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Effect of LX4211 on Glucose Homeostasis and Body Composition in Preclinical Models

David R. Powell, Christopher M. DaCosta, Melinda Smith, Deon Doree, Angela Harris, Lindsey Buhring, William Heydorn, Amr Nouraldeen, Wendy Xiong, Padmaja Yalamanchili, Faika Mseeh, Alan Wilson, Melanie Shadoan, Brian Zambrowicz, Zhi-Ming Ding

Lexicon Pharmaceuticals, Inc., The Woodlands, Texas, USA (DRP, CMD, MS, DD, AH, LB, AN, WX, FM, AW, MS, BZ, ZMD); Lexicon Pharmaceuticals, Inc., Princeton, New Jersey, USA (WH, PY).

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Corresponding Author:

David R. Powell, MD

Lexicon Pharmaceuticals, Inc.

8800 Technology Forest Place

The Woodlands, TX 77381

Telephone: 281-863-3060

Fax: 281-863-8115

Email: dpowell@lexpharma.com

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Abbreviations:

AMG, α-methylglucopyranoside

AUC, area under the curve

CL, clearance

C_{max}, plasma peak concentration

DIO, diet-induced obesity

DPP4, dipeptidylpeptidase 4

ED50, dose causing 50% maximal UGE in the first 24 hours following dosing

GLP-1, glucagon-like peptide 1

HFD, high fat diet

IC_{50}, concentration causing half-maximal inhibition

LBM, lean body mass

LC/MS, liquid chromatography/mass spectroscopy

LFD, low fat diet

OGTT, oral glucose tolerance test

QMR, quantitative magnetic resonance

RYGB, Roux-en-Y gastric bypass

SGLT1, sodium/glucose cotransporter 1

SGLT2, sodium/glucose cotransporter 2

T2DM, type 2 diabetes mellitus

T_{max}, time of plasma peak concentration

TZDs, thiazolidinediones

UGE, urinary glucose excretion

V_{ss}, volume of distribution

%F, oral bioavailability

Recommended section assignment: Endocrine and diabetes

Abstract

Treatments which lower blood glucose levels and body weight should benefit patients with type 2 diabetes mellitus (T2DM). We developed LX4211, an orally available small molecule that decreases postprandial glucose excursions by inhibiting intestinal SGLT1 and increases urinary glucose excretion (UGE) by inhibiting renal SGLT2. In clinical studies of patients with T2DM, LX4211 appears to act through dual SGLT1/SGLT2 inhibition to improve glycemic control and promote weight loss. Here, we present preclinical studies which explored the ability of LX4211 to improve glycemic control and promote weight loss. We found: 1) LX4211 inhibited in vitro glucose transport mediated by mouse, rat and dog SGLT1 and SGLT2; 2) a single daily LX4211 dose markedly increased UGE for > 24 hours in mouse, rat and dog; and 3) in the KKA^y mouse model of T2DM, LX4211 lowered A1C and postprandial glucose concentrations while increasing postprandial GLP-1 concentrations. Also, long-term LX4211 treatment: 1) decreased OGTT glucose excursions, increased OGTT 30 minute insulin concentrations and increased pancreatic insulin content in KKA^y mice; and 2) decreased weight gain in dogs and rats but not in KKA^y mice, while increasing food consumption in dogs, rats and KKA^y mice; in these KKA^y mice, calories lost through UGE were completely offset by calories gained through hyperphagia. These findings suggest that LX4211 improves glycemic control by dual SGLT1/SGLT2 inhibition in mice as in humans, and that the LX4211-mediated weight loss observed in patients with T2DM may be attenuated by LX4211-mediated hyperphagia in some of these individuals.

4

Introduction

An estimated 26 million people in the United States have diabetes, with the prevalence projected to increase to 44 million by 2034 (Huang et al., 2009). Patients with type 1 and type 2 diabetes mellitus (T2DM) are at increased risk for the microvascular complications of renal failure and retinopathy, and for macrovascular complications such as myocardial infarction and stroke. Available data suggest that lowering blood glucose and/or body weight decreases the risk of microvascular complications and may decrease the risk of macrovascular complications in individuals with T2DM (American Diabetes Association, 2013; National Institutes of Health, 1998; Reusch And Wang, 2011).

T2DM develops due to a defect in the ability of β-cells to compensate for insulin resistance with sufficient glucose-stimulated insulin secretion to normalize blood glucose levels. The natural history of T2DM is a progressive decline in β-cell function. Among other factors, high glucose levels appear to be a significant contributor to impaired β-cell function, a process referred to as glucotoxicity (DeFronzo, 2009; Wajchenberg, 2007).

The above observations suggest that treatments which lower blood glucose levels and body weight should be of value to patients with T2DM. Such effects are observed in patients treated with Roux-en-Y gastric bypass (RYGB) surgery; this treatment often reverses T2DM, even in individuals with long-standing disease (Cohen et al., 2012; Kashyap et al., 2013). Marked calorie restriction can duplicate this effect in patients with T2DM of shorter duration (Lim et al., 2011). In contrast to RYGB surgery and caloric restriction, the orally available glucose-lowering agents that have been

mainstays of anti-diabetic therapy for years, including metformin, sulfonylureas, thiazolidinediones and dipeptidylpeptidase (DPP)4 inhibitors, do not promote significant weight loss, and many promote weight gain, in patients with T2DM (American Diabetes Association, 2013; Nathan et al., 2009).

The diabetes-mediated impairment in β-cell insulin secretion can be reversed by lowering blood glucose levels. This was first demonstrated using phlorizin, an inhibitor of sodium/glucose cotransporter (SGLT)2-mediated renal glucose reabsorption, to increase urinary glucose excretion (UGE) in partially pancreatectomized rats (Rossetti et al., 1987).   Phlorizin was not developed as an antidiabetic agent due to lack of efficacy when given orally, but orally available compounds that increase UGE by inhibiting renal SGLT2 have been developed; these compounds lower blood glucose and body weight while potentially protecting pancreatic β-cells (Musso et al., 2012; Abdul-Ghani et al, 2012). In addition, we have developed LX4211, a dual SGLT1/2 inhibitor (Goodwin NC, et al., 2009; Zambrowicz et al., 2012) that has some structural similarity to phlorizin (Zambrowicz et al., 2012). In mice and humans, LX4211 not only increased UGE but also inhibited intestinal SGLT1, resulting in higher circulating glucagon-like peptide (GLP)-1 levels and lower postprandial glucose excursions (Powell et al., 2013a; Powell et al., 2013b; Zambrowicz et al., 2013a; Zambrowicz et al, 2013b). This dual inhibition, which resulted in improved glycemic control and weight loss in clinical studies of patients with T2DM (Zambrowicz et al., 2012; Rosenstock et al., 2012), provides two complementary mechanisms, each of which is insulin-independent, for improving glycemic control while potentially lowering demands on β-cells in individuals with T2DM. The studies presented here provide further evidence that

LX4211 is a dual SGLT1/SGLT2 inhibitor, examine the effect of LX4211 on UGE, food

consumption and weight gain in multiple species, and demonstrate the ability of LX4211

to improve glycemic control while increasing pancreatic β-cell insulin stores in the KKA^{y}

mouse model of T2DM.

Methods

    Animals. All reported studies were carried out in strict accordance with the

recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. The protocols for all studies performed at Lexicon

Pharmaceuticals were approved by the Lexicon Institutional Animal Care and Use

Committee (OLAW Assurance Number, A4152-01; AAALAC International Accreditation

Number, 001025). The protocols for all studies performed at Covance Laboratories

were approved by the Covance Institutional Animal Care and Use Committee (Animal

Welfare Assurance No. A3218-01; USDA Registration No. 35-R-0030).

    All mouse and some rat studies were performed at Lexicon Pharmaceuticals.

KK.Cg-Ay/J heterozygous mice (KKA^y mice; The Jackson Laboratory, Bar Harbor, ME)

were obtained at 4 weeks of age and fed high fat diet containing 45% kcal as fat (45%

HFD; D12451, Research Diets, New Brunswick, NJ). C57BL/6-*Tyr*c-Brd (C57) weanling

mice from an in-house colony were fed either 45% HFD for at least 20 weeks prior to

study to induce diet-induced obesity (DIO), or were fed a standard rodent chow diet

(5010, LabDiet, PMI Nutrition International, St. Louis, MO) containing 23% calories as

fat. Obese Prone Crl:CD rats (OP-CD rats; Charles River Laboratories International,

Wilmington, MA) were obtained at 4 weeks of age and fed HFD containing 60% kcal as

fat (60% HFD; D12492, Research Diets). Crl:CD Sprague-Dawley Rats (S-D rats;

Charles River) were obtained at 4 weeks of age and fed either chow diet or low fat diet

containing 10% kcal as fat (LFD; D12450B, Research Diets). All mice and rats were

maintained in a temperature-controlled environment on a fixed 12 hour light/12 hour

dark cycle and with free access to water and food.

Some rat and all dog studies were performed at Covance Laboratories (Madison, WI). During the 4 and 13 week rat toxicology studies, S-D rats were fed Teklad Certified Rodent Diet 8728C (Harlan Laboratories, Indianapolis, IN). During the 4 week dog toxicology study, beagle dogs were fed Teklad Certified Canine Diet #2027C (Harlan Laboratories). During the short-term dog pharmacokinetic/UGE studies, beagle dogs were fed non-certified Canine Diet 5L03 (PMI Nutrition International) and were provided certified canine treats, as appropriate. Rats and dogs were housed in temperature-controlled environments on a fixed 12 hour light/12 hour dark cycle and with free access to water and food.

Body composition measurements. Body composition of mice and rats was measured using quantitative magnetic resonance (QMR; ECHO Medical Systems, Houston, TX) technologies.

Glucose tolerance tests. Oral glucose tolerance tests (OGTTs) were performed on conscious, unanesthetized mice. After an overnight fast, mice were bled at baseline and then received 2 g/kg glucose by oral gavage. Whole blood samples obtained from the retro-orbital plexus at 0, 30, 60, 90 and 120 minutes were directly assayed for glucose levels by ACCU-CHEK Aviva glucometer (Roche, Indianapolis, IN); serum obtained at 0 and 30 minutes was used to measure insulin levels (Ultra Sensitive Rat Insulin ELISA Kit, Cat. # 90060, Crystal Chem, Downers Grove, IL).

Glucose levels and pH of gastrointestinal contents. For fed OP-CD rats, glucose concentration, pH and volume of cecal contents were measured as described previously (Powell et al., 2013a).

Pancreatic insulin content. At necropsy, the pancreas was isolated, weighed and snap-frozen with glass beads in bead beater tubes. At the time of assay, 295mL 95% EtOH was mixed with 5mL 12N HCl (37.5%) to make acidified EtOH (aEtOH). Chilled (4^oC) aEtOH was added to each tube at the ratio of 1g pancreas/10mL (1:10 w/v). After homogenizing for 3 minutes at 4^oC, the mixture was incubated at 4^oC for 48 hours and then centrifuged at 3000rpm for 30 minutes at 4^oC. The supernatant was then assayed for insulin (Ultra Sensitive Rat Insulin ELISA Kit. Crystal Chem), and total pancreatic insulin content was calculated.

24-hour UGE. *Mice:* 24 to 35 week old male DIO (30-51 g) C57 mice were individually housed in metabolic cages (Nalge Nunc International, Rochester, NY) and fed 45% HFD homogenized in water at a 2:1 (wt:wt) ratio; this paste diet prevented contamination of urine with crumbled diet. After an acclimatization period, each mouse received, by oral gavage, a single dose of either vehicle or vehicle containing LX4211. For this and all other studies, the gavage volume was 5 mL/kg and the vehicle was aqueous 0.1% v/v Tween 80 unless stated otherwise. Complete 24-hour urine samples were collected during the 24 hours after dosing (day 1) and, for some mice, on days 2 and 3. In a separate study, 12 to 13 week old female (38-55 g) KKA^y mice had UGE measured by the same protocol except that they received LX4211 daily. *Rats:* 11-15-week-old male (367-531 g) S-D rats were individually housed in metabolic cages (Nalge

Nunc International) and fed LFD homogenized in water at a 1:1 (wt:wt) ratio. After an acclimatization period, each rat received, by oral gavage, a single dose of either vehicle or LX4211. Complete 24-hour urine samples were collected on days 1 and 2 and, for some rats, on days 3 and 4. In separate studies, 9 week old male OP-CD rats had UGE measured by the same protocol except that the rats received daily doses of either vehicle or LX4211. For the above mouse and rat studies, the volume of each 24-hour urine collection was recorded and then the urine sample was centrifuged and analyzed for glucose concentration (Cobas Integra 400 Clinical Chemistry Autoanalyzer, Roche Diagnostics, Indianapolis, IN). *Dogs:* Adult, non-naïve male (8-12 kg) beagle dogs were fasted overnight and then individually housed in cages designed for separation of urine and feces and for collection of urine. Each dog received, by oral gavage, a single dose of either vehicle or LX4211. All dogs were provided free access to food 4 hours after dosing. Complete 24-hour urine samples were collected on day 1 and, for some dogs, on day 2. After the volume of each 24-hour urine collection was recorded, the urine sample was centrifuged and frozen at -70°C for later batch analysis of glucose concentrations (Cobas Integra 400 Clinical Chemistry Autoanalyzer, Roche Diagnostics). For all species, the absolute quantity of glucose excreted per day per animal was calculated based on urine volume and urine glucose concentration.

Pharmacokinetics. Adult male C57 mice (25-35 g) and male S-D rats (250-450 g) were maintained on chow diet. Adult male beagle dogs are the same animals used in the UGE studies described above; they were fasted overnight before dosing and were

fed 6 hours after dosing. All animals had free access to water and were conscious through the study.

For intravenous administration (IV), LX4211 was dissolved in 15% Captisol to form a clear solution and was administered at a dose of 1 mg/kg to all species. For oral administration (PO), LX4211 was gavaged to mice and rats at a dose of 10 mg/kg and to dogs at 3 mg/kg. Following intravenous injection, serial blood samples were collected in EDTA-containing tubes through 6 hours in rodents and up to 48 hours in dogs. Following oral administration, serial blood samples were collected through 24 hours in rodents and up to 48 hours in dogs. The plasma fraction was immediately separated by centrifugation at 4 ^{o}C, and then stored at -20 ^{o}C until sample analysis. Bioanalysis of plasma samples for quantitating plasma LX4211 concentrations was conducted using liquid chromatography/mass spectroscopy (LC/MS).

Pharmacokinetic data analysis. All plasma concentration versus time data for LX4211 was analyzed using non-compartmental model 200 (for oral administration) and 201 (for intravenous administration) of WinNonlin (version 5.0, Pharsight, Inc. Mountain View, CA). The half-life during the terminal phase was calculated from the elimination rate constant (λ) determined by the linear regression analysis of the log-linear part of the plasma concentration curve. The AUC_{0-t} was calculated using linear up/log down trapezoidal method up to the last measured concentration at time t. $AUC_{0-\infty}$ was calculated as $AUC_{0-t} + C_t/\lambda$. Clearance (CL) was calculated by dose/$AUC_{0-\infty}$. Other pharmacokinetic parameters included plasma peak concentration (C_{max}), the time of

12

C_{max} (T_{max}) and volume of distribution at steady state (V_{ss}). Oral bioavailability (%F) was calculated by the following equation:

%F = (AUC$_{0-\infty}$ PO/AUC$_{0-\infty}$ IV) x (Dose IV/Dose PO)

KKA^y diabetic mouse studies. Female KKAy mice were individually housed and fed 45% HFD. In Study 1, 10 week-old mice were acclimatized for 2 weeks and then, based on A1C and OGTT glucose AUC values, were randomized into 2 groups that began to receive, as dietary admixture, either vehicle or LX4211 at a dose of 1 mg/kg/day. In Study 2, 6-7 week-old mice were acclimatized for 1 week and then, based on A1C and body weight data, were randomized into 3 groups that began to receive, as dietary admixture, either vehicle or LX4211 dosed at 1 or 10 mg/kg/day. Measurements performed on the mice included UGE while in metabolic cages, body composition by QMR, body weight and food consumption. Glycemic control was evaluated by A1C levels (Study 1, Cobas Integra 400; Study 2, A1c Now+ System, Bayer HealthCare, Tarrytown, NY), fed glucose levels (Cobas Integra 400) and OGTT glucose excursions. β-cell insulin reserves were evaluated in both studies at necropsy by measuring pancreatic insulin content. On the final day of Study 2, the mice received an oral 2 g/kg glucose bolus and then, 2 hours later at necropsy, plasma was obtained to measure total GLP-1 (Glucagon-Like Peptide-1 Total ELISA Kit, catalog #EZGLP1T-36K, Millipore) concentrations as described previously (Powell et al., 2013b).

13

OP-CD obese rat study. Beginning at 8 weeks of age, male OP-CD rats were individually housed in metabolic cages and fed 60% HFD. Food consumption and body weight were measured daily Monday through Friday. Starting at 9 weeks of age, rats were dosed by oral gavage once daily with either vehicle or 30 mg/kg LX4211. Complete 24-hour urine samples were collected on dosing days 2-4, and 24-hour fecal collections on dosing day 4, after which the rats were removed from the metabolic cages. Urine samples were used to calculate UGE as described above. Fecal samples were weighed, homogenized with MilliQ water (2 g water to 1 g feces) using a bead beater at 4°C and then centrifuged at 4°C; the supernatant was analyzed for pH and glucose concentration, and fecal glucose content was calculated from the product of glucose concentration and volume of supernatant. The rats were necropsied in the fed state on day 25 of dosing; glucose levels and pH were measured in cecal contents, and carcasses were analyzed for body composition by QMR, as described above.

Toxicology studies. Body weight and food consumption data, obtained as part of GLP studies exploring the toxicology of LX4211, were analyzed from dosing groups that showed no significant adverse effects of LX4211 treatment. For all 3 toxicology studies, the dosing vehicle was 0.25% (w/v) methylcellulose in water. *Four week rat toxicology study:* Male (220-310 g) S-D rats, 6 to 7 weeks of age at study onset, began to receive single daily 10 mL/kg oral gavage doses of either vehicle (15 rats) or vehicle containing 10 (10 rats), 30 (10 rats) or 100 (15 rats) mg/kg of LX4211. Dosing continued for 4 weeks; during this time, the rats were housed individually in stainless steel cages. Body weights were recorded before the first dose was given on the first day of dosing, and

weekly thereafter. Food consumption was measured weekly during the dosing phase of the study. *Thirteen week rat toxicology study:* Male (310-450g) S-D rats, 11 to 12 weeks of age at study outset, began to receive single daily 10 mL/kg oral gavage doses of either vehicle (15 rats) or vehicle containing 3 (10 rats), 10 (10 rats), 30 (15 rats) or 100 (15 rats) mg/kg of LX4211. Dosing continued for 13 weeks; during this time, the rats were housed individually in stainless steel cages. Body weights and food consumption were monitored as in the 4 week rat toxicology study. *Four week dog toxicology study:* 13 male (9-10 kg) and 13 female (6-8 kg) purebred beagle dogs, 6.5 to 7.5 months of age at study outset, received single daily oral gavage doses of either vehicle (10 dogs) or vehicle containing 30 (6 dogs) or 100 (10 dogs) mg/kg of LX4211. Dosing continued for 28 days; during this time, the dogs were housed individually in stainless steel cages, and offered diet, for at least 6 hours/day. Body weights and food consumption were monitored as in the 4 week rat toxicology study.

SGLT2 and SGLT1 Cell Lines. The full-length coding sequences of rat SGLT2 and SGLT1 (accession numbers NM_022590.2 and BC081827), and dog SGLT2 and SGLT1 (accession numbers NM_001007142 and NM_001007141), containing HA-tags at the N-terminus, were cloned into the mammalian expression vector pIRESpuro2 (Clontech, Mountain View, CA). HEK293 cells (ATCC, Manassas, VA) were transfected with the HA-SGLT2-pIRESpuro2 and HA-SGLT1-pIRESpuro2 vectors and bulk stable cell lines were selected in the presence of 0.5 μg/mL of puromycin. All HA-SGLT2- and HA-SGLT1-expressing cell lines were maintained in DMEM media containing 10% FBS, 1% GPS and 0.5 μg/mL of puromycin. These HEK293 cell lines were used in

15

experiments to determine the IC_{50} (concentration causing half-maximal inhibition) of LX4211 for SGLT2- and SGLT1-mediated glucose transport.

α-Methylglucopyranoside (AMG) Uptake Assay. When expressed in cells, SGLT2 and SGLT1 mediate sodium-coupled uptake of D-glucose or AMG, a non-metabolizable glucose analogue specific for sodium-dependent glucose transporters. The ability of LX4211 to inhibit SGLT2- and SGLT1-mediated glucose transport was estimated by measuring SGLT2- or SGLT1-mediated ^{14}C-AMG uptake in the presence of increasing compound concentration, as described previously (Powell et al., 2013b).

Statistics. Data are presented as mean ± standard deviation (SD). Comparisons between two groups were analyzed by unpaired Student's T-test. Comparisons among three or more groups were analyzed by one-way ANOVA with post-hoc analysis performed by the Bonferroni method, except for the 13 week rat study, where post-hoc comparisons between each LX4211-treated group with the vehicle group were made using Dunnett's test. Values were considered statistically significant when $P < 0.05$.

Results

LX4211 Inhibition of SGLT1 and SGLT2 in vitro

The structure of LX4211 is presented in Figure 1A. LX4211 has been shown to inhibit glucose transport by HEK293 cells over-expressing mouse or human SGLT1 or SGLT2 (Zambrowicz et al., 2012; Powell et al., 2013b). As is shown in Table 1, LX4211 also inhibits glucose transport mediated by rat and dog SGLT1 and SGLT2.

LX4211 Pharmacokinetics

The major pharmacokinetic parameters of LX4211 in the mouse, rat and dog are summarized in Table 2 and Table 3. Plasma concentration-time profiles are presented in Figure 1B and 1C. Following an intravenous injection of LX4211, a low plasma clearance (CL) was observed in all species (Table 2), with the lowest CL in the dog (4.6 mL/min/kg). At steady state, LX4211's volume of distribution appeared comparable across species (Table 2). Following oral administration, LX4211 was extensively absorbed by the mouse and dog with oral bioavailabilities (%F) of 98% and 71%, respectively (Table 3). The T_{max} in the rat following oral dosing was approximately 3 hours, and the plasma levels remained high even at 6 hrs post dosing. Thus it was not possible to determine a true elimination rate, or $AUC_{0-\infty}$, and therefore %F could not be accurately determined for the rat. However, it is clear that the bioavailability in the rat is quite high.

LX4211 effect on UGE

17

For all species tested, LX4211 caused a dose-dependent increase in glucose excretion over the 24 hours following dosing (Figure 2). In mice, the minimal dose that increased UGE was 0.1 mg/kg, the maximal 24-hour UGE was 628 mg/day and the ED50 (daily dose causing 50% maximal glucose excretion in the first 24 hours following dosing) was estimated to be 1.8 mg/kg (Figure 2A). As shown in Figure 2B, UGE was close to maximal for 2 days after mice received single 10 mg/kg or 60 mg/kg doses of LX4211. In rats, the lowest dose tested (0.3 mg/kg) increased UGE, the maximal 24-hour UGE was 5.1 g/day and the ED50 was estimated to be 0.75 mg/kg (Figure 2C). As shown in Figure 2D, UGE was close to maximal for at least 2 days after rats received a single 50 mg/kg dose of LX4211. In dogs, the minimal dose that increased UGE was 0.1 mg/kg, the maximal 24-hour UGE was 36.7 g/day, and the ED50 was estimated to be 0.2 mg/kg (Figure 2E). As shown in Figure 2F, UGE was close to maximal for at least 2 days after dogs received a single 3 mg/kg dose of LX4211.

LX4211 effect on glucose homeostasis

The KKA^y mouse model of T2D was used in two independent studies to test the effects of LX4211. In Study 1 (Figure 3A), LX4211 at a dose of 1 mg/kg significantly lowered both A1C (Figure 3B) and fed glucose levels (Figure 3C) relative to vehicle-treated control mice. During an OGTT, LX4211 also significantly lowered glucose excursions (Figure 3D) while significantly increasing insulin concentrations between 0 and 30 minutes (Figure 3E). At necropsy, total pancreatic insulin content was significantly greater in the LX4211-treated mice (Figure 3F). As expected, UGE was significantly increased by LX4211 (Figure 3G). In Study 2 (Figure 4A), LX4211 at doses

18

of 1 and 10 mg/kg significantly lowered both A1C (Figure 4B) and fed glucose levels

(Figure 4C). During an OGTT, each LX4211 dose significantly lowered glucose

excursions (Figure 4D), and insulin concentrations were better maintained in the group

receiving 10 mg/kg of LX4211 relative to vehicle-treated controls (Figure 4E). Total

pancreatic insulin content was significantly greater in both LX4211 treatment groups

relative to vehicle-treated controls (Figure 4F). UGE was significantly and comparably

increased by the two LX4211 doses (Figure 4G).

LX4211 effect on body composition

Long-term treatment with LX4211 had no effect on either body weight or body fat

of KKA^y mice from the two independent studies (Figure 5A-D; Supplementary Table 1).

However, LX4211 increased food consumption in these mice (Figure 5E-F). In Study 2,

which showed a significant increase in food consumption, the estimated mean increase

in energy intake by LX4211-treated mice (11.8 kjoules/mouse) was comparable to the

estimated mean energy lost as urinary glucose by these mice (10.6 kjoules/mouse).

Lean rats and dogs treated with LX4211 for 4 weeks gained significantly less body

weight despite consuming significantly more food (Figure 6A-D). To examine this effect

more closely, lean rats were treated with increasing doses of LX4211 for 13 weeks.

Body weight gain in these rats was impaired by LX4211 treatment (Figure 6E;

Supplementary Table 2); although the differences in body weight gain did not achieve

statistical significance, they were accompanied by significant, dose-dependent

increases in food consumption (Figure 6F; Supplementary Table 2).

In a study using OP-CD obese rats (Figure 7A), treatment with 30 mg/kg LX4211 induced a robust increase in UGE (Figure 7B). After 4 weeks of treatment, LX4211-treated rats gained significantly less body weight, body fat and LBM relative to vehicle treated controls (Figure 7C-E; Supplementary Table 1) despite comparable food intake (Figure 7F).

LX4211 effect on intestinal glucose absorption

After 4 weeks of LX4211 treatment, OP-CD obese rats showed a significant increase in cecal glucose content and a decrease in cecal pH (Figure 7G-H). Although GLP-1 levels were not measured in these rats, total GLP-1 levels were significantly increased in LX4211-treated KKA[y] mice 2 hours after an oral challenge with 2 g/kg glucose (Figure 4H).

Discussion

LX4211 is a non-selective dual SGLT1 and -2 inhibitor in all species studied to date, and is orally bioavailable in these same species (Zambrowicz et al., 2012; Powell et al., 2013b; data presented here). The prolonged exposure observed after oral administration of LX4211 to mice, rats and dogs is consistent with what has been reported in humans, as is the prolonged increase in UGE (Zambrowicz et al., 2012). These observations support the relevance of LX4211 studies in mouse, rat and dog preclinical models.

KKA^y mice are quite hyperglycemic despite hyperinsulinemia and β-cell insulin degranulation, indicating that their β-cells cannot fully compensate for the increased demand created by severe insulin resistance (Iwatsuka et al., 1970). We used this T2DM model to test the effect of LX4211 on glycemic control. We found that glycemic control was improved in 2 independent studies where KKA^y mice received LX4211; A1C and fed glucose values were much lower in LX4211-treated mice, similar to findings in patients with T2DM (Zambrowicz et al., 2012), and glucose excursions during OGTTs were significantly decreased during LX4211 treatment in mice, as they were in humans (Zambrowicz et al., 2012). Despite their decreased glucose excursions, the LX4211-treated mice had significantly greater glucose-stimulated insulin release compared to vehicle-treated control mice. In each of the 2 studies, this was associated with a significant increase in pancreatic insulin content; the mice were in the fed state in both instances, and in fact the mice from study 2 had also received an oral glucose challenge 2 hours prior to necropsy. The combination of decreased glucose excursions,

21

increased insulin response and increased β-cell insulin reserves in multiple cohorts of

LX4211-treated postprandial mice suggests that LX4211 is having a beneficial effect on

β-cell function. This beneficial effect may be due, in part, to the ability of LX4211 to

lower blood glucose by increasing UGE and by delaying intestinal glucose absorption,

two insulin-independent mechanisms of action that should unload β-cells by decreasing

the demand for insulin. TZDs also lower blood glucose by insulin-sparing mechanisms,

raising the possibility that, like TZDs, LX4211 may preserve β-cell function during long-

term treatment (DeFronzo, 2009; Wajchenberg, 2007; Kahn et al., 2011). Consistent

with these observations, 1) SGLT2 deficiency preserved β-cell function in *db/db* mice

(Jurczak et al., 2011); 2) phlorizin-mediated SGLT2 inhibition markedly improved

multiple measures of β-cell function in partially pancreatectomized rats (Rossetti et al.,

1987); and 3) β-cell function and islet morphology were significantly improved in ZDF

rats treated with the SGLT2 inhibitor dapagliflozin (Macdonald et al., 2010).

Improved glycemic control in LX4211-treated KKA[y] mice may be due not only to

increased UGE but also to other mechanisms. In healthy mice, LX4211 treatment is

associated with blunted OGTT glucose excursions along with increased cecal glucose

and circulating GLP-1 levels, and with lower cecal pH. This is due to LX4211-mediated

inhibition of intestinal SGLT1, resulting in delayed absorption of intestinal glucose and

subsequent appearance of glucose in the cecum, where it undergoes fermentation to

short-chain fatty acids which are powerful mediators of GLP-1 release (Powell et al.,

2013b; Zambrowicz et al., 2013a). Healthy humans and patients with T2DM also show

LX4211-mediated decreases in postprandial glucose excursions accompanied by

increases in circulating GLP-1 levels (Zambrowicz et al., 2012; Zambrowicz et al.,

2013a; Zambrowicz et al., 2013b). In data presented here, postprandial OP-CD obese

rats treated with LX4211 also show greater cecal glucose content and lower cecal pH,

further supporting the likelihood that this is a general mechanism across species.   In

addition, the finding that LX4211-treated KKA[y] mice had blunted glucose excursions

and increased GLP-1 levels after oral glucose challenge suggests that this mechanism

is active in diabetic mice as it is in diabetic humans, and may have contributed to the

overall improvement in glycemic control observed in the KKA[y] mice. Further, the

increased GLP-1 levels that accompanied LX4211 treatment may also help to preserve

β-cell function (DeFronzo, 2009; Wajchenberg, 2007; Bunck et al., 2011).

Body weight and body fat values were comparable between LX4211-treated and

untreated KKA[y] mice in 2 independent studies. Although mice receiving LX4211 lost

large amounts of glucose in their urine, the caloric losses were offset by an increase in

food consumption. These results were consistent with our findings in SGLT2 knockout

mice (Powell et al., 2013a). In comparison, KKA[y] mice treated with the SGLT2

inhibitors T-1095 or sergliflozin showed a slight but significant decrease in weight gain

which was associated with a slight increase in food consumption in the sergliflozin-

treated mice (Oku et al., 1999; Katsuno et al., 2009). In general, our studies of LX4211-

treated rats and dogs show less weight gain despite increased food consumption,

results that are consistent with DIO rat studies reporting weight loss induced by long-

term dapagliflozin treatment that was partially offset by increased food consumption

(Devenny et al., 2012). Taken together, these results suggest that the ability of LX4211

to lower body weight and body fat in preclinical models depends on the extent to which

LX4211-mediated hyperphagia compensates for the increased loss of glucose calories

in urine. Weight loss is consistently reported in clinical trials evaluating patients with T2DM who received LX4211 (Zambrowicz et al., 2012; Rosenstock et al., 2012) or other SGLT2 inhibitors (Abdul-Ghani et al., 2012). The effect of LX4211 on food consumption in patients with T2DM has not yet been carefully monitored; such studies will be required to determine whether hyperphagia attenuates the degree of weight loss in some LX4211-treated patients as it does in some LX4211-treated preclinical models.

In summary, data presented here show that LX4211 is a dual SGLT1/SGLT2 inhibitor in multiple preclinical species as it is in humans; this suggests that the response to LX4211 in these species may be predictive of the response to LX4211 in humans. This hypothesis is supported by the ability of LX4211 to markedly lower A1C and postprandial glucose excursions while increasing UGE and GLP-1 levels in diabetic mice as in humans. Based on these considerations, our data suggest that LX4211 may maintain β-cell insulin reserves in patients with T2DM, and that the LX4211-mediated weight loss observed in these patients may be attenuated by LX4211-mediated hyperphagia in at least some of these individuals.

Authorship Contributions

Participated in research design: Powell, DaCosta, Smith, Heydorn, Nouraldeen, Yalamanchili, Mseeh, Wilson, Shadoan, Zambrowicz, Ding

Conducted experiments: DaCosta, Smith, Doree, Harris, Buhring, Xiong, Mseeh, Shadoan

Contributed new reagents or analytic tools: Mseeh

Performed data analysis: Powell, DaCosta, Smith, Doree, Heydorn, Nouraldeen, Yalamanchili, Mseeh, Wilson, Shadoan, Ding

Wrote or contributed to the writing of the manuscript: Powell, Mseeh, Wilson, Zambrowicz, Ding

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Figure legends

Figure 1. Pharmacokinetic studies of LX4211 in three preclinical species. A) Chemical structure of LX4211, (2S,3R,4R,5S,6R)-2-(4-chloro-3-(4-ethoxybenzyl)phenyl)-6-(methylthio)tetrahydro-2H-pyran-3,4,5-triol**. Plasma concentrations of LX4211 (mean ± SD) were measured at various times following **B)** an intravenous dose of 1 mg/kg to each species, and **C)** an oral dose of 10 mg/kg to mice and rats, and an oral dose of 3 mg/kg to dogs.

Figure 2. LX4211 increases UGE in mice, rats and dogs. UGE was measured over 24 hour intervals as described in methods. In **A)** mice, **C)** rats and **E)** dogs, UGE was measured during the first 24 hours after treatment with vehicle or increasing doses of LX4211. After animals received a single dose of LX4211 on Day 1, UGE was also measured in: **B)** mice during 3 successive 24-hour intervals; **D)** rats during 2-4 successive 24-hour intervals; and **F)** dogs during 2 successive 24-hour intervals. In panels A, C and E, ** $P < 0.01$; *** $P < 0.001$ vs. the vehicle-treated group.

Figure 3. LX4211 improves glucose homeostasis in KKA^y diabetic mice – Study 1. A) Schematic outline of study design. UGE, 24-hour UGE measured in metabolic cages; FC, food consumption; BW, body weight; BG, fed blood glucose; Ins, fed serum insulin. **B) A1C.** A time course of change in A1C levels. **C) Fed blood glucose.** A time course of fed blood glucose levels. **D) OGTT glucose data.** Blood glucose excursions from the week 9 OGTT. **E) OGTT insulin data.** Change in serum insulin levels between the 0 and 30 minute time points of the week 9 OGTT. **F) Total**

pancreatic insulin. At necropsy, the pancreas of each mouse was isolated, and pancreatic insulin was extracted and measured as described in Methods. **G) 24-hour UGE.** Multiple 24-hour UGE measurements were made on mice in metabolic cages both before and after the start of LX4211 dosing. In panels B-G, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. the vehicle-treated group.

Figure 4. LX4211 improves glucose homeostasis in KKA^y diabetic mice – Study 2. A) Schematic outline of study design. UGE, 24-hour UGE measured in metabolic cages; FC, food consumption; BW, body weight; Fed G, fed serum glucose. **B) A1C.** Change in A1C levels between baseline (week 2) and week 6 of the study. **C) Fed serum glucose.** Fed serum glucose levels obtained at necropsy. **D) OGTT glucose data.** Blood glucose excursions from the week 6 OGTT. **E) OGTT insulin data.** Change in serum insulin levels between the 0 and 30 minute time points of the week 6 OGTT. **F) Total pancreatic insulin.** At necropsy, the pancreas of each mouse was isolated, and pancreatic insulin was extracted and measured as described in Methods. **G) 24-hour UGE.** The mean value of two 24-hour UGE measurements made on consecutive days in mice acclimatized in metabolic cages during study week 7 or 8. **H) Plasma Total GLP-1 levels.** Total GLP-1 levels measured at necropsy, 2 hours after a 2 g/kg oral glucose bolus. In panels B-H, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. the vehicle-treated group.

Figure 5. The effect of LX4211 on body composition and food consumption in KKA^y diabetic mice. In KKAy Study 1, mice were evaluated for: **A) change in body**

weight between the start of LX4211 treatment and necropsy; **B) change in body fat** between QMR measurements made in week 1 and week 9; and **C) food consumption,** reported as the mean daily intake measured during the 8 days that the mice were maintained in metabolic cages while receiving LX4211 or vehicle. In KKAy Study 2, mice were evaluated for: **D) change in body weight** between measurements made on the first day of LX4211 or vehicle treatment and on multiple days during the first 6 weeks of treatment; **E) body fat** measured by QMR during study week 9; and **F) food consumption,** reported as the mean daily intake during the first 6 weeks of treatment with LX4211 or vehicle. In panel F, ** $P < 0.01$; *** $P < 0.001$ vs. the vehicle-treated group.

Figure 6. Effects of LX4211 on body weight of lean rats and dogs. In rats, **A) change in body weight** and **B) food consumption** were measured during a 4-week toxicology study and then compared between the vehicle-treated group (N=15) and pooled data from groups receiving LX4211 at doses of 10 (N=10), 30 (N=10) or 100 (N=15) mg/kg/day. In dogs, **C) change in body weight** and **D) food consumption** were also measured during a 4-week toxicology study and then compared between the vehicle-treated group (N=10) and pooled data from groups receiving LX4211 at doses of 30 (N=6) or 100 (N=10) mg/kg/day. In rats, **E) body weight** and **F) food consumption** were measured during a 13-week toxicology study and then compared between the vehicle-treated group (N=15) and individual groups receiving LX4211 at doses of 3 (N=10), 10 (N=10), 30 (N=15) or 100 (N=15) mg/kg/day; the key for all groups in panels E and F is found in panel E. No significant adverse effects of LX4211 treatment were

observed at these doses in any of the three studies. In panels A-D, * $P < 0.05$; ** $P <$ 0.01; *** $P < 0.001$ vs. the vehicle-treated group. In panel F, * P < 0.05 for vehicle vs. all other groups; ^ P < 0.05 for vehicle vs. the 10, 30 and 100 mg/kg/d groups.

Figure 7. Effects of LX4211 on OP-CD obese rats. A) Schematic outline of study design. UGE, 24-hour UGE measured in metabolic cages; FC, food consumption; BW, body weight. **B) UGE,** presented as mean 24-hour UGE values estimated from collections made on 3 consecutive days in metabolic cages. **C) Change in body weight** between the start of LX4211 treatment and necropsy. **D) Body fat** and **E) lean body mass**, measured by QMR at the end of the study. **F) food consumption,** reported as the mean daily intake during the 25 days of treatment with LX4211 or vehicle. Cecal contents were isolated from fed rats at the time of necropsy and used to measure **G) cecal glucose**, reported here as the total amount of glucose recovered, and **H) cecal pH**, which represents a direct pH measurement of the cecal contents. In panels B-E, G and H, * $P < 0.05$; *** $P < 0.001$ vs. the vehicle-treated group.

Table 1. LX4211 inhibits SGLT-mediated glucose transport

Species	SGLT1 IC50 (nM)	N	SGLT2 IC50 (nM)	N
Human [a]	36 ± 9	9	1.8 ± 0.4	8
Mouse [b]	62 ± 26	8	0.6 ± 0.2	8
Rat	104 ± 38	11	0.5 ± 0.3	10
Dog	86 ± 14	8	1.4 ± 0.5	8

[a] Data are from reference 19
[b] Data are from reference 21
N, number of determinations

Table 2. Pharmacokinetic parameters after a 1 mg/kg IV dose of LX4211

Species	N	Half-life (hr)	$AUC_{0-\infty}$ (nM*hr)	CL (mL/min/kg)	Vss (L/kg)
Dog		7.27 ± 1.39	8849 ± 1796	4.6 ± 0.9	1.98 ± 0.34
Rat		0.90 ± 0.09	1847 ± 132	21.3 ± 1.5	1.40 ± 0.07
Mouse		1.39 ± 0.45	1940 ± 331	20.6 ± 3.0	2.07 ± 0.14

N, number of animals; CL, clearance; Vss, volume at steady state

Table 3. Pharmacokinetic parameters after a 3 or 10 mg/kg PO dose of LX4211

Species	N	Dose (mg/kg)	Half-life (hr)	T_{max} (hr)	C_{max} (nM)	$AUC_{0-\infty}$ nM*hr	%F
Dog		3	7.38 ± 1.17	0.65 ± 0.34	2502 ± 325	18775 ± 4132	71 ± 16
Rat		10	ND	3.00 ± 1.15	1116 ± 378	ND	ND
Mouse		10	6.52 ± 0.82	0.31 ± 0.13	4373 ± 408	19079 ± 1580	98 ± 8

N, number of animals; %F, % bioavailable; ND, not determined

Figure 1

A

B

C

Figure 2

A

B

C

E

F

Figure 3

A

KKAy Mouse Study 1

Study Week

1 2 3 4 5 6 7 8 9 10

Begin LX4211

BW, FC
OGTT
QMR

BW, FC
BG, Ins
A1C
UGE

BW, FC
BG, Ins
A1C
UGE

BW, FC
BG, Ins
A1C

BW, FC
UGE

BW, FC
BG, Ins
A1C
OGTT
QMR

Necropsy

Standard
Cages

Metabolic
Cages

Standard
Cages

Metabolic
Cages

Standard
Cages

B

C

D

E

F

G

Figure 4

Figure 5

A

B

C

D

E

F

Figure 6

A

Rat

Change in Body Weight (g)

Vehicle LX4211

**

B

Rat

Food Consumption (g/week)

Vehicle LX4211

***

C

Dog

Change in Body Weight (kg)

Vehicle LX4211

*

D

Dog

Food Consumption (g/week)

Vehicle LX4211

**

E

Rat

Body Weight (g)

Time (Day)

- 0 mg/kg
- 3 mg/kg
- 10 mg/kg
- 30 mg/kg
- 100 mg/kg

F

Rat

Food Comsuption (g/week)

Time (Weeks)

Figure 7

A

OP-CD Rat Study

Effect of LX4211 on Glucose Homeostasis and Body Composition in Preclinical Models

David R. Powell, Christopher M. DaCosta, Melinda Smith, Deon Doree, Angela Harris, Lindsey Buhring, William Heydorn, Amr Nouraldeen, Wendy Xiong, Padmaja Yalamanchili, Faika Mseeh, Alan Wilson, Melanie Shadoan, Brian Zambrowicz, Zhi-Ming Ding

Lexicon Pharmaceuticals, Inc., The Woodlands, Texas, USA (DRP, CMD, MS, DD, AH, LB, AN, WX, FM, AW, MS, BZ, ZMD); Lexicon Pharmaceuticals, Inc., Princeton, New Jersey, USA (WH, PY)

Supplementary Table 1. Body composition of KKA^y mice and OP-CD obese rats after long-term treatment with LX4211

Model	Study Week	Treatment	N	Body weight (g)	Body fat (g)	LBM (g)	Body fat (%)
KKA^y mice	0	Vehicle	6	37.5 ± 1.5	15.0 ± 0.8	22.5 ± 0.9	40.0 ± 0.9
(Study 1)	0	LX4211, 1 mg/kg	6	37.9 ± 1.4	15.5 ± 1.0	22.4 ± 0.5	40.8 ± 1.3
	5	Vehicle	6	44.9 ± 2.3	21.0 ± 1.3	23.8 ± 1.3	46.9 ± 1.4
	5	LX4211, 1 mg/kg	6	45.5 ± 4.8	20.7 ± 3.6	24.8 ± 1.5	45.3 ± 3.4
KKA^y mice	5	Vehicle	15	50.0 ± 2.7	23.5 ± 2.2	26.5 ± 0.7	47.0 ± 1.9
(Study 2)	5	LX4211, 1 mg/kg	14	51.0 ± 2.6	23.9 ± 1.7	27.0 ± 1.2	46.9 ± 1.6
	5	LX4211, 10 mg/kg	15	50.0 ± 3.1	23.2 ± 2.4	26.7 ± 1.2	46.4 ± 2.4
OP-CD rats	4	Vehicle	8	489 ± 36	72 ± 11	418 ± 37	14.7 ± 2.5
	4	LX4211, 30 mg/kg	8	454 ± 29*	59 ± 9*	395 ± 21*	13.0 ± 1.3

LBM, lean body mass; N, number of mice; * different from vehicle, p < 0.05

Supplementary Table 2. Body weight and food consumption of lean Sprague-Dawley rats treated with LX4211 for 13 weeks

LX4211 (mg/kg/d)	Body Weight (g) Day 1	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43
0	390 ± 32	425 ± 35	455 ± 36	479 ± 39	500 ± 44	517 ± 47	538 ± 52
3	396 ± 19	418 ± 24	442 ± 28	465 ± 33	480 ± 37	493 ± 43	511 ± 51
10	398 ± 21	425 ± 18	448 ± 21	467 ± 24	490 ± 29	505 ± 37	521 ± 44
30	392 ± 30	416 ± 30	438 ± 30	460 ± 36	478 ± 39	495 ± 41	515 ± 49
100	387 ± 34	411 ± 38	438 ± 41	458 ± 53	479± 54	492 ± 55	508 ± 59

	Day 50	Day 57	Day 64	Day 71	Day 78	Day 85	Day 92
0	543 ± 51	557 ± 55	575 ± 58	580 ± 59	598 ± 62	592 ± 66	604 ± 70
3	526 ± 50	536 ± 52	548 ± 51	553 ± 55	562 ± 57	565 ± 60	577 ± 59
10	530 ± 41	540 ± 44	555 ± 51	561 ± 56	569 ± 56	569 ± 57	581 ± 57
30	522 ± 48	535 ± 53	550 ± 55	557 ± 56	573 ± 60	573 ± 58	581 ± 60
100	513 ± 62	518 ± 64	533 ± 69	532 ± 70	543 ± 75	548 ± 80	563 ± 80

LX4211 (mg/kg/d)	Food Consumption (g) Day 1-8	Day 8-15	Day 15-22	Day 22-29	Day 29-36	Day 36-43	Day 43-50	Day 50-57
0	212 ± 17	222 ± 17^	215 ± 19*	223 ± 15*	216 ± 17^	224 ± 22^	207 ± 17*	227 ± 20*
3	218 ± 20	240 ± 14	243 ± 19	251 ± 19	242 ± 21	248 ± 26	242 ± 29	246 ± 29
10	238 ± 24	259 ± 31	258 ± 30	271 ± 29	264 ± 31	274 ± 35	261 ± 27	271 ± 27
30	230 ± 26	248 ± 27	258 ± 32	269 ± 26	274 ± 27	283 ± 37	267 ± 24	277 ± 33
100	221 ± 31	261 ± 29	263 ± 32	286 ± 37	283 ± 33	294 ± 31	277 ± 28	286 ± 35

* Vehicle group different from all other groups, p < 0.05

^ Vehicle group different from 10, 30 and 100 mg/kg/d groups, p < 0.05