serum starvation, cells were incubated with [^{14}C]-glucose with or without tofogliflozin at 37°C for 4 h. [^{14}C]-CO_2 content in air exhausted from the culture medium was trapped on a LumaPlate-96 (PerkinElmer, Waltham, MA, USA) and glucose oxidation activity was calculated.

Hepatic glucose production was evaluated in rat primary hepatocytes isolated from the liver of SD rats with standard methods (69). The hepatocytes were cultured in a medium containing 1.25% BSA, 5% fetal bovine serum, 20 nM insulin, and 20 mM glucose. The cells were then incubated at 37°C for 2 h in a buffer containing 0.5 nM glucagon (Peptide Institute, Osaka, Japan) in the presence or absence of tofogliflozin. Glucagon-induced glucose production was calculated by measuring glucose concentration in the medium with an Amplex Red Glucose Assay Kit (Invitrogen).

GSIS was evaluated in pancreatic islets isolated from Wistar rats with standard methods (70). After the incubation of islets with 3.3 or 16.7 mM glucose in the presence or absence of tofogliflozin at 37°C for 1 h, insulin concentrations in the media were measured and GSIS was calculated from the difference between the insulin concentrations in the supernatant of the 3.3 and 16.7 mM glucose-stimulated islets.

Glucosidase inhibition

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

Activities of rat intestinal glucosidases were measured by using extracts of rat intestinal acetone powder (Sigma-Aldrich) as partially purified glucosidases based on a previously reported method (71). Partially purified glucosidases from XPEMB1 cells (human skin fibroblast cells; ATCC) were used to measure the activities of human lysosomal glucosidases (72). Each of 4-methylumbelliferyl α-D-glucopyranoside (Sigma-Aldrich), 4-methylumbelliferyl ß-D-glucopyranoside (Sigma-Aldrich), 4-methylumbelliferyl ß-D-galactopyranoside (Sigma-Aldrich), 4-methylumbelliferyl α-D-mannopyranoside (Sigma-Aldrich), and 4-methylumbelliferyl ß-D-mannopyranoside (Sigma-Aldrich) was used as a substrate for the reaction of α-glucosidase, ß-glucosidase, ß-galactosidase, α-mannosidase, and ß-mannosidase, respectively (73). A reaction mixture containing partially purified glucosidases, 250 mM sodium citrate pH4.2, and 40–160 μM substrate, with or without tofogliflozin, was incubated at room temperature for 1–3 h. Enzyme activity was detected by measuring 4-methylumbelliferone concentration in the reaction mixture in terms of fluorescence (excitation wavelength 360 nm/emission wavelength 450 nm).

Binding on various receptors, channels, and transporters

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

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

Receptor					
A1	human	GABA	human	NOP	human
A2A	human	GAL1	human	PAC1	human
A3	human	GAL2	human	PPAR	human
a1	rat	PDGF	mouse	PCP	human
a2	rat	CXCR2	human	EP4	human
b1	human	CCR1	human	TP	human
b2	human	H1	human	IP	human
AT1	human	H2	human	P2X	rat
AT2	human	MC4	human	P2Y	rat
BZD (central)	rat	MT1	human	5-HT1A	human
BZD (peripheral)	rat	M1	human	5-HT1B	rat
BB	rat	M2	human	5-HT2A	human
B2	human	M3	human	5-HT2B	human
CGRP	human	M4	human	5-HT2C	human
CB1	human	M5	human	5-HT3	human
CCK1	human	NK1	human	5-HT5A	human
CCK2	human	NK2	human	5-HT6	human
D1	human	NK3	human	5-HT7	human
D2S	human	Y1	human	s	rat
D3	human	Y2	human	sst	mouse
D4.4	human	NTS1	human	GR	human
D5	human	DOP	human	VPAC1	human
ETA	human	KOP	human	V1a	human
ETB	human	MOP	human		

Channel	
Ca2+ channel	rat
KV channel	rat
SKCa channel	rat
Na+ channel	rat
Cl- channel	rat

Transporter	
norepinephrine transporter	human
dopamine transporter	human
5-HT transporter	human

Inhibition (%) is expressed as percentage of inhibition of control-specific binding in the presence of tofogliflozin (10 μmoL/L).

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

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle (0.5% carboxymethylcellulose sodium salt (CMC); 5 mL/kg) was orally administered to non-fasted ZDF rats (male, 10 weeks of age). Blood samples were collected from the tail vein immediately before administration (0 h) and at 1, 2, 4, 6, 8, 12, and 24 h after administration, and plasma glucose levels and concentrations of tofogliflozin in the plasma were measured. Urine samples were collected from the rats' metabolic cages every 4 h from immediately after administration to 12 h after administration, and the urine volume and the UGC were measured. Renal glucose clearance was determined by dividing the amount of UGE for each urine collection period (4-h cumulative urine sample) by the plasma glucose area under the curve (Glu AUC) for each respective 4-h period (Glu AUC_{0-4h}, Glu AUC_{4-8h},

or Glu AUC_{8-12h}). These Glu AUCs were determined by using the trapezoidal rule. Blood and urinary glucose concentrations were measured by the hexokinase method (Autosera S GLU; Sekisui Medical Co., Tokyo, Japan). Plasma tofogliflozin concentrations were measured with an LC-MS/MS system (high performance liquid chromatography [Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA]; tandem quadrupole mass spectrometer [API 4000:S112; Applied Biosystems, Foster City, CA, USA]). Pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-24}, $t_{1/2}$) were obtained from the quantitative values of each rat by using WinNonlin ver.4.1 (Pharsight, St. Louis, MO, USA).

Plasma glucose lowering effects in diabetic mice (db/db mice)

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

Postprandial plasma glucose lowering effects in diabetic rats (GK rats)

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

Plasma glucose lowering effects and improvement of glucose intolerance by long-term administration in diabetic mice (db/db mice)

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

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

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

Statistical analysis

Data are presented as mean ± S.D. in in vitro experiments and mean ± SEM in in vivo experiments. Statistical analysis was performed by using SAS System for

Windows, Release 8.02 (SAS Institute Japan, Tokyo, Japan). Statistical significance was determined by the parametric Dunnett's multiple comparison.

2. Results

In vitro SGLT2 inhibition and SGLT2 selectivity

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

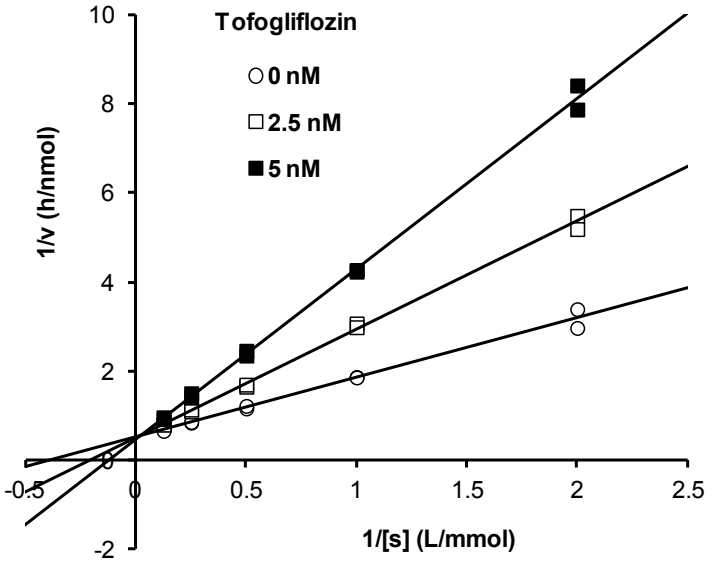


Fig. I-2 Inhibition of hSGLT2 by tofogliflozin.

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

Next, the inhibitory activities of tofogliflozin and other SGLT inhibitors against the 7 human SGLTs (hSGLT1, hSGLT2, hSGLT3, hSGLT4, hSGLT5, hSGLT6, and hSMIT1) were compared in SGLT-overexpressing cells (CHO, HEK293, or COS-7) by measuring sodium-dependent sugar (AMG, fructose, or MI) uptake (Table I-2). Tofogliflozin showed the highest selectivity toward hSGLT2 versus hSGLT1, hSGLT6, and hSMIT1. The compounds with the highest selectivity toward hSGLT2 versus hSGLT3, hSGLT4 and hSGLT5 were dapagliflozin, luseogliflozin, and PF-04971729, respectively, indicating that among the SGLT inhibitors tested tofogliflozin was the most selective SGLT2 inhibitor.

Table I-2 SGLT2 IC_{50} values and SGLT2 selectivity of SGLT2 inhibitors.

Inhibitor	IC_{50} (nM)	SGLT2 selectivity					
	hSGLT2	hSGLT1	hSGLT3	hSGLT4	hSGLT5	hSGLT6	hSMIT1
Tofogliflozin	2.9 ± 0.7	2900	19000	1500	540	6200	28000
Dapagliflozin	1.3 ± 0.2	610	190000	3000	210	1300	22000
Canagliflozin	6.7 ± 2.9	290	52000	2800	180	200	5600
Ipragliflozin	2.8 ± 0.5	860	7700	4500	87	3500	21000
Empagliflozin	3.6 ± 1.6	1100	62000	2200	110	1100	8300
Luseogliflozin	3.1 ± 0.1	1600	8100	9800	280	220	7800
PF-04971729	1.4 ± 0.1	1300	>71000	2300	3400	980	26000
Phlorizin	16.4 ± 5.2	11	1300	490	36	1000	25000

SGLT2 selectivity is expressed as the ratio of IC_{50} of a compound against hSGLT1, hSGLT3, hSGLT4, hSGLT5, hSGLT6, or hSMIT1 to its IC_{50} against hSGLT2.

In addition to the selectivity of tofogliflozin toward hSGLT2 versus that toward other human SGLTs, the inhibitory activities of tofogliflozin against rat and mouse SGLT1 and SGLT2 were compared. Tofogliflozin inhibited both rat and mouse SGLT2 at lower concentrations than phlorizin: IC_{50} values of tofogliflozin against rat and mouse SGLT2 were 14.5 ± 1.9 and 5.0 ± 1.2 nM, and those of phlorizin were 48.2 ± 11.4

and 16.8 ± 5.6 nM, respectively. Moreover, tofogliflozin was more selective than phlorizin: the selectivity of tofogliflozin toward SGLT2 was 560 times that toward rSGLT1 and 360 times that toward mSGLT1 (IC_{50} for rSGLT1, 8200 ± 1900 nM; IC_{50} for mSGLT1, 1800 ± 870 nM), whereas the selectivity of phlorizin toward SGLT2 was 20 times that toward rSGLT1 and 19 times that toward mSGLT1 (IC_{50} for rSGLT1, 970 ± 180 nM; IC_{50} for mSGLT1, 310 ± 110 nM).

Effects on non-SGLT-related reactions and functions

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

Table I-3 Inhibition activity of tofogliflozin against glucose-related cellular functions.

Action	Source	Detection	Tofogliflozin (μmol/L)	Inhibiton (%, Mean±SD)	n
Glucose uptake	XM13A1 cells (human myoblast)	[^{3}H] labeled 2-deoxy-glucose uptake	100	3.3, 0.8	2
	L6 cells (rat myoblast)		100	13.0 ± 2.1	3
	3T3-L1 cells (mouse adipocyte)		100	24.3 ± 6.6	3
Glucose oxidation	XM13A1 cells	[^{14}C]-CO_2 production from D-[^{14}C] glucose	100	19.3, 20.2	2
Glycogen synthesis	XM13A1 cells	Incorporation of ^{14}C into glycogen from D-[^{14}C] glucose	100	23.8, 13.7	2
Hepatic glucose production	Primary rat hepatocytes	Glucose production to culture medium	100	-8.3	1
Glucose stimulated Insulin secretion	Isolated rat pancreatic islets	Insulin secretion to culture medium	10	-4.4 ± 16.0	3

Glucosidases also play critical roles in glucose metabolism; therefore, the effects of tofogliflozin on several glucosidase activities were also evaluated by using purified human pancreatic α-amylase, partially purified rat intestinal glucosidases, or partially purified human lysosomal glucosidases from XPEMB1 cells. Tofogliflozin (100 μM) exhibited no marked inhibition against these enzymes (Table I-4).

Table I-4 Inhibition activity of tofogliflozin against glucosidase reactions.

Action	Source	Detection	Tofogliflozin (μmol/L)	Inhibiton (%, Mean±SD)	n
Glucosidase reaction		Digestion of substrate			
Human α-amylase	Purified amylase from human pancreas	Starch	100	-9.3 ± 10.8	3
Rat glucoamylase	Partially purified glucosidases from rat intestinal acetone powder	Starch	100	4.7 ± 0.4	3
Rat sucrase		Sucrose	100	-0.2 ± 3.1	3
Rat trehalose		Trehalose	100	5.2 ± 15.1	3
Rat isomaltose		Isomaltose	100	7.8 ± 1.1	3
Human α-glucosidase	Partially purified glucosidases from XPEMB1cells (human skin fibroblast)	4MU-α-D-glucopyranoside	100	-7.0 ± 19.0	3
Human β-glucosidase		4MU-β-D-glucopyranoside	100	-11.5 ± 4.8	3
Human β-galactosidase		4MU-β-D-galactopyranoside	100	-2.4 ± 2.3	3
Human α-mannosidase		4MU-α-D-mannopyranoside	100	-5.6 ± 12.6	3
Human β-mannosidase		4MU-β-D-mannopyranoside	100	-9.0 ± 2.7	3

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

At 10 μM of tofogliflozin no obvious effect was detected in any of these assays. Therefore, the IC_{50} values of tofogliflozin against these reactions were over 600 times the IC_{50} value of tofogliflozin against SGLT2.

Table I-5 Inhibition activity of tofogliflozin on the specific ligand binding to receptors, channels, and transporters.

Receptor

Target	Origin	Inhibition (%)	Target	Origin	Inhibition (%)	Target	Origin	Inhibition (%)
A1	human	-5	GABA	human	-10	NOP	human	-2
A2A	human	15	GAL1	human	0	PAC1	human	-9
A3	human	-10	GAL2	human	-7	PPAR	human	1
a1	rat	4	PDGF	mouse	-6	PCP	human	-7
a2	rat	-9	CXCR2	human	1	EP4	human	-15
b1	human	-8	CCR1	human	13	TP	human	19
b2	human	-2	H1	human	-6	IP	human	-9
AT1	human	-32	H2	human	6	P2X	rat	-2
AT2	human	-4	MC4	human	1	P2Y	rat	7
BZD (central)	rat	14	MT1	human	2	5-HT1A	human	-8
BZD (peripheral)	rat	1	M1	human	12	5-HT1B	rat	-9
BB	rat	-6	M2	human	8	5-HT2A	human	17
B2	human	0	M3	human	6	5-HT2B	human	46
CGRP	human	15	M4	human	-5	5-HT2C	human	-11
CB1	human	4	M5	human	5	5-HT3	human	8
CCK1	human	-23	NK1	human	-3	5-HT5A	human	0
CCK2	human	10	NK2	human	-16	5-HT6	human	6
D1	human	-12	NK3	human	15	5-HT7	human	-1
D2S	human	0	Y1	human	-32	s	rat	11
D3	human	0	Y2	human	-3	sst	mouse	7
D4.4	human	-3	NTS1	human	-1	GR	human	-9
D5	human	2	DOP	human	-1	VPAC1	human	-4
ETA	human	-2	KOP	human	7	V1a	human	13
ETB	human	3	MOP	human	-4			

Channel

Target	Origin	Inhibition (%)
Ca^{2+} channel	rat	1
KV channel	rat	-5
SKCa channel	rat	2
Na^{+} channel	rat	18
Cl^{-} channel	rat	5

Transporter

Target	Origin	Inhibition (%)
norepinephrine transporter	human	2
dopamine transporter	human	8
5-HT transporter	human	-5

Details of the methodologies for each assay can be found at http://www.cerep.fr.

Increase of renal glucose clearance and reduction of plasma glucose levels in ZDF rats

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

Changes in plasma tofogliflozin concentrations in these rats were examined over time (Fig. I-3C). After administration, plasma tofogliflozin concentration rapidly increased in a dose-dependent manner. At 3 mg/kg, plasma tofogliflozin concentration

peaked (C_{max}: 1.39 μg/mL, 3600 nM) at 2 h after administration (T_{max}: 1.42 h), then the concentration decreased ($t_{1/2}$: 2.9 h) to 0.069 μg/mL (180 nM) at 12 h (Table I-6).

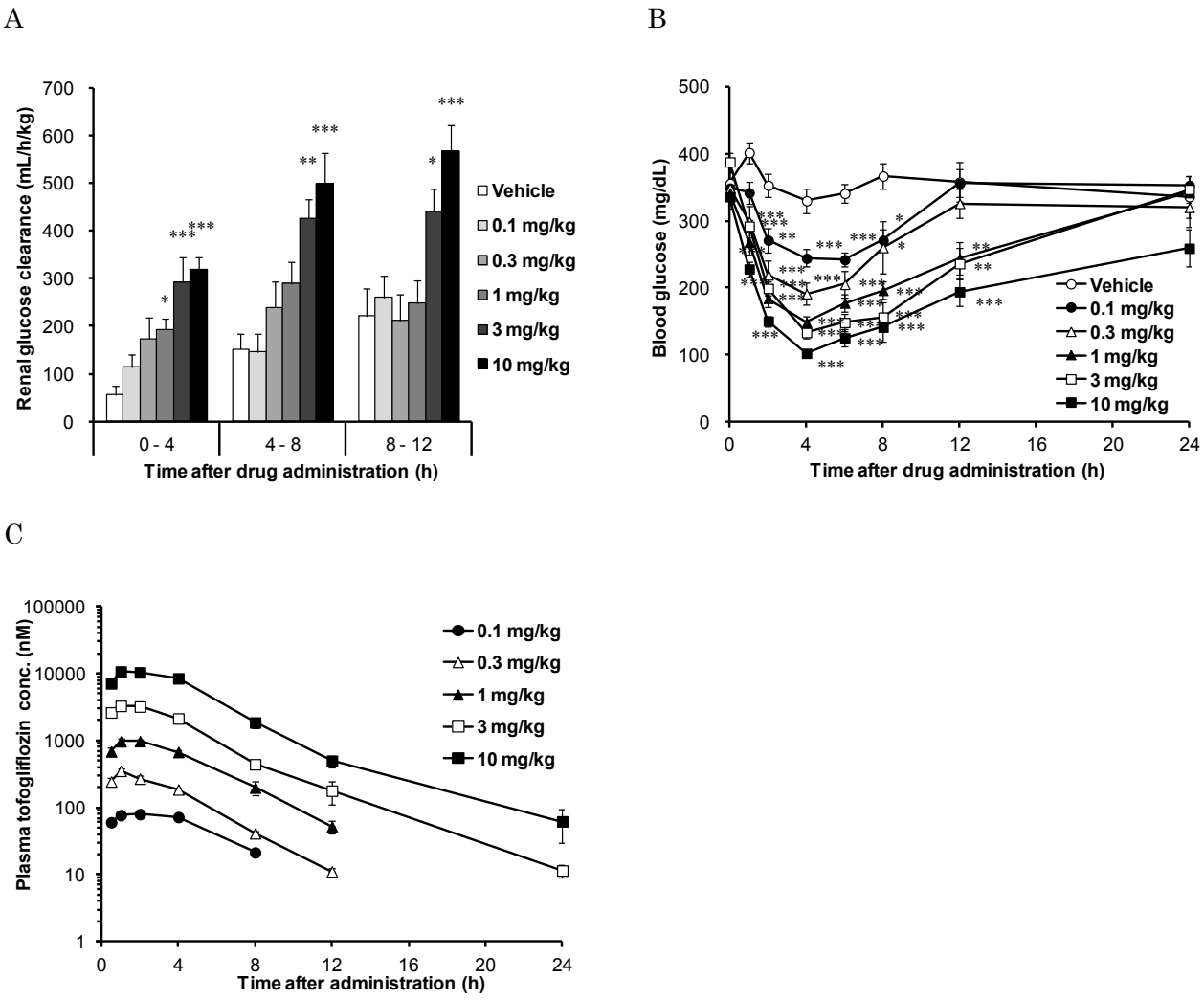


Fig. I-3 Effects of single oral administration on plasma glucose level and renal glucose clearance in ZDF rats.

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle was administered to ZDF rats by oral gavage. A) Renal glucose clearance was calculated as described in the text every 4 h for 12 h after drug administration. B) Plasma glucose levels were determined before (0 h) and at 1, 2, 4, 6, 8, 12 and 24 h after drug administration under non-fasting conditions. C) Plasma tofogliflozin concentrations were measured at 0.5, 1, 2, 4, 8, 12, and 24 h after drug administration. Data are expressed as mean ± SEM (n=6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle-treatment group by Dunnett's multiple comparison test.

Table I-6 Tofogliflozin pharmacokinetic parameters in ZDF rats and db/db mice.

Animal	Dose (mg/kg)	n	C_{max} (μg/mL)	T_{max} (h)	AUC_{0-24} (μg・h/mL)	$t_{1/2}$ (h)
ZDF rats	0.1	6	0.03	2.50	0.20	NC
	0.3	6	0.14	1.00	0.59	2.4
	1	6	0.42	2.00	2.10	2.8
	3	6	1.39	1.42	6.49	2.9
	10	6	4.63	2.33	22.97	3.0
db/db mice	0.1	3	0.03	0.25	0.07	2.6
	0.3	3	0.08	0.50	0.25	2.4
	1	3	0.35	0.25	0.97	2.7
	3	3	1.17	0.25	3.16	3.1
	10	3	4.38	0.25	10.10	2.7

NC: Not calculated

Improvement of hyperglycemia in db/db mice

The acute effect of tofogliflozin on plasma glucose levels in non-fasted db/db mice was then tested to confirm the glucose-lowering effect also in mice. Tofogliflozin rapidly reduced plasma glucose levels in a dose-dependent manner, and the reduction of plasma glucose levels was maintained until 12 h after drug administration at dosages of 1 mg/kg or higher (Fig. I-4). The maximum glucose lowering effects were observed at 4 h after administration in the 0.1 to 1 mg/kg groups and 6 h after administration in the 3 and 10 mg/kg groups. As was the case with the ZDF rats, the plasma glucose levels of db/db mice also fell within the normal range at the highest dose but were not lowered to below 100 mg/dL.

The plasma tofogliflozin concentration in db/db mice increased rapidly just after the administration, and the increment was dose-dependent. Pharmacokinetic parameters at 10 mg/kg tofogliflozin are as follows: T_{max} 0.25 h, C_{max} 4.38 μg/mL and $t_{1/2}$ 2.7 h (Table I-6).

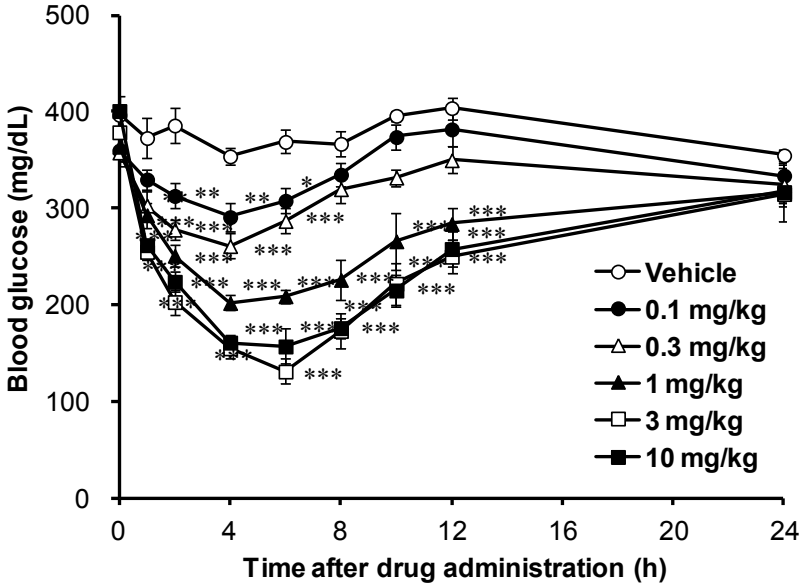


Fig. I-4 Effects of single oral administration on plasma glucose level in db/db mice.

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) or vehicle was administered to db/db mice by oral gavage. Plasma glucose levels were determined before (0 h) and at 1, 2, 4, 6, 8, 10, 12, and 24 h after drug administration under non-fasting conditions. Data are expressed as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett's multiple comparison test.

Suppression of postprandial hyperglycemia in GK rats

In addition to the acute hypoglycemic effect in diabetic animals under non-fasted conditions, we examined the suppressive effect of tofogliflozin on postprandial glucose increase using GK rats, a non-obese animal model of T2D with glucose intolerance. In these animals, voglibose (0.1, 0.3, or 1 mg/kg), an α-glucosidase inhibitor used for the improvement of postprandial hyperglycemia in the clinic, suppressed plasma glucose increase dose-dependently from 15–30 min to 4 h after meal loading (Fig. I-5A). The suppressive effects of tofogliflozin (1, 3, or 10 mg/kg) on glucose increase after meal loading (Fig. I-5B) were comparable to those of voglibose, indicating that tofogliflozin improves postprandial hyperglycemia.

A B

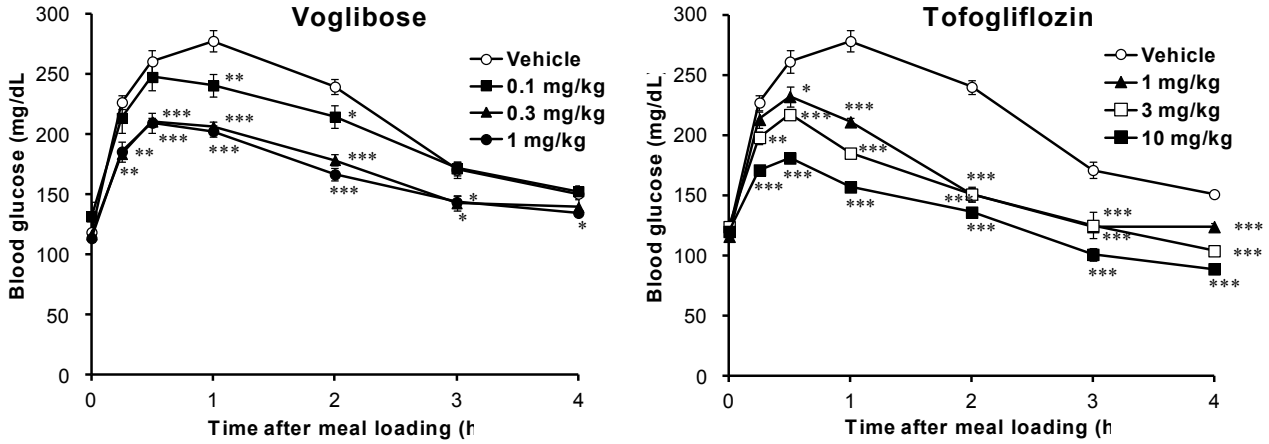


Fig. I-5 Effects of single oral administration on postprandial plasma glucose levels during meal tolerance test in GK rats.

GK rats were fasted overnight, and voglibose (0.1, 0.3, or 1 mg/kg), tofogliflozin (1, 3, or 10 mg/kg) or vehicle was administered by oral gavage 5 min before meal administration. Plasma glucose levels were determined before (0 h) and at 0.25, 0.5, 1, 2, 3, and 4 h after meal administration. Data are expressed as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett's multiple comparison test.

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

To examine the long-term effects of tofogliflozin on hyperglycemia and glucose tolerance in db/db mice, tofogliflozin was orally administered once daily for 4 weeks at the doses at which acute plasma glucose reduction was observed (0.1–10 mg/kg). Tofogliflozin dose-dependently reduced glycated Hb after 4-week administration, and the glycated Hb levels at doses of 0.3 mg/kg and over were significantly lower than in the group treated with vehicle only (Fig. I-6A). During the 4-week administration, no difference in food intake or body weight due to the tofogliflozin treatment was observed. Although the plasma IRI levels of the vehicle group decreased from 23.0 ±

2.0 ng/mL on day −1 to 6.6 ± 0.7 ng/mL on day 28, tofogliflozin significantly prevented the decrease of IRI levels at doses of 3 and 10 mg/kg (Fig. I-6B).

Four days after the final administration, an OGTT was performed to test whether long-term glucose control by tofogliflozin improves glucose tolerance. In the groups treated with 0.3 mg/kg or more of tofogliflozin, plasma glucose levels at 4 h after glucose loading were reduced compared with the vehicle group (Fig. I-6C); moreover, plasma glucose AUC_{0-4h} of the 3 and 10 mg/kg groups were lower than that of the vehicle group (Fig. I-6D). Therefore, long-term administration of tofogliflozin improved hyperglycemia and thereby ameliorated glucose intolerance of the obese diabetic mice.

Effects on UGE and plasma glucose levels in normal rats

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

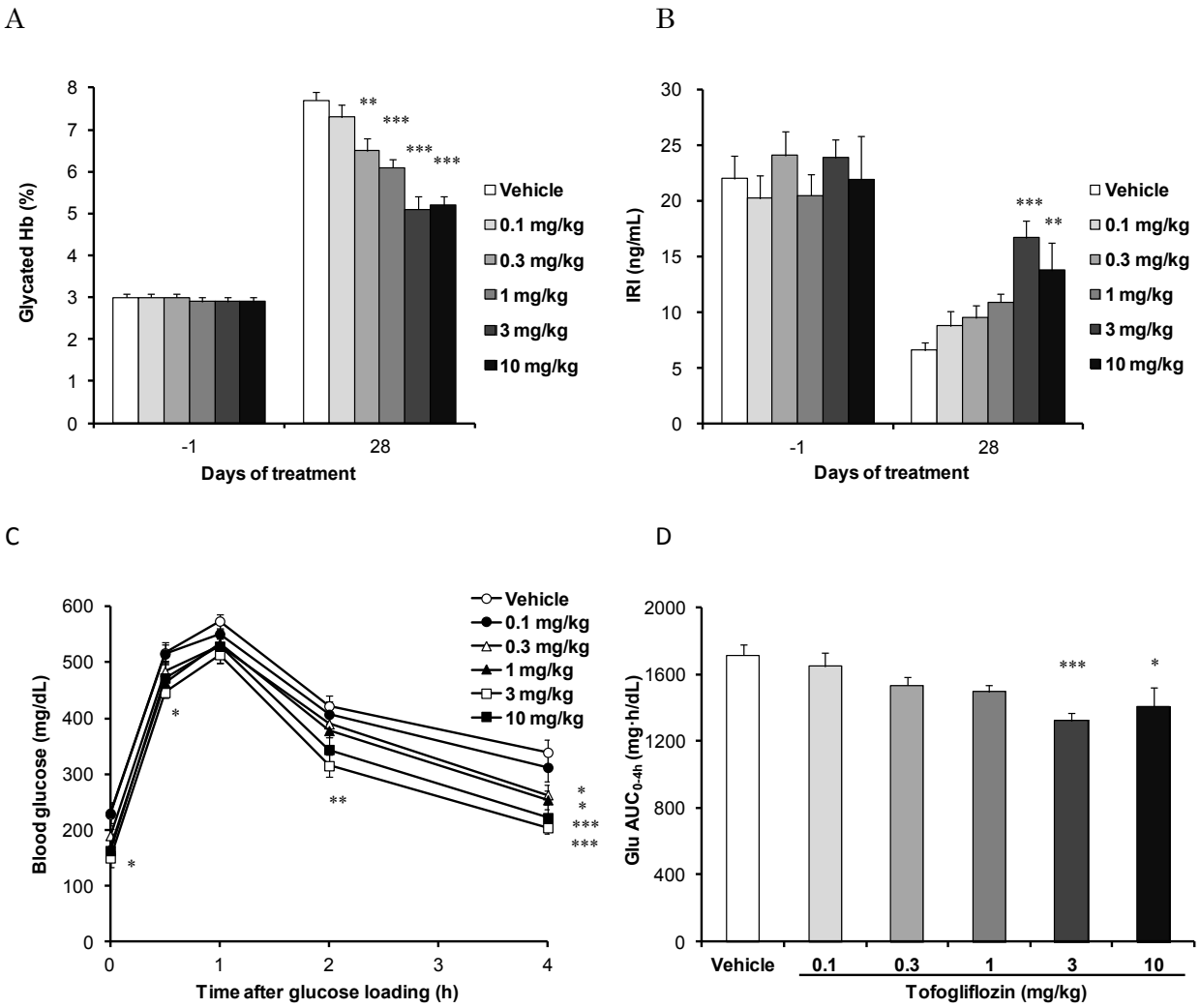


Fig. I-6 Effects of 4-week repetitive administration on glycated hemoglobin (Hb) in db/db mice.

Tofogliflozin (0.1, 0.3, 1, 3, or 10 mg/kg) was administered once daily between 17:00 and 19:00 by oral gavage for 4 weeks. Blood was collected between 09:00 and 12:00 on day −1 and day 28. A) Plasma glycated Hb levels. B) Plasma IRI levels. C) Changes in plasma glucose level in the OGTT 4 days after final administration in mice fasted overnight. Plasma glucose levels were determined before (0 h) and at 0.5, 1, 2, and 4 h after oral glucose administration (3 g/kg). D) Plasma glucose AUC0–4h (Glu AUC0–4h) calculated by the trapezoidal rule. Data are expressed as mean ± SEM (n=10). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treatment group by Dunnett's multiple comparison test.

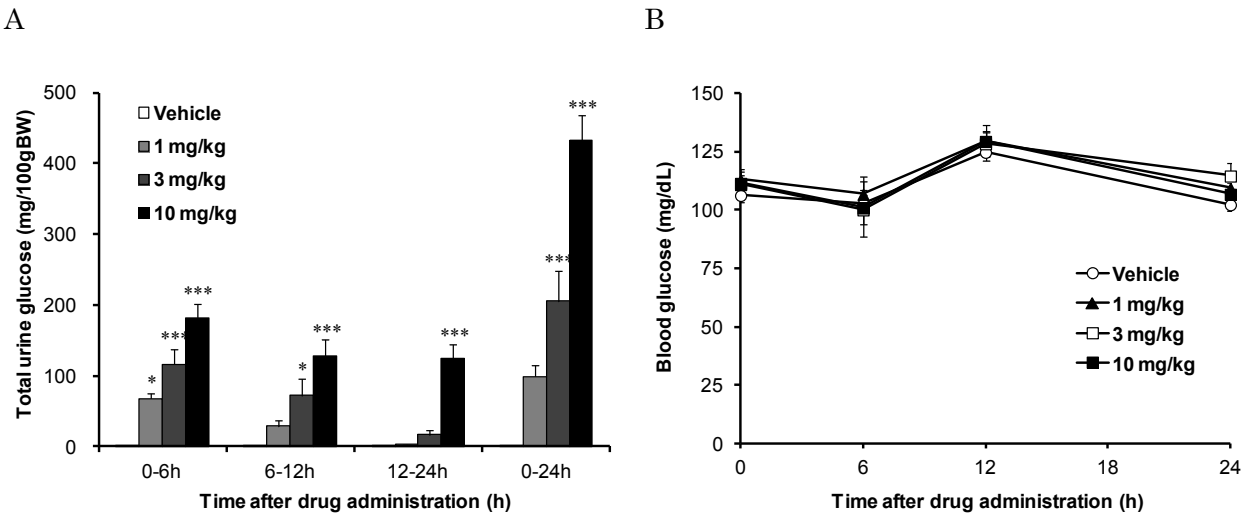


Fig. I-7 Effects of single oral administration on UGE in SD rats.

Tofogliflozin (1, 3, or 10 mg/kg) or vehicle was administered to SD rats by oral gavage. The amounts of UGE were calculated from urine volume and urine glucose concentration at 0–6, 6–12, and 12–24 h after drug administration under non-fasting conditions. A) Total urine glucose. B) Plasma glucose level. Data are expressed as mean ± SEM (n=6). *p < 0.05, ***p < 0.001 versus vehicle-treatment group by Dunnett's multiple comparison test.

3. Discussion

Pharmaceutical companies have been trying to find potent and highly bioavailable SGLT2 inhibitors, and several SGLT2 inhibitors are currently being launched for diabetes treatment. Dapagliflozin, one of such compounds, induced UGE and reduced plasma glucose and HbA1c levels in patients with T2D (18), indicating that SGLT2 inhibition is a practical drug target for the treatment of diabetes.

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

As shown in this study, tofogliflozin is a potent SGLT2 inhibitor and the most selective among the compounds in this class. The highly selective inhibition of SGLT2 by tofogliflozin is an important characteristic because safe and efficacious antidiabetics are needed and SGLT family members are thought to play a number of important physiological roles.

Acarbose, an antidiabetic in the class of α-glucosidase inhibitors, increases gastrointestinal events such as flatulence, abdominal pain, and diarrhea (74). Since tofogliflozin did not inhibit intestinal glucosidases in vitro, it may have a low-risk of adverse gastrointestinal effects. In addition, the absence of any inhibitory activity of tofogliflozin against lysosomal glucosidases suggests a low-risk of undesirable side effects, because lysosomal glucosidase deficiency is known to be responsible for various lysosomal diseases (75, 76). The results of comprehensive in vitro binding assays to molecular targets (71 receptors, 5 ion channels, 3 transporters; shown in Table I-5) also suggest no off-target effects of tofogliflozin. Although a low safety concern is suggested from the high selectivity of tofogliflozin to SGLT2, more extensive and

comprehensive analyses in long-term clinical studies with a large number of patients will be needed to confirm the safety of tofogliflozin.

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

Moreover, we could account for the plasma glucose lowering effects of tofogliflozin by the amount of UGE induced in ZDF rats. Tofogliflozin increased UGE in the period 0–4 h after administration, and the calculated maximum difference in UGE between the vehicle and tofogliflozin groups was approximately 8 mg/kg/min. It is reported that endogenous glucose production is approximately 14 mg/kg/min in ZDF rats as compared with approximately 5–6 mg/kg/min in non-diabetic rats (77), and the difference was comparable to the UGE induced by tofogliflozin as described above.

On the other hand, plasma glucose levels are influenced by various factors such as insulin secretion and the insulin sensitivity of skeletal muscle, liver, and adipose tissue. We observed that tofogliflozin induced acute glucose-lowering effects without

an increase in plasma insulin levels in ZDF rats (data not shown). These in vivo observations together with the results of the in vitro experiments discussed above suggest that tofogliflozin's glucose-lowering effects are independent of pancreatic insulin secretion and insulin sensitivity. These experimental data indicated that by inducing UGE with selective inhibition of SGLT2, tofogliflozin could substantially improve hyperglycemia in diabetic rodent models.

It is believed that daily management of plasma glucose levels could improve the pathological condition of diabetes. In our experiments on db/db mice, administration of tofogliflozin for 4 weeks reduced glycated Hb levels and prevented IRI reduction. Furthermore glucose tolerance was improved in mice treated with 3 or 10 mg/kg tofogliflozin. These results suggest that long-term treatment with tofogliflozin may improve the pathological condition of diabetes by better preservation of β-cell function. Since glucotoxicity contributes to the deterioration of β-cell function through oxidative stress under hyperglycemic conditions (78), and since tofogliflozin lowers plasma glucose but has no direct effect other than SGLT2 inhibition, the preservation of β-cell function with tofogliflozin might be due to the reduction of oxidative stress through the sustained glucose-lowering effects.

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

Actually, in ZDF rats in the 10 mg/kg tofogliflozin group, the maximum unbound tofogliflozin concentrations in plasma were estimated to be more than 100 times the

rSGLT2 IC_{50} value and one-quarter of the rSGLT1 IC_{50} value, by which renal glucose reabsorption via SGLT2 should be almost completely inhibited and that via SGLT1 should be almost completely maintained. Under these conditions, although the plasma glucose level was decreased to within the normal range with the increase of renal glucose clearance, no hypoglycemia was observed.

Additionally in SD rats, although UGE was induced with tofogliflozin dose-dependently, no significant reduction in plasma glucose levels was observed with tofogliflozin even at 10 mg/kg. In separate pharmacokinetic experiments using SD rats treated with tofogliflozin at 10 mg/kg, the maximum unbound tofogliflozin concentrations in plasma were estimated to be 85 times the rSGLT2 IC_{50} value and one-seventh the rSGLT1 IC_{50} value (data not shown).

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

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

V. Mechanism of Body Weight Reduction in Obese Animal Models

1. Materials and Methods

The lists of the reagents, animals, apparatuses and schedules for each experiment are summarized in Table II-1.

Reagents and chemicals

Tofogliflozin was synthesized (59) in our laboratories at Chugai Pharmaceutical Co. Pioglitazone hydrochloride (pioglitazone) was purchased from Sequoia Research Products Ltd (Pangbourne, UK). We prepared a powdered high-fat diet (HFD, 60% kcal fat, D-12492 [Research Diets, Inc., New Brunswick, NJ, USA]) containing 0.05% tofogliflozin (HFD/TOFO), rodent diet (CE-2 [Clea Japan]) containing 0.015% or 0.0015% tofogliflozin (CE-2/TOFO), CE-2 containing 0.02% pioglitazone (CE-2/PIO), and CE-2 containing 0.02% pioglitazone plus 0.0015% tofogliflozin (CE-2/PIO+TOFO).

Animals

Male Wistar rats (Jcl:Wistar) and KKAy mice (KK-Ay/TaJcl) purchased from Clea Japan were housed under a 12-h/12-h light/dark cycle (lights on 7:00 AM–7:00 PM) with controlled room temperature (20–26°C) and humidity (35–75%), and allowed free access to food (CE-2) and water. All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical Co., Ltd. The protocols were approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical.

Table II-1 The lists of the reagents, animals, apparatuses and schedules for experiments

DIO rats	Animal		Male Wistar rats (Jcl), 8 weeks, n=7		
	Diet		Week -4 - -1	Week 1- 9	
		ND group	ND: D-12450B (Research Diet)	ND	
		HFD group	HFD: D-12492 (Research Diet)	HFD	
		TOFO group	HFD	HFD/TOFO (0.05%)	
	Body weight		Periodically		
	Food intake		Periodically		
	Urinary glucose		Week 1, 7		Autosera S GLU (Sekisui Medical)
	Hematocrit		Week 5, 9		Hematcrit Capillary VC-H075P (Terumo)
	Rectal temp.		Week 7, 9		Microprobe Thermomerter BAT-12 (Physitemp Instruments)
	Plasma TG		Week 9		L Type Wako TG・M (Wako)
	Plasma TC		Week 9		L Type Wako CHO・M (Wako)
	Plasma FFA		Week 9		NEFA-HA Test Wako (Wako)
	Plasma Glc		Week 9		Autosera S GLU (Sekisui Medical)
	Plasma total ketone bodies		Week 9		Auto Wako Total Ketone bodies (Wako)
	CT scan		Week 8		MicroCT Scanner eXplore Locus (General Electric Company)
	Indirect calorimetry		Week 9		O_2/CO_2 Metabolic Measuring System MM208 (Muromachi Kikai)
	Locomotor activity		Week 9		Supermex Miltichannel activity-counting System Animex Auto MK-110 Muromachi Kikai)
	Plasam insulin		Week 9		Rat insulin ELISA kit (Morinaga)
	Plasma leptin		Week 9		Rat leptin ELISA kit (Morinaga)
	Tissue weight		Week 10		
	CD14, CD68 mRNA		Week 10		7900HT Fast Real Time PCR system (Applied Biosystems)
	Size of adipocyte		Week 10		HE stainning of mesentric adipose tissue slice, NIS-Elements D2.20 SP1 (Nikon)
	Infirtration of macrophage into adipose tissue		Week 10		Immunohistochemical stainning of mesenteric adipose tissue slice with anti-rat CD68 antibody, MCA341R (AbD Serotec) eSlide Capture Device Aperio ScanScope (Aperio Technologies)
KKAy mice					
Exp.	Animal		Male KKAy mice (Clea Japan), 8 weeks, n=12		
	Diet		Week 1 - 5		
		Control group	CE-2 (Clea Japan)		
		TOFO group	CE-2/TOFO (0.015%)		
	Body weight		Periodically		
	Food intake		Periodically		
	Plasma glucose		Day -2, 14, 28		Blood Glucose Monitoring System ACCU-Check Aviva (Roche Diagnostics)
	Glycated hemoglobin		Day -2, 28		Auto Wako HbA1c (Wako)
	Plasma TG		Day 28		L Type Wako TG・M (Wako)
	Plasma TC		Day 28		L Type Wako CHO・M (Wako)
	Plasma insulin		Day 28		Mouse insulin ELISA kit (Morinaga)
	Plasma adiponectin		Day 28		Mouse/Rat adiponectin ELISA kit (Otsuka Pharmaceutical)
	Liver weight		Day 35		
	Liver TG content		Day 35		Triglyceride E-test kit (Wako)
	Urinary glucose		Day 29 - 30		Autosera S GLU (Sekisui Medical)
Exp. 2	Animal		Male KKAy mice (Clea Japan), 8 weeks, n=8, 16		
	Diet		Day 1 - 20		
		Control group	CE-2		
		TOFO group	CE-2/TOFO (0.015%)		
	Body weight		Periodically		
	Plasam glucose		Day -1, 2, 14, 20		Blood Glucose Monitoring System ACCU-Check Aviva (Roche Diagnostics)
	Body water content		Day 3, 20		Dead body was dried at 65 ℃
Exp. 3	Animal		Male KKAy mice (Clea Japan), 8 weeks, n=6		
	Diet		Week 1- 4		
		Control group	CE-2		
		PIO group	CE-2/PIO (0.02%)		
		TOFO group	CE-2/TOFO (0.0015%)		
		PIO+TOFO group	CE-2/PIO+TOFO		
	Body weight		Periodically		
	Food intake		Periodically		
	Plasma glucose		Day -2, 14, 28		Blood Glucose Monitoring System ACCU-Check Aviva (Roche Diagnostics)
	Glycated hemoglobin		Day -2, 28		Auto Wako HbA1c (Wako)

<Effect of tofogliflozin in DIO rats>

General methods

Twenty-one male Wistar rats (8 weeks old), randomly allocated into 3 groups matched for plasma glucose and body weight, were housed individually with free access to food and water. ND group and HFD group were fed for 13 weeks a powdered normal diet (ND, 10% kcal fat, D-12450B [Research Diets, Inc.]) and powdered HFD, respectively. TOFO group was fed HFD for 4 weeks and HFD/TOFO for an additional 9 weeks. Week 1 was defined as when feeding with HFD/TOFO started. Body weight and food consumption were measured periodically. Hematocrit was measured in blood collected via the jugular vein by Hematocrit Capillary (VC-H075P [Terumo Co., Tokyo, Japan]) at Week 5 and 9. Rectal temperature was measured with a microprobe thermometer (BAT-12 [Physitemp Instruments, Inc., Clifton, NJ, USA]). Plasma tofogliflozin concentration was measured in blood collected via the jugular vein or the tail vein as described previously (21). To determine the following biochemical parameters, blood was sampled via the jugular vein of non-fasted rats then centrifuged to obtain plasma samples between 9:00 and 12:00. Plasma insulin and leptin concentrations were measured with ELISA kits (Morinaga Institute of Biological Science, Inc.). Plasma glucose, TG, total cholesterol (TC), and total TKB were measured with an automated analyzer (TBA-120FR [Toshiba Medical Systems Co., Tochigi, Japan]). At the end of the study (Week 10), rats were euthanized by exsanguination under anesthesia between 13:00 and 17:00, and adipose tissues (mesenteric, epididymal, inguinal, and retroperitoneal adipose tissue) and skeletal muscle (soleus) were isolated. The tissues were weighed and stored at −80°C or in 10% formalin solution until use.

Body composition by micro-computed tomography (microCT)

Rats were anesthetized with isoflurane, and body composition evaluated with a microCT scanner (eXplore Locus [General Electronic Company]) and image analysis

software (MicroView [General Electronic Company]) (79). The CT value of olive oil (Wako Pure Chemical Industries, Ltd.) was used as standard density for adipose tissue. The CT value of a standard phantom with calibration cells containing calcium hydroxyapatite (Kyoto Kagaku, Kyoto, Japan) at a concentration equivalent to 400 mg/cm^3 was used as standard density for bone. A voxel with a CT value higher than that of the standard phantom was defined as bone. A voxel with CT value between that of the standard phantom and olive oil was defined as lean body mass.

Quantitative RT-PCR for CD14 and CD68 mRNA

Total RNA was isolated from the frozen mesenteric adipose tissue with TRIzol reagent (Life Technologies Co., Carlsbad, CA, USA) and an RNeasy 96 kit (Qiagen, Venlo, Netherlands). The relative amounts of CD14 and CD68 mRNA in the total RNA were calculated with 18S rRNA as an internal control by a real-time RT-PCR quantitative system (7900HT Fast Real Time PCR system [Applied Biosystems]). The RT-PCR reaction was performed with a QuantiTect Probe RT-PCR kit (Qiagen) and probes (TaqMan Gene Expression Assays for rat, CD14: Rn00572656_g1, CD68: Rn01495634_g1, 18S rRNA: Hs99999901_s1 [Applied Biosystems]).

Cell size of adipocytes

Mesenteric adipose tissue was fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in a paraffin block. The paraffin block was sliced 7 μm thick and stained with hematoxylin-eosin (HE) by Sapporo General Pathology Laboratory Co. (Sapporo, Japan). In 2 microscopic images selected at random per section, the cell size (surface area) of all adipocytes (approximately 150 cells) in the images was measured with image analysis software (NIS-Elements D2.20 SP1 [Nikon Co., Tokyo, Japan]).

Immunohistochemical staining

The block of paraffin-embedded mesenteric adipose tissue was sliced 4 μm thick and stained with anti-rat CD68 (ED-1) monoclonal antibody (monocyte/macrophage marker; No. MCA341R [AbD Serotec, Kidlington, UK]) as a primary antibody, anti-mouse/rabbit IgG-HRP (LSAB2 System-HRP, No. K0609 [Dako Denmark A/S, Glostrup, Denmark]) as a secondary antibody, and a chromogenic substrate for HRP (DAB+, Liquid, K3468 [Dako Denmark A/S]) by Sapporo General Pathology Laboratory Co. In 5 microscopic images selected at random per section, the CD68-positive area was calculated as a percentage of total area with an eSlide Capture Device (Aperio ScanScope CS [Aperio Technologies, Vista, CA]) and image analysis software (Image scope [Aperio Technologies]).

UGE

Rats were housed individually in metabolic cages for 24 h and urine samples were collected and urine volume measured. UGC was measured by the hexokinase method (Autosera S GLU [Sekisui Medical Co.]).

Indirect calorimetry and locomotor activity

At Week 9, rats were housed in metabolic chambers (AC-001R [Muromachi Kikai Co., Tokyo, Japan]) with free access to food and water for 24 h. Air was sampled every 5 min and consumed oxygen (VO_2 [mL/min]) and produced carbon dioxide (VCO_2 [mL/min]) were measured by an O_2/CO_2 metabolic measuring system (MM208 [Muromachi Kikai Co.]). From these we calculated RQ (= VCO_2/VO_2) and energy expenditure (EE = $1.07 \times RQ \times VO_2 + 3.98 \times VO_2$ [cal/min]). At the same time, locomotor activity was monitored every minute by a Supermex multichannel activity-counting system (Animex Auto MK-110 [Muromachi Kikai Co.]).

<Effect of tofogliflozin in KKAy mice>

Experiment 1: Effect on plasma glucose, body weight, and liver TG content

Twenty-four male KKAy mice (8 weeks old), randomly allocated into 2 groups matched for plasma glucose and body weight, were fed CE-2 or CE-2/TOFO (0.015%) for 5 weeks. The mice were caged individually with free access to food and water. The day on which the mice began to be fed CE-2/TOFO is defined as Day 1. Body weight and food consumption were measured periodically. Blood was sampled via the tail vein, and plasma glucose level was measured by plasma glucose monitoring system (ACCU-Check Aviva [Roche Diagnostics]). Glycated Hb level was measured with an automated analyzer (Auto Wako HbA1c [Wako Pure Chemical Industries]). Plasma TG and TC levels were measured with the automated analyzer. Plasma insulin and adiponectin levels were measured with ELISA kits (insulin: Morinaga Institute of Biological Science, Inc.; adiponectin: Otsuka Pharmaceutical co., Tokyo, Japan). Between Day 29 and Day 30, mice were housed individually in metabolic cages for 24 h, and the urine volume and UGC were measured. At the end of the study (Day 35), mice were euthanized by exsanguination under anesthesia, then their livers were isolated, weighed, and stored at −80°C until use. The liver was homogenized in methanol and a 2-fold volume of chloroform was added and mixed then centrifuged at 1200 *g* for 10 min. The supernatant was collected, dried, and dissolved in isopropyl alcohol containing 10% Triton X-100. TG concentration in the solution was measured with a Triglyceride E-test kit [Wako Pure Chemical Industries]), and liver TG content (mg/g tissue) was calculated.

Experiment 2: Effect on body water content

Thirty-two male KKAy mice (8 weeks old) were randomly allocated into 2 groups (CE-2 and CE-2/TOFO [0.015%]) matched for plasma glucose and body weight. On Day 3 or Day 20, body weight (BW) was measured and the mice were euthanized by cervical

dislocation. The dead body was dried at 65°C for 17 days and dry body weight (DBW) was measured. Body water content (BWC) of the mouse was calculated as follows:

$$BWC\ (\%) = 100 \times (BW - DBW) / BW$$

Experiment 3: Effect on body weight gain induced by pioglitazone

Twenty-four male KKAy mice (8 weeks old), randomly allocated into 4 groups matched for plasma glucose and body weight, were fed CE-2, CE-2/PIO, CE-2/TOFO (0.0015%), or CE-2/PIO+TOFO for 4 weeks. Body weight, food consumption, plasma glucose, and glycated Hb levels were measured by the same methods as in Experiment 1.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with SAS System for Windows, Release 8.02 (SAS Institute Japan). Statistical significance was determined by Student's *t*-test, Dunnett's multiple comparison test, or analysis of covariance (ANCOVA). Multi regression analysis was performed with Microsoft Excel 2007 SP3 (Microsoft Corporation, Redmond, WA).

2. Results

Body weight gain and fat accumulation in DIO rats

Body weight increased more rapidly (Fig. II-1A) in HFD group (134.4 ± 9.6 g/9 weeks) than in ND group (106.6 ± 2.8 g/9 weeks), and the final body weight was greater in HFD group (540 ± 18.5 g) than in ND group (483.9 ± 7.7 g). HFD feeding increased total fat mass by about 46% (Fig. II-1B). On the other hand, there was no change in bone mass (Fig. II-1C) or lean body mass (Fig. II-1D) between the groups. HFD feeding increased weights of all adipose tissues (Fig. II-1E) without increasing soleus weight (Fig. II-1F).

In rats fed HFD/TOFO (TOFO group), body weight increased more slowly than in HFD group, and the final body weight was approximately 30 g lower in TOFO group than in HFD group (Fig. II-1A) which was almost equivalent to the HFD-induced body weight gain versus ND group (about 28 g) mentioned above.

The enlargement of total fat mass decreased by 14.6% in TOFO group compared to HFD group (Fig. II-1B). In contrast, there was no change in bone mass or lean body mass between HFD and TOFO groups. Tofogliflozin administration prevented HFD-induced enlargement of adipose tissues without reducing soleus weight (Fig. II-1F).

Body weight correlated positively with fat mass ($R^2 = 0.799$, $P = 4.7\times10^{-8}$; Fig. II-1G); however, there was no correlation with bone mass (Fig. II-1H) or lean body mass (Fig. II-1I). Multiple regression analysis performed with body weight gain between Week 1 and Week 8 as a dependent variable, and fat, bone, and lean body mass as independent variables (Table II-2) found that body weight gain was mainly accounted for by increased fat mass ($P = 7.01\times10^{-6}$).

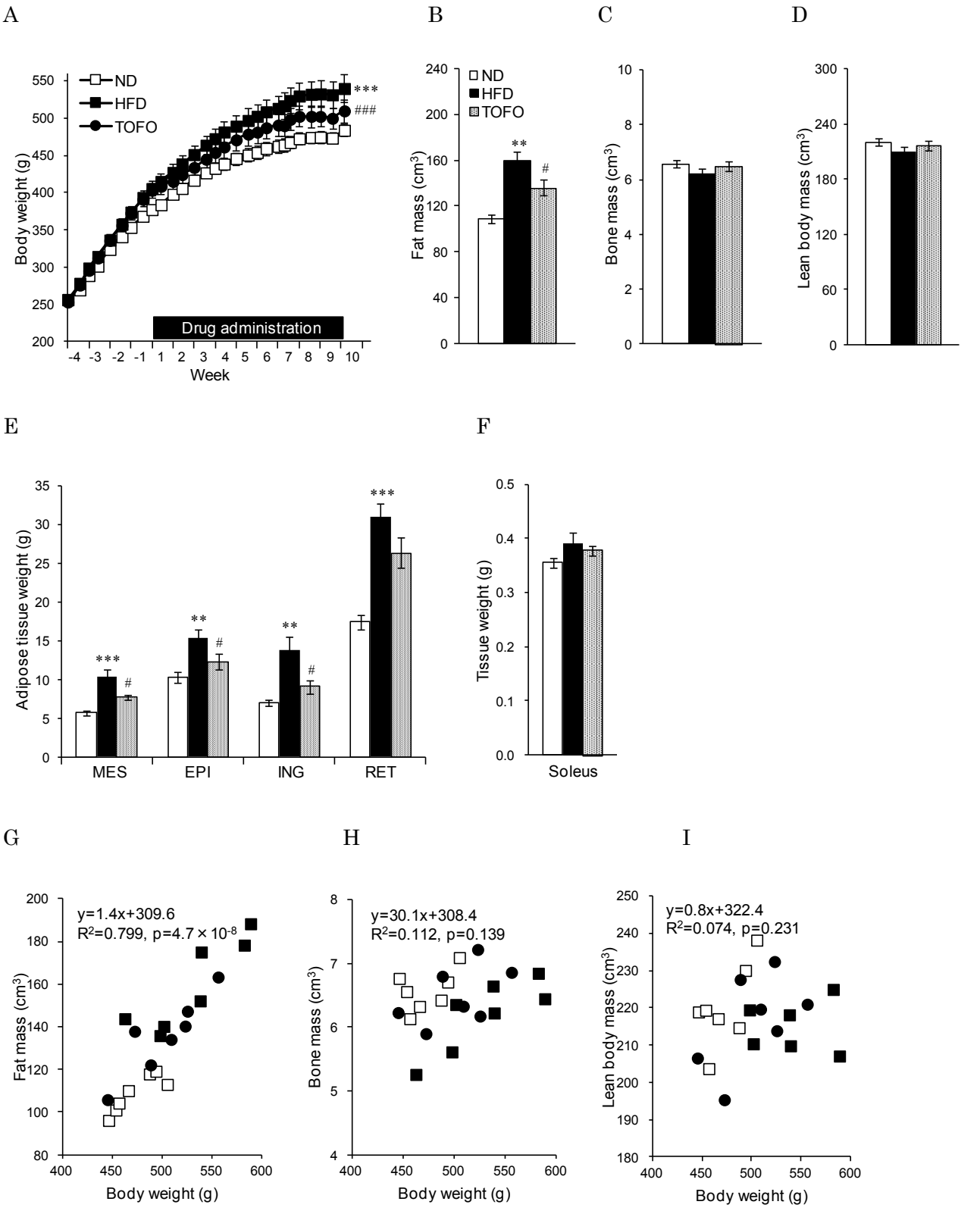


Fig. II-1 Effects of long-term administration of tofogliflozin on body weight and body composition of DIO rats.

A) Time course of body weight of rats fed ND, HFD, or HFD/TOFO for 9 weeks. B–D) Fat (B), bone (C), and lean body mass (D) calculated from microCT scanner images from the level of the

5th cervical vertebra to the 2nd caudal vertebra at Week 8. E–F) The weight of adipose tissues (E) and soleus (F) measured for mesenteric (MES), epididymal (EPI), inguinal (ING), and retroperitoneal (RET) adipose tissues and the soleus isolated at Week 10. G–I) Body weight plotted against fat mass (G), bone mass (H), or lean body mass (I). Spearman correlation coefficient between body weight and each parameter is shown in the graph. Data (A–F) are expressed as mean ± SEM (n = 7). **P < 0.01 or ***P < 0.001 versus ND group, and #P < 0.05 or ###P < 0.001 versus HFD by ANCOVA (A) or Student's t-test (B–F).

Table II-2 Result of multiple regression analysis of 3 independent variables (fat mass, bone mass, lean body mass) compared to body weight gain between Week 1 and Week 8

Model's properties	Independent variables	β	t	*p*	Partial R^2
R^2=0.783, adj. R^2=0.745	Fat mass	0.67	7.59	7.45×10^{-7}	0.799
F=10.18, df=3	Bone mass	5.41	0.61	0.550	0.112
$p=7.01\times10^{-6}$	Lean body mass	0.18	0.59	0.564	0.074
	(constant)	-56.63	-1.44		

β is standardized regression coefficient. Partial R2 is the partial correlation of each independent variable with body weight gain.

Cell size of adipocytes, and monocyte/macrophage infiltration into mesenteric adipose tissue in DIO rats

To evaluate the effects of tofogliflozin on the development of adipose tissue in vivo, the cell size of mesenteric adipose tissue was compared among treatment groups (Fig. II-2A). Cell size distribution was shifted to the larger direction in HFD group compared to ND group (Fig. II-2B). Mean cell size was larger in HFD group than in ND group (Fig. II-2C). The mRNA levels of the monocyte/macrophage markers CD14 and CD68 in mesenteric adipose tissue in HFD group increased to 4.5 times and 2.4 times (respectively) the levels in ND group (Fig. II-2D). Immunohistochemical staining

indicated that the area positive for CD68 (ED-1) was larger in HFD group than in ND group (Fig. II-2E, F).

In TOFO group, the shift in adipose cell size distribution to the larger direction induced by HFD feeding was suppressed (Fig. II-2B), resulting in a significant reduction in mean adipose cell size in TOFO group as compared with HFD group (Fig. II-2C). In addition, tofogliflozin administration decreased CD14 and CD68 mRNA levels as compared with HFD group (Fig. II-2D; CD14: $P = 0.033$, CD68: $P = 0.074$), and reduced the CD68 (ED-1) positive area in mesenteric adipose tissue (Fig. II-2E, F).

Total calorie balance in DIO rats

The plasma tofogliflozin concentrations in TOFO group were 658 ± 40 ng/mL at Week 1, 558 ± 33 ng/mL at Week 5, and 509 ± 50 ng/mL at Week 9. Tofogliflozin administration induced UGE at both Week 1 and Week 7 by 628 ± 28 and 674 ± 32 mg/day/100 g BW (Fig. II-3A), which are estimated as calorie losses of 9.7 ± 0.6 and 12.6 ± 0.6 kcal/day, respectively. At the same time, calorie intake via food consumption from Week 1 to Week 9 in TOFO group (5718 ± 187 kcal) was greater by about 10% than that in HFD group (5049 ± 214 kcal, Fig. II-3B). On the other hand, no changes in rectal temperature (Fig. II-3C), locomotor activity (Fig. II-3D), or energy expenditure (Fig. II-3E) were observed between HFD and TOFO groups.

Thus, tofogliflozin administration increased both calorie loss by promoting UGE and calorie intake by increasing food consumption without altering energy utilization (Table II-3). We assumed that the calorie loss (approximately 13 kcal/day) was greater than the increase in calorie intake (approximately 10 kcal/day), and the deduced total calorie balance decreased by approximately 3 kcal/day in TOFO group as compared to HFD group.

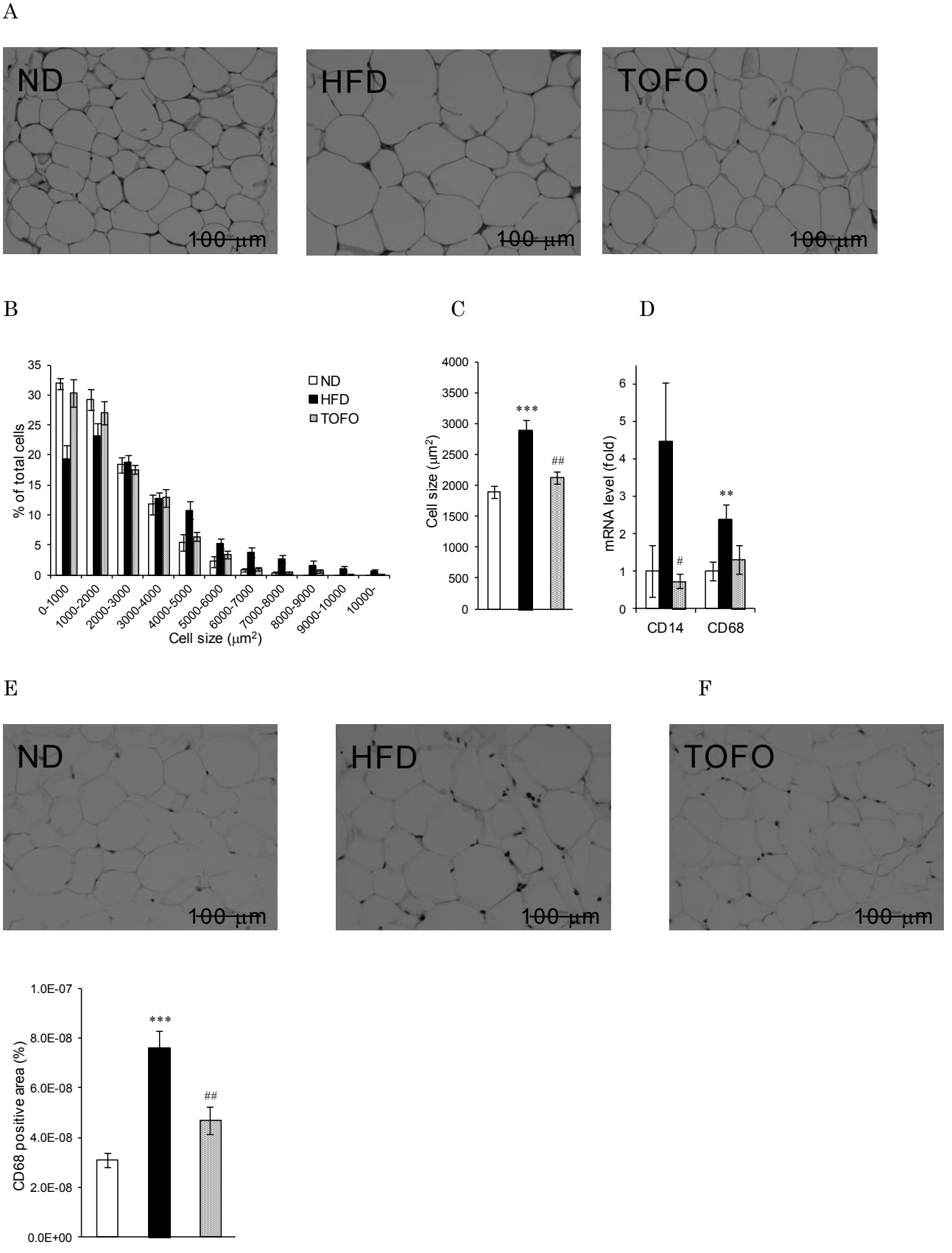


Fig. II-2 Effects of long-term administration of tofogliflozin on the cell size of adipocytes and infiltration of inflammatory cells into mesenteric adipose tissue in DIO rats.

A) Representative images of HE-stained sections of mesenteric adipose tissue isolated at Week 10. B, C) Distribution (B) and mean value (C) of cell size of adipocytes. D) mRNA level of CD14 and CD68 in mesenteric adipose tissue relative to level in ND group. E) Representative images of sections stained with anti-rat CD68 (monocyte/macrophage marker) monoclonal antibody. F) Percentage of CD68-positive area in microscopic images. Data (B, C, D, and F) are expressed as mean ± SEM (n = 7). ***P < 0.001 versus ND group, and #P < 0.05 or ##P < 0.01 versus HFD by Student's t-test.

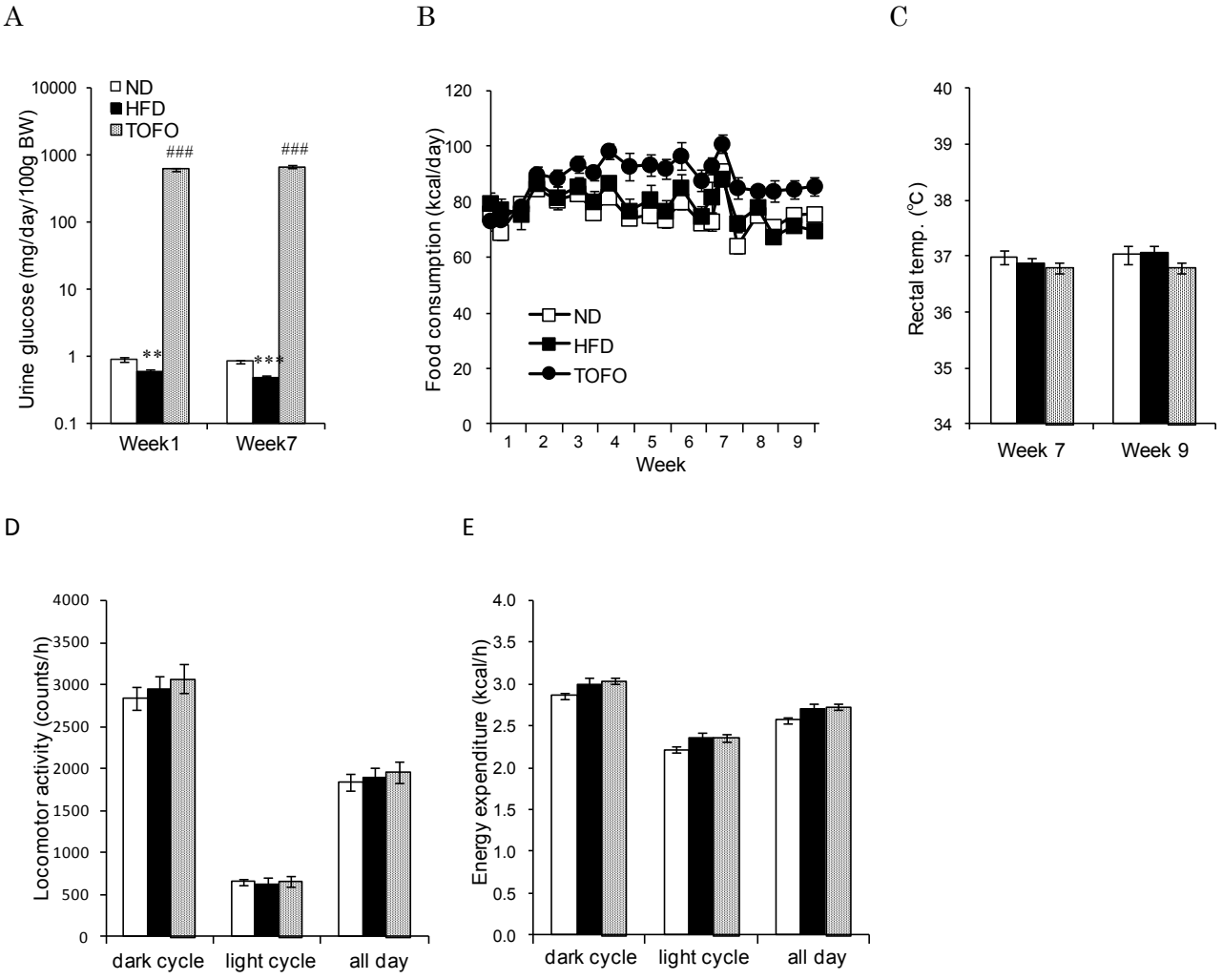


Fig. II-3 Effects of long-term administration of tofogliflozin on energy balance and metabolism of DIO rats.

A) Urinary glucose excretion at Week 1 and Week 7. B) Food consumption. C) Rectal temperature at Week 7 and Week 9. D–F) Locomotor activity (D), energy expenditure (E), and respiratory

quotient (F) measured with metabolic chamber at Week 9. Data are expressed as mean ± SEM (n = 7). Statistical comparisons (A, C–E) were performed by Student's t-test test: *P < 0.05, **P < 0.01, ***P < 0.01 versus ND group, and #P < 0.05, ##P < 0.01, ###P < 0.001 versus HFD.

Table II-3 Effect of long-term administration of tofogliflozin on deduced total calorie balance in DIO rats

Group	Estimated energy balance (kcal/day)			
	Food intake	Energy expenditure	Urinary glucose excretion	Total
ND	77.7 ± 1.7	-61.0 ± 0.8	0.0 ± 0.0	16.6 ± 1.7
HFD	81.0 ± 3.5	-64.2 ± 1.6	0.0 ± 0.0	16.8 ± 3.0
TOFO	90.8 ± 3.1	-64.8 ± 0.9	-12.6 ± 0.6	13.4 ± 2.3

Metabolic balance between carbohydrate oxidation and fatty acid oxidation, and other biochemical parameters in DIO rats

RQ values of ND group were stable at around 0.95 in both light and dark periods. In contrast, RQ values of HFD group were decreased to around 0.85 in both light and dark periods (Fig. II-4A), indicating an increased rate of fatty acid oxidation with HFD. Plasma TG level was lower in HFD group than in ND group (Fig. II-4B), whereas TKB level was higher in HFD group than in ND group (Fig. II-4C). There was no difference in plasma TC level among the groups (Fig. II-4D). Plasma leptin level was greater in HFD group than in ND group (Fig. II-4E). Plasma insulin level in HFD group and ND group was 2.3 ± 0.31 ng/mL and 1.9 ± 0.2 ng/mL, respectively (ND versus HFD: $P = 0.295$, Fig. II-4F). There were no differences between HFD and ND groups in plasma glucose levels at Week 9 (Fig. II-4G) or hematocrit levels at Week 5 and Week 9 (Fig. II-4H).

RQ value was further decreased by tofogliflozin to around 0.80 (Fig. II-4A). Tofogliflozin also reduced plasma TG level (Fig. II-4B), but increased TKB level (Fig. II-4C) as compared with HFD group. Plasma TC level was not changed by tofogliflozin (Fig. II-4D). In TOFO group, the HFD-induced increase of plasma leptin was prevented (Fig. II-4E) and a tendency to decreasing plasma insulin (1.7 ± 0.2 ng/mL, Fig. II-4F) was observed. There were no differences between HFD and TOFO groups in plasma glucose (Fig. II-4G), which may be due to the compensatory increase in endogenous

glucose production in the TOFO group responding to the increased UGE (37), or hematocrit levels (Fig. II-4H).

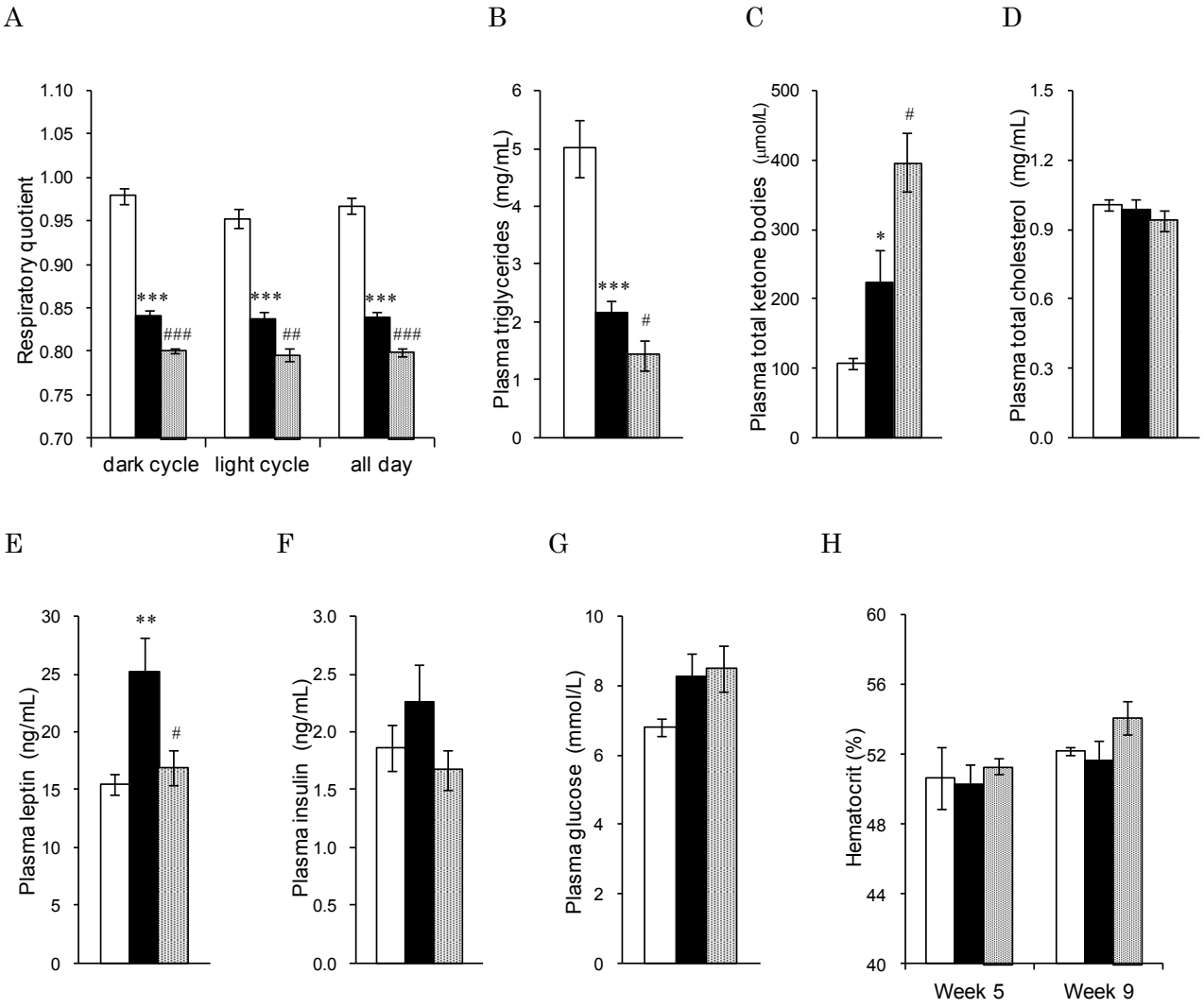


Fig. II-4 Effects of long-term administration of tofogliflozin on metabolism of DIO rats.

A) Respiratory quotient measured with metabolic chamber at Week 9. B–G) Plasma TG (B), total ketone bodies (C), total cholesterol (D), leptin (E), insulin (F), and glucose (G) levels determined at Week 9. H) Hematocrit level evaluated at Week 5 and Week 9. Data are expressed as mean ± SEM (n = 7). Statistical comparisons were performed by Student's t-test test: *P < 0.05, **P < 0.01, ***P < 0.01 versus ND group, and #P < 0.05, ##P < 0.01, ###P < 0.001 versus HFD.

Body weight gain and hyperglycemia in KKAy mice

Experiment 1: Body weight, plasma glucose, and biochemical parameters in long-term treatment

Plasma tofogliflozin concentrations in TOFO group were 546 ± 69 ng/mL on Day 1 and 402 ± 37 ng/mL on Day 28. Although an apparent UGE was observed in control group of KKAy mice on Day 29, tofogliflozin administration increased UGE by about 2.5 times that of control group (Fig. II-5A). In control group, plasma glucose level was maintained above 22 mmol/L (Fig. II-5B), and glycated Hb level increased from Day −2 to Day 28 by about 1.8-fold (Fig. II-5C). Tofogliflozin administration reduced plasma glucose to below 11 mmol/L, and prevented the increase in glycated Hb. Body weight of control group increased by 8.6 ± 0.5 g over 35 days (Fig. II-5D). Although food consumption increased approximately 10% in TOFO group (948 ± 18 kcal/35 days) compared to control group (858 ± 23 kcal/35 days, Fig. II-5E), tofogliflozin administration attenuated body weight gain (Fig. II-5D).

There were no differences in plasma TG and TC level between the two groups on Day 28 (Fig. II-5F, G). Although plasma insulin level in control group increased from Day −2 to Day 28 by about 4.5-fold, tofogliflozin administration prevented this hyperinsulinemia (Fig. II-5H). Plasma adiponectin level was greater in TOFO group than in control group (Fig. II-5I). The hypertrophy of liver with steatosis observed in control group was suppressed by tofogliflozin administration (Fig. II-5J, K).

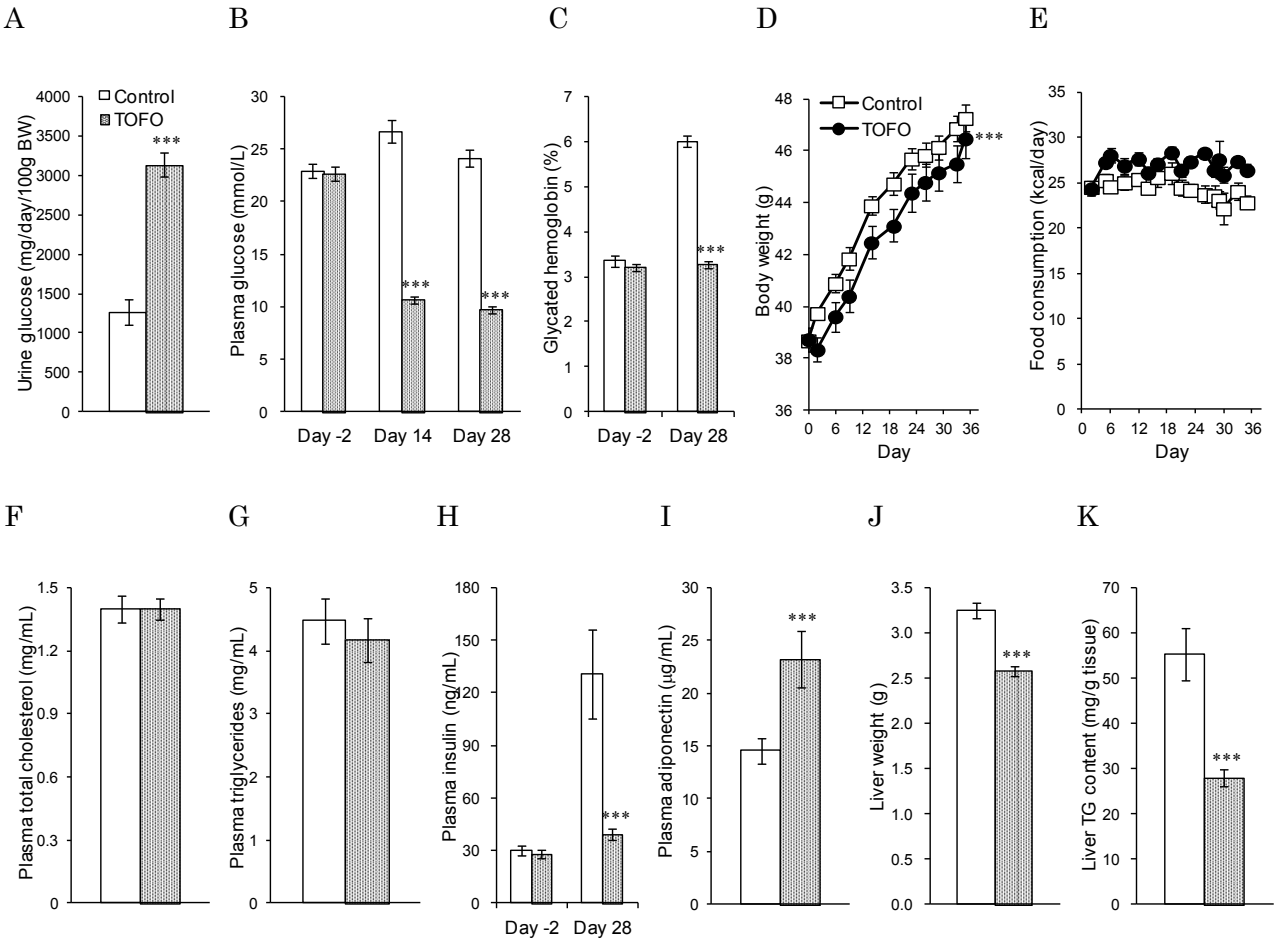


Fig. II-5 Effects of long-term administration of tofogliflozin on the plasma glucose and body weight of KKAy mice.

A) Urinary glucose excretion for 24 h from Day 29 to 30. B) Plasma glucose levels on Day −2, Day 14, and Day 28. C) Glycated hemoglobin (%) on Day −2 and Day 28. D) Time course of body weight. E) Food consumption between Day 0 and 35. F–I) Plasma triglycerides (F), total cholesterol (G), insulin (H), and adiponectin (I) determined on Day 28. J, K) The weight (J) and triglyceride (TG) content (K) of the liver sampled on Day 35. Data are expressed as mean ± SEM (n = 12). Statistical comparisons were performed by Student's t-test (B-K), or by ANCOVA (A). *P < 0.05 and ***P < 0.001 versus control group.

Experiment 2: Body water content with 3- or 20-day treatment

Tofogliflozin administration again reduced plasma glucose to below 12 mmol/L throughout the course of the experiment (Fig. II-6A). Tofogliflozin administration also decreased body weight gain, resulting in a significant difference in body weight between control and TOFO groups (Fig. II-6B) on Day 3 and Day 20. However, there was no significant reduction in body water content on Day 3 or Day 20 in TOFO group (Fig. II-6C, D) as compared with control group.

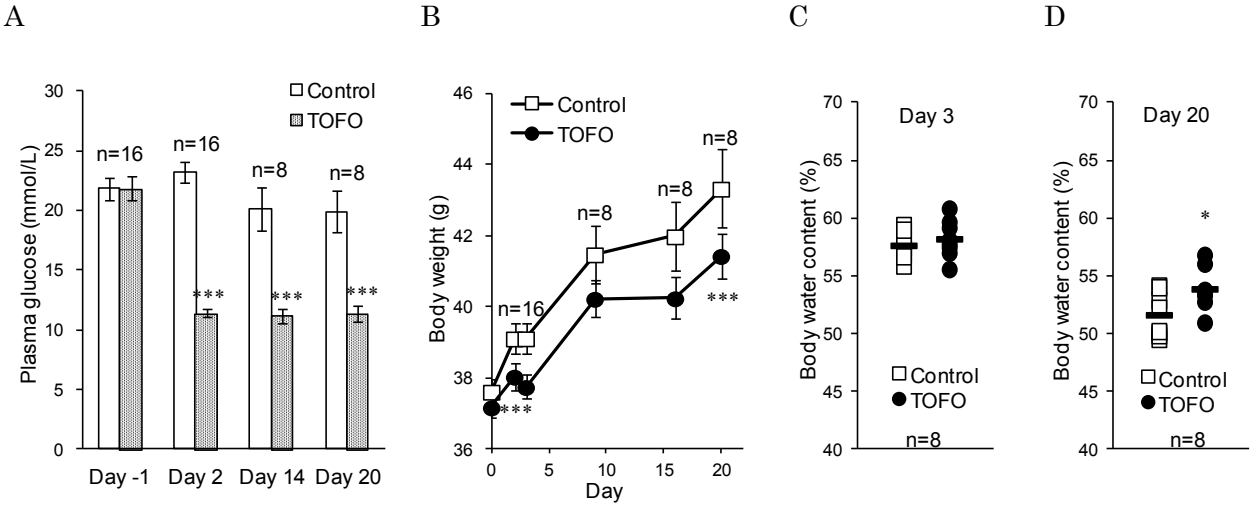


Fig. II-6 Effects of long-term administration of tofogliflozin on the body water content of KKAy mice.

A) Plasma glucose levels on Day −1, Day 2, Day 14, and Day 20. B) Time course of body weight. C, D) Body water content of mice on Day 3 or Day 20. Data are expressed as mean ± SEM (n = 8 or 16). Statistical comparisons were performed by Student's t-test (B-D), or by ANCOVA (A). *P < 0.05 and ***P < 0.001 versus control group.

Experiment 3: Effect on body weight gain induced by 28-day pioglitazone treatment

Similar to Experiment 1, the body weight of control group increased by 8.2 ± 0.2 g over 28 days (Fig. II-7A). Tofogliflozin administration again attenuated body weight gain (7.0 ± 0.4 g/28 days) as compared with control group. Pioglitazone administration increased the body weight more rapidly by 15.5 ± 0.6 g over 28 days as compared with control group. The combined administration of pioglitazone and tofogliflozin reduced the body weight gained with pioglitazone (Fig. II-7A).

Pioglitazone administration reduced plasma glucose (Fig. II-7B) and glycated Hb levels (Fig. II-7C) on Day 28 as compared with control group. Tofogliflozin administration also reduced plasma glucose and glycated Hb levels, but the plasma glucose lowering effects of tofogliflozin (0.0015%) at Day 14 and Day 28 were slightly smaller than those of pioglitazone (0.02%). Combined administration of pioglitazone and tofogliflozin additively reduced plasma glucose and glycated Hb levels, resulting in a significant reduction in plasma glucose levels in PIO+TOFO group versus TOFO group at Day 14 and Day 28 (Fig. II-7B), and in glycated Hb levels in PIO+TOFO group versus PIO and TOFO groups at Day 28 (Fig. II-7C). In this experiment, food consumption was not changed in PIO group (652 ± 12 kcal/28 days), TOFO group (700 ± 14 kcal/28 days), or PIO+TOFO group (705 ± 20 kcal/28 days) as compared with control group (664 ± 18 kcal/28 days), and food consumption was not changed in PIO+TOFO group as compared with PIO or TOFO groups (Fig. II-7D).

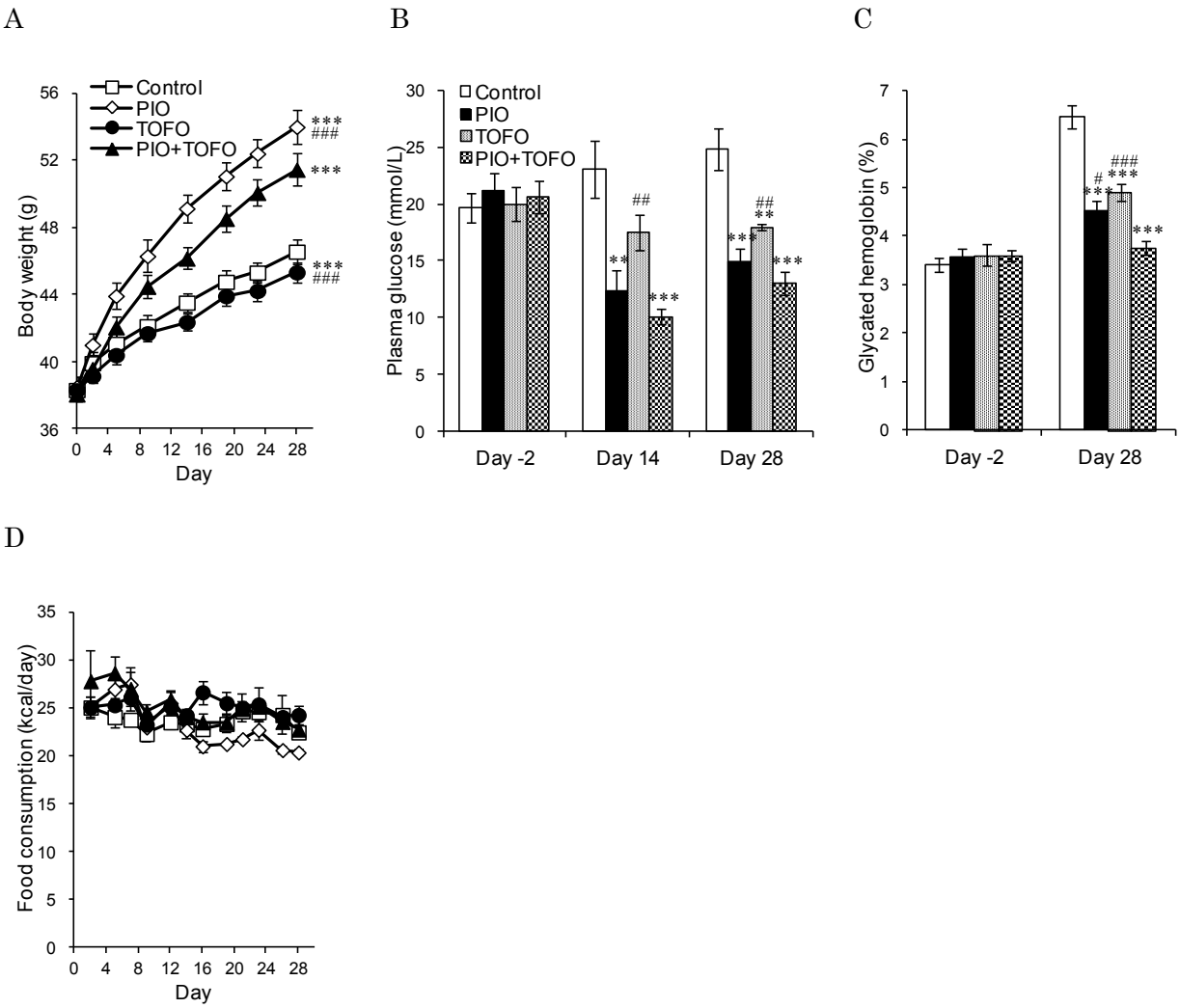


Fig. II-7 Effects of long-term administration of tofogliflozin on pioglitazone-induced body weight gain of KKAy mice.

A) Time course of body weight. B) Plasma glucose levels on Day −2, Day 14, and Day 28. C) Glycated hemoglobin (%) on Day −2 and Day 28. D) Food consumption between Day 0 and 28. Data are expressed as mean ± SEM (n = 6). Statistical comparisons were performed by Dunnett's multiple comparison test (B, C) or by ANCOVA (A). **P < 0.01, ***P < 0.001 versus control group; #P < 0.05, ##P < 0.01, ###P < 0.001 versus PIO+TOFO group.

3. Discussion

In this study, excess body weight gain was attenuated with tofogliflozin in both obese DIO rats and diabetic KKAy mice. In DIO rats treated with tofogliflozin, enlargement of fat mass was suppressed without reduction of lean body mass or bone mass. In addition, suppression of body weight with tofogliflozin was accompanied with amelioration of hepatic steatosis in KKAy mice without a change in body water content. These results were consistent with other preclinical studies (80, 81, 82) and a clinical study (83) with SGLT2 inhibitors.

Although tofogliflozin reduced body weight in clinical studies (38, 39), its mechanism was unclear. In db/db mice, an animal model of T2D, although tofogliflozin treatment improved hyperglycemia, the body weight was paradoxically increased as compared with the untreated control group (84), which was possibly due to preserved insulin secretion in tofogliflozin group, similarly to other SGLT2 inhibitors (85). Thus, it is difficult to evaluate the mechanisms of body weight reduction with SGLT2 inhibitors when using diabetic animal models with dysfunctional insulin secretion.

Therefore, we used DIO rats and KKAy mice in which insulin secretion is maintained, and successfully found that tofogliflozin ameliorated body weight gain. Moreover, body weight gain induced by pioglitazone was suppressed in KKAy mice treated with tofogliflozin.

In DIO rats treated with tofogliflozin, calorie loss by UGE exceeded the increased calorie intake, and deduced total calorie balance was lower than in HFD group. Accumulated total calorie loss calculated by deduced total calorie balance was approximately 190 kcal/head between Week 1 and Week 8, which was equivalent to the decreased fat mass (23.2 cm^3) with tofogliflozin. These results suggest that tofogliflozin induces total calorie loss by increasing UGE, and attenuates obesity predominantly by reducing fat accumulation. A similar mechanism for body weight reduction was proposed in the preclinical study with another SGLT2 inhibitor (81).

In this study, body weight reduction was not associated with increased hematocrit in DIO rats or decreased body water content in KKAy mice, suggesting that body weight reduction was not caused by dehydration in these animal models.

In both DIO rats and KKAy mice treated with tofogliflozin, food consumption increased. Tofogliflozin prevented fat accumulation and reduced plasma leptin level in DIO rats, and improved hyperglycemia and hyperinsulinemia in KKAy mice. Reduction of insulin and leptin—hormones regulating appetite and food consumption (86) —might increase food consumption; however, tofogliflozin increased food consumption even in db/db mice (84) and KKAy mice—animal models with low sensitivity to insulin and leptin—implying that other mechanisms might contribute to the regulation of their food consumption. Further analysis will be needed to clarify the mechanisms underlying the increased food consumption to compensate for the calorie loss and plasma glucose reduction.

One of the possible compensatory mechanisms for reduced plasma glucose level is lipolysis in adipose tissues, which generates glycerol and free fatty acids (FFAs). Released glycerol is used for the endogenous glucose production in the liver, and the FFAs are used for ATP production with β-oxidation, leading to the increased production of ketone bodies (85, 87) which are important energy substrates for the skeletal muscle, heart and brain.

Compared with HFD group, in DIO rats treated with tofogliflozin, RQ decreased by approximately 0.04, plasma TKB increased to approximately 180 μmol/L, and volume and weight of adipose tissues were reduced. These results suggest that above-mentioned compensatory metabolic changes were induced by the treatment with tofogliflozin. The same phenomena were observed in other preclinical studies with SGLT2 inhibitors (80, 81, 82). In particular, in DIO mice treated with tofogliflozin, increased fatty acid oxidation was observed with the upregulated expression of liver genes linked to stimulation of fatty acid oxidation such as PPARα or carnitine palmitoyltransferase 1α in the liver (80). A slight increase in plasma TKB was also

reported in T2D patients treated with SGLT2 inhibitors (88). These results indicate that treatment with SGLT2 inhibitors induces a metabolic shift from carbohydrate oxidation to fatty acid oxidation in order to compensate for glucose loss, thereby promoting fat utilization.

Obesity-induced fat accumulation in adipose tissues and liver, inflammation in adipose tissues, increased fatty acid synthesis in the liver, and increased TG level in the plasma are associated with insulin resistance (25, 89). Amelioration of these pathological conditions observed in DIO rats and KKAy mice treated with tofogliflozin suggests that long-term treatment with tofogliflozin may improve insulin resistance in patients with T2D.

Extreme liver steatosis is also associated with insulin resistance, inflammation and fibrosis in the liver, or nonalcoholic steatohepatitis (NASH) (90). In KKAy mice, tofogliflozin improved hepatic steatosis, which is consistent with the effects seen in DIO mice treated with tofogliflozin (80) and in NASH animal models treated with another SGLT2 inhibitor (91). These results suggest that long-term administration of SGLT2 inhibitors may also prevent the progression of NASH in T2D patients with obesity.

Pioglitazone, a PPARγ agonist, improves insulin resistance and hyperglycemia in patients with T2D, but induces body weight gain (30) with an increase in fat mass and fluid retention. Although pioglitazone induced body weight gain in KKAy mice, this effect was suppressed by tofogliflozin. In addition, combination therapy with tofogliflozin and pioglitazone improved hyperglycemia more effectively than did monotherapy with either tofogliflozin or pioglitazone alone. This combination therapy might be an attractive choice for T2D treatment.

The present study indicated that tofogliflozin promotes UGE and calorie loss, and prevents body weight gain predominantly by ameliorating fat accumulation in adipose tissue and liver. Tofogliflozin may have the potential to prevent or improve not only hyperglycemia but also insulin sensitivity by suppressing TG accumulation and

inflammation in adipose tissue and liver. Further studies are required to evaluate the effect of long-term treatment with tofogliflozin on insulin resistance in patients with T2D.

VI. Mechanism of Inducing Urinary Tract Infection in Novel Animal Model

1. Materials and Methods

Chemicals

Dapagliflozin (DAPA) (60), canagliflozin (CANA) (61), and tofogliflozin (TOFO) (20) were all synthesized in our laboratories at Chugai Pharmaceutical Co., Ltd.

Animals

Female C57BL/6J mice (C57BL/6JJcl) were purchased from CLEA Japan. Mice were housed under a 12-h/12-h light/dark cycle (lights on 7:00 AM–7:00 PM), with controlled room temperature (20–26°C) and humidity (35–75%), and were allowed *ad libitum* access to a diet of standard laboratory chow (CE-2 pellets; CLEA Japan). All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical Co., Ltd. The protocols were approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical.

Urinary glucose excretion in mice

Mice (7 weeks of age) were randomly allocated into treatment groups matched for body weight. The mice were treated by single oral gavage with an SGLT2 inhibitor (dapagliflozin, canagliflozin, or tofogliflozin) or vehicle (0.5% CMC; 20 mL/kg). Urine samples were collected from mice housed in metabolic cages twice every 12 h until 12 or 24 h after the administration, and urine volume was measured. Urine samples were stored at −30°C until use. UGC was measured by the hexokinase method (Autosera S GLU; Sekisui Medical Co.) with an automated analyzer (TBA-120FR; Toshiba Medical Systems).

Culture of *Candida albicans*

Candida albicans (strain U-50-1) isolated from the urine of a leukemic patient (92) was cultured in Sabouraud dextrose broth (SDB; Becton, Dickinson and Company, Sparks, MD, USA) at 28°C overnight with shaking. Cells in the culture medium were collected by centrifugation and suspended and diluted 1:10 in phosphate buffered saline with 0.01% gelatin (G-PBS). The number of viable cells in the suspension was determined by counting the number of colonies on Sabouraud dextrose agar (SDA) plates 2 or 3 days after the incubation at 28°C and expressed as CFU. The final inocula ranged from 4 to 7 x 10^6 CFU/mL.

Mouse model of ascending urinary tract infection (UTI model)

C57BL/6J mice (7 weeks of age) were randomly allocated into treatment groups matched for body weight on Day −1. On day 0 of the experiment, mice under anesthesia with isoflurane (Escain; Mylan Inc., Osaka, Japan) were challenged transurethrally with *C. albicans* in 0.05 mL of G-PBS by use of stainless steel needles. Needles with smooth points were made from hypodermic needles (20 mm in length and 0.4 mm in width at the outer diameter; Minatoya/Goto Shigeo Shoten, Tokyo, Japan), autoclaved, and firmly attached to 1-mL disposable syringes. Mice were inoculated by inserting the needle into the urethral opening, with the tip approximately a half of the needle deep, and slowly pushing the syringe plunger. In preliminary experiments, maximal yeast colonization in kidney was seen around Day 6 in this spontaneously resolving model of UTI; therefore, Day 6 was chosen as the end point in all studies. Depending on the treatment regimen, the mice were treated with an SGLT2 inhibitor or vehicle (0.5% CMC; 5 mL/kg) daily from Day −1 to Day 0, Day 1, or Day 5, then euthanized by cardiac exsanguination under anesthesia with isoflurane on Day 6. The kidneys were isolated aseptically and homogenized in G-PBS. The homogenates were diluted serially with G-PBS and plated onto SDA plates. The plates were incubated at 28°C for 2 or 3

days, colonies were counted and the number of yeast cells was expressed as CFU per kidney.

Statistical analysis

Data are presented as mean ± SEM Statistical analysis was performed using SAS System for Windows, Release 8.02 (SAS Institute Japan). Statistical significance was determined by the parametric Dunnett's multiple comparison test. $P<0.05$ was regarded as statistically significant.

2. Results

Effect of single oral administration of dapagliflozin on UGE

Dapagliflozin induced UGE dose-dependently in female C57BL/6J mice. A significant increase in total UGE was observed between 0 and 24 h after administration at doses of 1 and 10 mg/kg (Fig. III-1A). The UGC also was significantly increased in the periods 0–12 h and 12–24 h after administration of doses of 1 and 10 mg/kg (Fig. III-1B).

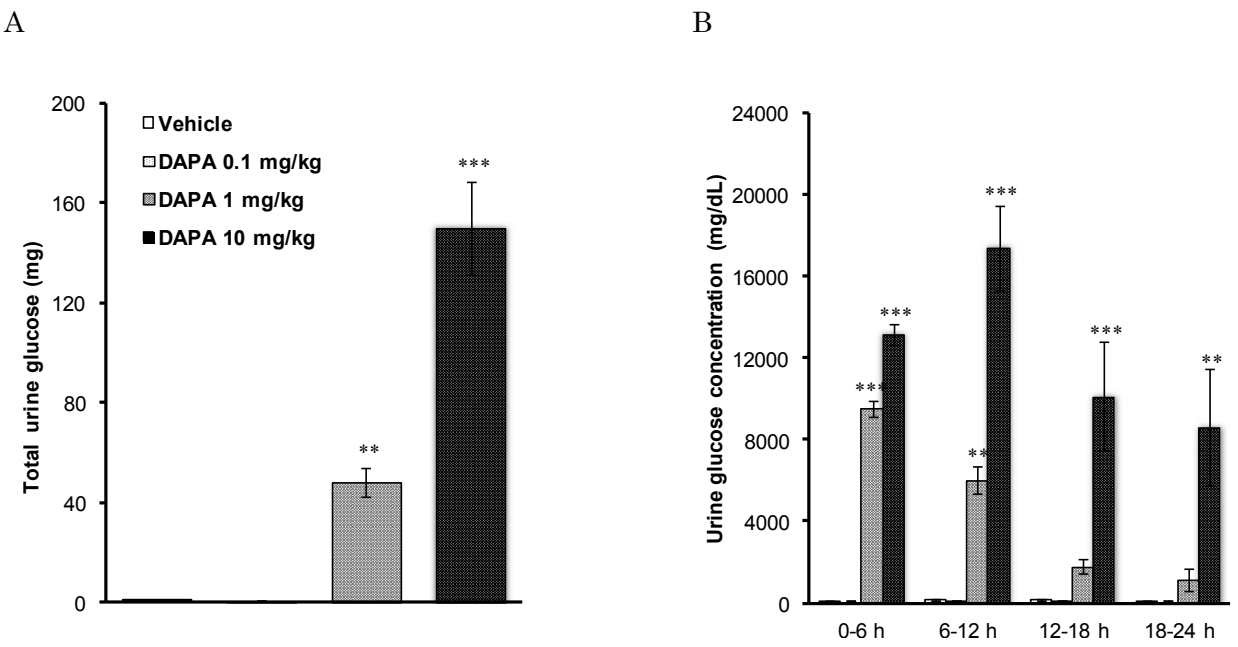


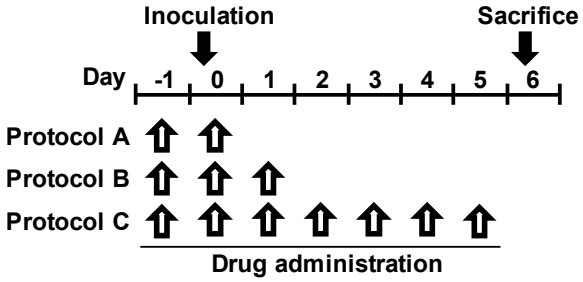
Fig. III-1 Effect of single oral gavage of dapagliflozin (DAPA) on urinary glucose excretion in female C57BL/6J mice.

A) Total urine glucose level in the vehicle-treated group was 1.0 ± 0.2 mg. B) Urine glucose concentrations (UGC) of the vehicle-treated group at 0–12 h and 12–24 h were 351 ± 71 and 238 ± 69 mg/dL, respectively. Urine samples were collected from mouse metabolic cages twice every 12 h until 12 or 24 h after the administration. The amounts of urine glucose were calculated from urine volume and urine glucose concentration. Data are expressed as mean ± SEM (n = 6). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. the vehicle-treated group by Dunnett's multiple comparison test.

Effect of duration of dapagliflozin treatment on UTI

Mice were treated with dapagliflozin (10 mg/kg/day) or vehicle 2, 3, or 7 times from Day −1. All mice were inoculated with 3.5×10^5 CFU of *C. albicans* on Day 0 (Fig. III-2A). Although the number of yeast cells in kidney of the vehicle-treated group (7-day treatment, 4.19 ± 0.24 $\log_{10}$ CFU per kidney) was lower than the number of inoculated cells on Day 0, the recovery of *C. albicans* from kidney in each of the three dapagliflozin-treated groups was significantly higher than in the vehicle-treated group (2-day dapagliflozin treatment, 6.11 ± 0.25; 3-day treatment, 6.73 ± 0.06; 7-day treatment, 7.38 ± 0.06 $\log_{10}$ CFU per kidney). The yeast cell counts in the dapagliflozin-treated groups increased depending on the duration of treatment (Fig. III-2B), indicating that there was an ascending infection by *C. albicans* in the urinary tract in the dapagliflozin groups.

A



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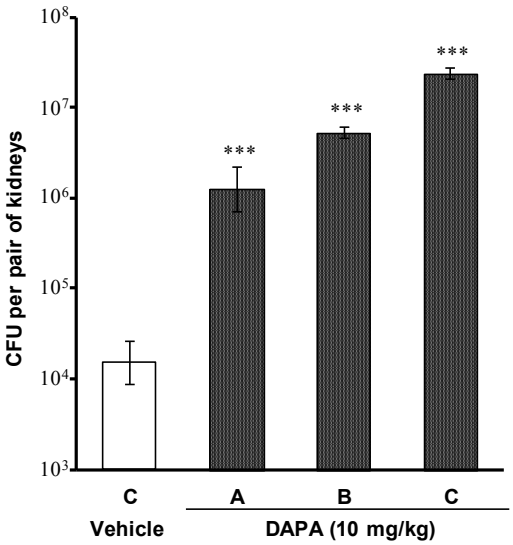


Fig. III-2 Effect of duration of dapagliflozin (DAPA) treatment on UTI in C57BL/6J mice.

A) Protocols. B) Effect on UTI. Female mice were treated by oral gavage with DAPA (10 mg/kg) or vehicle daily from Day −1 to Day 0 (2 days, Protocol A), Day 1 (3 days, Protocol B), or Day 5 (7 days, Protocol C). Mice were euthanized and kidneys were isolated on Day 6. The number of

viable *C. albicans* cells in kidney was determined by colony counting. Data are expressed as mean ± SEM (n = 3–4). ***, P < 0.001 vs. vehicle group by Dunnett's multiple comparison test.

Effect of dapagliflozin dose on UTI

Mice were treated with dapagliflozin (0.1, 1, or 10 mg/kg/day) or vehicle three times from Day −1 to Day 1 (Fig. III-2A, Protocol B). The recovery of *C. albicans* from kidney in the vehicle-treated group was 3.49 ± 0.43 $\log_{10}$ CFU per kidney, which was nearly identical to the count in the 7-day vehicle treatment group in Protocol C (Fig. III-2A). Dapagliflozin treatment dose-dependently increased the yeast cell counts (0.1 mg/kg dapagliflozin, 3.53 ± 0.68; 1 mg/kg dapagliflozin, 5.35 ± 0.49; 10 mg/kg dapagliflozin, 6.41 ± 0.12 $\log_{10}$ CFU per kidney) (Fig. III-3).

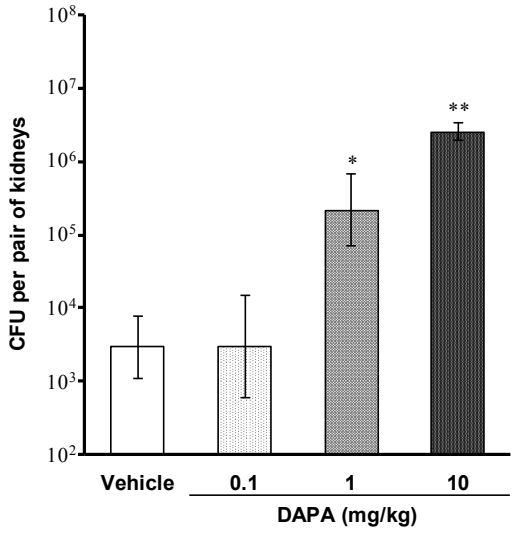


Fig. III-3 Dose-dependent effect of dapagliflozin (DAPA) on UTI in C57BL/6J mice.

Female mice were treated by oral gavage with DAPA (0.1, 1, or 10 mg/kg) or vehicle daily from Day −1 to Day 1 (Protocol B). Mice were euthanized and kidneys were isolated on Day 6. The number of viable *C. albicans* cells in kidney was determined by colony counting. Data are expressed as mean ± SEM (n = 6–7). *, P < 0.05; **, P < 0.01 vs. vehicle-treated group by Dunnett's multiple comparison test.

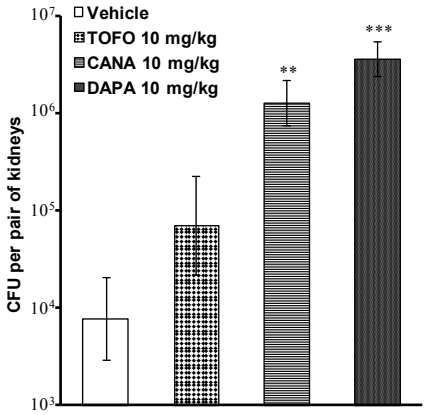
Effects of SGLT2 inhibitors on UTI and UGE

To clarify that the glucosuric effect of SGLT2 inhibition, rather than a dapagliflozin-specific effect, was important with respect to UTI, we examined the effects of tofogliflozin, canagliflozin, and dapagliflozin on UTI in Protocol B (Fig. III-2A). Mice were treated with SGLT2 inhibitor (10 mg/kg/day) or vehicle 3 times from Day −1 to Day 1.

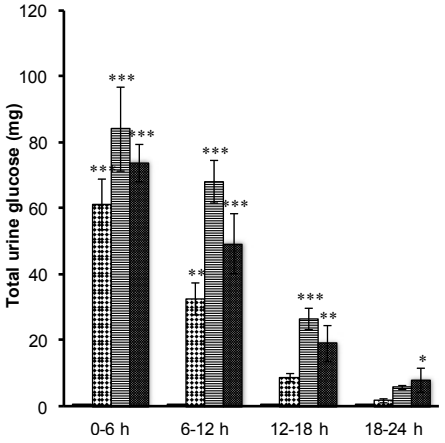
Both canagliflozin and dapagliflozin significantly increased the recovery of *C. albicans* from kidney (canagliflozin, 6.11 ± 0.23, dapagliflozin, 6.56 ± 0.18 $\log_{10}$ CFU per kidney, Fig. III-4A). In contrast, although tofogliflozin increased the yeast cell counts (tofogliflozin, 5.38 ± 0.71; vehicle, 4.08 ± 0.70 $\log_{10}$CFU per kidney), the difference was not statistically significant ($p=0.211$).

In a separate group of 7-week-old mice, the effects of the SGLT2 inhibitors (single oral gavage, 10 mg/kg) on UGE were compared. Significant increases in total UGE were observed during the period of 0–12 h after administration of tofogliflozin, 0–18 h after administration of canagliflozin, and 0–24 h after administration of dapagliflozin (Fig. III-4B). Although canagliflozin and dapagliflozin each significantly increased UGC for up to 24 h after the administration, tofogliflozin significantly increased UGC for up to 12 h (Fig. III-4C).

A



B



C

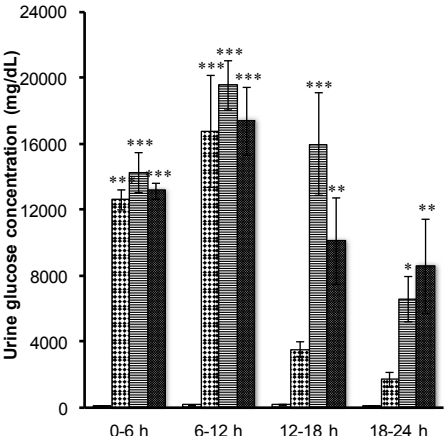


Fig. III-4 Effects of SGLT2 inhibitors on UTI and urinary glucose excretion in C57BL/6J mice.

A) Effect on UTI. Female mice were treated by oral gavage with tofogliflozin (TOFO; 10 mg/kg), canagliflozin (CANA; 10 mg/kg), dapagliflozin (DAPA; 10 mg/kg), or vehicle daily from Day −1 to Day 1 (Protocol B). Mice were euthanized and kidneys were isolated on Day 6. The number of viable *C. albicans* cells in kidney was determined by colony counting. B, C) Effect on urinary glucose excretion. Female mice were treated by single oral gavage with TOFO (10 mg/kg), CANA (10 mg/kg), DAPA (10 mg/kg), or vehicle. Urine samples were collected from mouse metabolic cages every 6 h until 24 h after the administration. The amounts of urine glucose were calculated from urine volume and urine glucose concentration. Data are expressed as mean ± SEM (n = 6-10). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle group by Dunnett's multiple comparison test.

3. Discussion

Although glucosuria enhances bacterial growth in vitro (50), it is not confirmed as a risk factor for the development of symptomatic UTI in women with diabetes (51). In addition, no evidence of ascending bacterial UTI was observed even with persistent glucosuria in SGLT2 knockout mice (93), suggesting a complex relationship between glucosuria and bacterial UTI. In this study, we have clarified the relationship between glucosuria and fungal UTI by using a newly established UTI model with normoglycemic mice, *C. albicans* as a uropathogen, and glucosuria induced with SGLT2 inhibitors.

Although the most common cause of UTI in diabetic patients is *Escherichia coli*, fungal infections with *Candida* species are also involved (48, 49, 94). Because a tendency towards increased prevalence of *Candida* species in the urine of diabetic patients treated with canagliflozin was reported (42), we used *C. albicans* as the pathogen for our mouse UTI model.

In the vehicle-treated groups in three separate experiments, the number of *C. albicans* in kidney 6 days after inoculation was lower than the inoculated cell counts, indicating that the ascending infection with *C. albicans* persisted in the urinary tract but was spontaneously resolved gradually over 1–2 weeks. In contrast, dapaglifozin increased the yeast cell counts in accordance with the duration of dapagliflozin treatment (Fig. III-2B) and dose (Fig. III-3), indicating that the ascending infection was exacerbated by dapagliflozin.

In a separate group of normal mice, dapagliflozin dose-dependently promoted glucosuria (Fig. III-1A). The nearly identical dose–response relationship observed between glucosuria (Fig. III-1A) and UTI (Fig. III-2B) suggests that the ascending infection with *C. albicans* was influenced by the degree of glucosuria.

In addition, although dapagliflozin (10 mg/kg) or canagliflozin (10 mg/kg) significantly increased the susceptibility of the mice to UTI, tofogliflozin (10 mg/kg) did not significantly increase the susceptibility to UTI (Fig. III-4A).

In contrast to the significant increases in UGC up to 24 h after administration of dapagliflozin or canagliflozin, a significant increase in UGC in the tofogliflozin-treated group lasted only up to 12 h (Fig. III-4C). The plasma half-life of the SGLT2 inhibitors in rats is estimated to be 4.6 h for dapagliflozin (60), 5.0 to 5.2 h for canagliflozin (61), and 2.4 to 3.0 h for tofogliflozin (21). Therefore, the persistent increases in UGC with SGLT2 inhibitors in mice may be associated with the pharmacokinetic profile of each SGLT2 inhibitor and not with a compound-specific off-target effect.

The above-mentioned differences among the tested SGLT2 inhibitors with respect to the duration of increased UGC and differences in the effects on the severity of UTI suggest that prolonged glucosuria above a certain level, which may be influenced by the pharmacokinetic profile of each compound, is critical to the impact of an SGLT2 inhibitor on the susceptibility of mice to UTI. At present, we hypothesize that the lower frequency of UTI with tofogliflozin treatment in this model can be ascribed to the shorter duration of increased UGC than with other SGLT2 inhibitors.

Although tofogliflozin showed a shorter plasma half-life than dapagliflozin and canagliflozin in rats (21, 60, 61), it induced glucosuria and improved hyperglycemia in diabetic animal models including ZDF rats for at least 12 hours with an oral dosing of 1 to 10 mg/kg (21). In addition, both dapagliflozin and canagliflozin also induced glucosuria robustly in ZDF rats at a dose of 1 to 10 mg/kg (95, 82) with improvements in hyperglycemia comparable with tofogliflozin. These results suggest that difference in the plasma half-life between tofogliflozin and other SGLT2 inhibitors may have a minor impact on their maximum plasma glucose-lowering effects, at least in ZDF rats. However, the duration of persistent glucosuria may also have an impact on the stability of long-term glycemic control. Therefore further evaluations will be required to understand the relationships between the duration of persistent glucosuria, the plasma glucose-lowering effects, and the risk of UTI with SGLT2 inhibitors.

A limitation in this UTI model is that it does not include the pathological conditions of T2D, which may also be associated with increased prevalence of infection (46, 52). Nevertheless, this model has merit for the purpose of specifically evaluating the effect of glucosuria on susceptibility to UTI. With further modifications, this mouse model will be a useful tool with which to study the pathophysiology of fungal UTI.

VII. COMPREHENSIVE DISCUSSIONS

In our preclinical analysis, tofogliflozin was a potent and competitive SGLT2 inhibitor, induced UGE and decreased both postprandial and causal plasma glucose level, and improved hyperglycemia and pathological conditions of T2D. Tofogliflozin also induced calorie loss via inducing UGE and ameliorated body weight gain, and prevented or improved lipids accumulation in adipose tissues (obesity) and liver (hepatic steatosis), and insulin resistance in the obese animals. These results indicate that tofogliflozin could prevent or improve hyperglycemia, pathological conditions of T2D such as insulin resistance, obesity, and hepatic steatosis in patients with T2D. Actually in 24 or 52-weeks clinical trials for patients with T2D, tofogliflozin induced UGE, reduced both causal and postprandial plasma glucose levels dose-dependently, and improved insulin resistance, obesity, and parameters of liver functions (38, 39). Also, tofogliflozin slightly reduced systolic and diastolic blood pressure, plasma uric acid level, and plasma LDL level, and increased plasma HDL level. Moreover, tofogliflozin without insulin actions could be used with all of oral antidiabetic drugs, and the combination therapies improved hyperglycemia compared to each monotherapy.

Preclinical studies indicated that tofogliflozin was a highly selective SGLT2 inhibitor with the weak actions toward the other SGLT subtypes, reactions for glucose metabolism and various molecular targets. Although tofogliflozin induced UGE in normal rats, it was not observed hypoglycemia. Moreover, experimental results in UTI animal model constructed in our laboratory suggested that prolonged glucosuria would be critical to the impact of a SGLT2 inhibitor on the susceptibility of mice to UTI. And high UGC has not been maintained until 24 h after dosing tofogliflozin with a shorter plasma half-life compared to other SGLT2 inhibitors. With the highly selectivity and the short half life, it is suggested that tofogliflozin would induce fewer unexpected side-effects, hypoglycemia or UTI. Tofogliflozin actually was well tolerated with a

treatment discontinuation rate of <6% over 52 weeks in the clinical trial (38, 39). Adverse events that led to discontinuation occurred at similar rates in tofogliflozin and placebo groups. Most adverse events were mild or moderate in severity. Main adverse events were pollakiuria and thirst, an incidence of >5% with tofogliflozin. The incidence of hyperketonemia in tofogliflozin groups appeared to be dose-dependent. Also the incidence of hypoglycemia was similar between the treatment groups, but it was higher in the subgroups of patients treated with tofogliflozin-sulfonylurea combination. Genital and urinary tract infections were induced with more than 5% incidence, but all events were transient.

The results of the clinical trials indicated that tofogliflozin is an effective and tolerable antidiabetic agent both monotherapy and in combination with other oral antidiabetic agents. But we cannot answer the several questions in terms of efficacy with more long-term treatment, improvement or prevention of Type 1 diabetes, obesity, fatty liver, or dyslipidemia, efficacy in T2D patients with renal dysfunction, body weight reduction in patients who is lean, dehydration induced by pollakiuria, and incidence of cardiovascular diseases. Dr. Kaku *et al.* wrote that there were some limitations in these clinical trials, so larger number of patients enrolled, longer study duration, and patients with more various race or diseases are required to answer these questions, and further studies after the launch would clarify more optical treatment regimens using tofogliflozin for patients with various pathological features.

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