LX4211 Increases Serum GLP-1 and PYY Levels by Reducing SGLT-1-mediated Absorption of Intestinal Glucose

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Running Title: SGLT1 Inhibition Increases Serum GLP-1 and PYY Levels

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Abbreviations:
aGLP-1, active GLP-1
AMG, α-methylglucopyranoside
GI, gastrointestinal
GLP-1, glucagon-like peptide 1
GIP, glucose-dependent insulino tropic polypeptide
HFD, high fat diet
LFD, low fat diet
OGTT, oral glucose tolerance test
PYY, peptide YY
SCFA, short-chain fatty acid
SGLT1, sodium/glucose cotransporter 1
SGLT2, sodium/glucose cotransporter 2
SI, small intestine
tGIP, total GIP
tGLP-1, total GLP-1
T2DM, type 2 diabetes mellitus
-/-, knockout mice
+/-, wild type littermate mice

Recommended section assignment: Endocrine and diabetes
Abstract

LX4211, a dual sodium/glucose cotransporter 1 (SGLT1) and SGLT2 inhibitor, is thought to decrease both renal glucose reabsorption by inhibiting SGLT2 and intestinal glucose absorption by inhibiting SGLT1. In clinical trials in patients with type 2 diabetes mellitus (T2DM), LX4211 treatment improved glycemic control while increasing circulating levels of glucagon-like peptide (GLP)-1 and peptide YY (PYY). To better understand how LX4211 increases GLP-1 and PYY levels, we challenged SGLT1 knockout (−/−) mice, SGLT2 −/− mice, and LX4211-treated mice with oral glucose. LX4211-treated mice and SGLT1 −/− mice had increased levels of plasma GLP-1, plasma PYY and intestinal glucose during the 6 hours after a glucose-containing meal, as reflected by area-under-the-curve (AUC) values, whereas SGLT2 −/− mice showed no response. LX4211-treated mice and SGLT1 −/− mice also had increased GLP-1 AUC, decreased glucose-dependent insulinotropic polypeptide (GIP) AUC, and decreased blood glucose excursions during the 6 hours after a challenge with oral glucose alone. However, GLP-1 and GIP levels were not increased in LX4211-treated mice, and were decreased in SGLT1 −/− mice, 5 minutes after oral glucose, consistent with studies linking decreased intestinal SGLT1 activity with reduced GLP-1 and GIP levels five minutes after oral glucose. These data suggest LX4211 reduces intestinal glucose absorption by inhibiting SGLT1, resulting in net increases in GLP-1 and PYY release and decreases in GIP release and blood glucose excursions. The ability to inhibit both intestinal SGLT1 and renal SGLT2 provides LX4211 with a novel dual mechanism of action for improving glycemic control in patients with T2DM.
Introduction

Over 20 million people in the United States have Type 2 diabetes mellitus (T2DM), and the number is projected to double by 2034 (Huang et al, 2009). Treatments that control blood glucose in these patients decrease complications (Holman et al, 2008; Ray et al, 2009; UK Prospective Diabetes Study (UKPDS) Group, 1998a; UK Prospective Diabetes Study (UKPDS) Group, 1998b). Metformin is standard first line therapy, but, in most patients, glycemic control worsens after a few years of metformin monotherapy (Turner et al, 1999; Wallace and Matthews, 2002). Combining metformin with other approved therapies can improve glycemic control but often leads to side-effects, including weight gain and hypoglycemia which may increase risk of cardiovascular events; these concerns underscore the need to develop new agents that safely and effectively lower blood glucose in patients with T2DM (Nathan et al, 2009; Rodbard et al, 2009).

We have developed LX4211, an oral molecule that is a dual inhibitor of sodium/glucose cotransporter 1 (SGLT1) and SGLT2 (Goodwin et al, 2009; Zambrowicz et al, 2012). In a phase 2 clinical trial (Zambrowicz et al, 2012), LX4211 significantly improved glycemic control, lowered triglycerides and produced downward trends in both weight and blood pressure over 4 weeks of once-daily dosing in patients with T2DM. LX4211 acts by inhibiting renal SGLT2 to increase urinary glucose excretion, and is also thought to act by inhibiting intestinal SGLT1 to reduce absorption of dietary glucose. A consequence of LX4211 treatment should therefore be increased levels of both glucose in the intestine and short chain fatty acids (SCFA) – the bacterial fermentation products of glucose – in the colon, each of which can trigger release of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from intestinal L-cells (Cherbut et al, 1998; Dumoulin et al, 1998; Elliott et al, 1993; Fu-Cheng et al, 1995; Herrmann et al, 1995; Kim et al, 2005; Lin et al, 2012; Tolhurst et al, 2012; Yoder et al, 2010; Wu et al, 2012; Zhou et al, 2006;
Zhou et al, 2008). Based on similarity of this gastrointestinal (GI) response to that triggered by ingesting dietary-resistant starch (Zhou et al, 2008; Nilsson et al, 2008) and by roux-en-Y gastric bypass surgery (Cummings, 2009; Shin et al, 2010), GLP-1 and PYY release should begin soon after eating and last for hours, a pattern that was in fact observed in LX4211-treated patients with T2DM (Zambrowicz et al, 2011) who, importantly, did not have an increased frequency of GI side-effects. Increasing GLP-1 activity, either by injecting long acting GLP-1 analogs or by inhibiting dipeptidylpeptidase (DPP)4-mediated GLP-1 inactivation, is clearly associated with improved glycemic control in patients with T2DM (Richter et al, 2008; Shyangdan et al, 2011).

Inhibitors of intestinal SGLT1 were recently shown to decrease blood glucose excursions after oral glucose challenge (Ikumi et al, 2008; Sakuma et al, 2010; Shibazaki et al, 2012), suggesting that such inhibitors may be effective anti-diabetic therapies. Also, one of these studies showed increased postprandial portal levels of active GLP-1 (aGLP-1) after chronic SGLT1 inhibition (Shibazaki et al, 2012), consistent with our observation that circulating total GLP-1 (tGLP-1) was increased for hours in SGLT1 knockout (-/-) mice after oral glucose challenge (Powell et al, 2013). Together, the above studies suggest that increased GLP-1 levels might contribute to the improved oral glucose tolerance observed with SGLT1 inhibition. However, these results appear inconsistent with studies showing that: 1) SGLT1 is required for glucose-mediated GLP-1 release by intestinal L-cells in vitro (Reimann et al, 2008); 2) mice pre-treated with delivery of the SGLT1 inhibitor phlorizin to their intestinal lumen had lower portal GLP-1 levels 5 minutes after oral glucose challenge (Moriya et al, 2009); and 3) plasma GLP-1 levels were lower 5 minutes post oral glucose challenge in SGLT1 -/- mice (Gorboulev et al, 2012). These results suggest that LX4211-associated postprandial increases in plasma GLP-1 and PYY might be mediated by another mechanism, perhaps involving LX4211 inhibition of SGLT2.
Also, SGLT1 -/- mice had lower total GIP (tGIP) levels 5 minutes after oral glucose challenge (Gorboulev et al, 2012), which suggests that LX4211 might lower postprandial GIP levels; this is important because decreased postprandial GIP levels may benefit patients with T2DM (Asmar et al, 2010; Baggio and Drucker, 2007; Chia et al, 2009; Flint et al, 1998; Reimann, 2010). The studies presented here were designed to address the roles played by SGLT1 and SGLT2 in LX4211-induced increases in postprandial GLP-1 and PYY levels and decreases in postprandial blood glucose excursions, and to explore the affect of LX4211 on postprandial GIP levels.

Materials and Methods

Mice. The Institutional Animal Care and Use Committee at Lexicon Pharmaceuticals approved all study protocols. General methods for mouse husbandry (Brommage et al, 2008), generation of SGLT1 -/- mice (Powell et al, 2013) and generation of SGLT2 -/- mice (Jurczak et al, 2011) have been described. Unless stated otherwise, all mice studied were of mixed genetic background (129/SvEvBrd and C57BL/6-Tyr c-Brd); genotyping was performed on tail DNA as described previously (Donoviel et al, 2001). Mice were fed either high fat diet which contains 45% kcal as fat (HFD; D12451, Research Diets, New Brunswick, NJ) or glucose-free HFD which contains 45% kcal from fat and 35% kcal from fructose (D08040105i, Research Diets).

24-Hour urinary glucose excretion (UGE). Five male albino C57BL/6-Tyr c-Brd mice, 7 months of age and maintained on HFD from weaning, were individually housed in Nalgene Metabolic Cages for Mice (product MTB-0311, Nalge Nunc International, Rochester, NY, USA) and acclimated overnight; at all times, these mice had free access to HFD and water. LX4211
was formulated in aqueous 0.1% v/v Tween 80 for oral administration at 5 mL/kg dose volume. On study day 1, after mice received vehicle by oral gavage between 2 and 3 pm, their urine was collected for the next 24 hours. On study day 2, after mice received 60 mg/kg LX4211 by oral gavage between 2 and 3 pm, their urine was again collected for the next 24 hours. After the volume of each 24-hour urine collection was recorded, the urine sample was immediately centrifuged and then analyzed for glucose concentration using a Cobas Integra 400 Clinical Chemistry Autoanalyzer (Roche Diagnostics, Indianapolis, IN). The volume and glucose concentration of each 24-hour urine sample was used to calculate the 24-hour UGE for each mouse on each day.

**Meal challenge.** Preliminary data suggested that a meal consisting of low fat diet (LFD, 10% kcal as fat, Research Diets D12450B) supplemented with glucose, prepared by adding 50g of LFD powder and 9.4 g of glucose to water with a final volume of 94 ml, was superior to LFD alone in terms of increasing circulating levels of GLP-1 and PYY (data not shown). As our standard meal challenge, mice received 25 ml/kg of this LFD and glucose mixture (9.2 g/kg glucose, 2.5 g/kg protein, 0.6 g/kg fat) by oral gavage. In some studies, glucose alone was provided as the meal challenge. For all meal challenge studies, GI contents and/or blood were obtained at necropsy in the absence of a meal challenge (baseline control) and/or at various times after the meal challenge. GI contents were analyzed for glucose and pH levels. The contents of the entire small intestine (SI, between stomach and cecum), cecum, and colon (between cecum and rectum) were individually collected, weighed, and frozen at -20°C for later analysis. For batched analysis, samples were thawed on wet ice, and water was added to each sample at the ratio (w/v) of 1:5. Samples were then homogenized using a Mini Beadbeater (Biospec Products,
Bartlesville, OK) at high speed for one minute. Homogenized samples were centrifuged at 4°C at 4750 rpm for 25 minutes. After the supernatant was removed and the volume recorded, 25 ul samples were assayed for glucose concentration by the Cobas Integra 400 Autoanalyzer (Roche Diagnostics). Total glucose content (mg) was calculated by multiplying the glucose concentration by the volume of the centrifuged supernatant. The remaining supernatant was thawed to room temperature and analyzed for pH using an FE20 pH meter (Mettler Toledo, Columbus, OH). Blood samples obtained by retro-orbital bleed from unanesthetized mice were used to simultaneously measure circulating levels of aGLP-1 (Glucagon-Like Peptide-1 Active ELISA Kit, catalog #EGLP-35K, Millipore, St. Charles, MO), tGLP-1 (Glucagon-Like Peptide-1 Total ELISA Kit, catalog #EZGLP1T-36K, Millipore), tGIP (Glucose-dependent insulinotropic peptide Total ELISA kit, catalog #EZRMGIP-55K, Millipore) and PYY (PYY ELISA Kit, catalog #48-PYYRT-E01.1, ALPCO, Salem, NH). For the aGLP-1 assay, a 500 ul aliquot of blood was collected into an ice-cold EDTA-containing tube. DPP IV inhibitor solution provided in the aGLP-1 ELISA kit was immediately added to the tube in a ratio of 10 µl DPP IV inhibitor/ml of blood. The sample was mixed and centrifuged immediately at 1000 g for 10 minutes at 4°C, followed by collection of plasma for aGLP-1 analysis. For tGLP-1 and PYY assays, an additional 300 µl aliquot of blood was collected into a separate EDTA-containing tube; this sample was mixed and centrifuged immediately at 1000 g for 10 minutes at 4°C, followed by collection of plasma for tGLP-1, tGIP and PYY analysis. For each assay, blood collection and handling, and all aspects of the assay protocol, were performed exactly as recommended by the kit manufacturer. Because of the large volume of blood drawn, an independent cohort of mice was used at each time point for each treatment group. Time-course data were converted to area-under-the-curve (AUC) values by trapezoidal summation using GraphPad Prism v4.03.
**Oral Glucose Tolerance Test.** Oral glucose tolerance tests (OGTTs) were performed on unanesthetized adult male mice fed HFD from weaning. After an overnight fast, mice had their blood collected from the retro-orbital plexus at time 0 and then received, by oral gavage, 2 g glucose/kg body weight. In a study performed on 19 week-old C57BL/6-Tyr-Brd mice, LX4211 or vehicle was coformulated with the glucose bolus, and blood collected at multiple subsequent time points was then assayed for whole blood glucose using an Accu-Chek Aviva glucometer (Roche Diagnostics, Indianapolis, IN). In a study performed on 10 week-old SGLT1 -/- mice, serum collected 30 and 60 minutes after the oral glucose bolus was assayed for glucose using a Cobas Integra 400 Analyzer (Roche Diagnostics). In each study, serum collected at 0 and 30 minutes was also assayed for insulin (Ultra Sensitive Rat Insulin ELISA Kit, Cat. # 90060, Crystal Chem, Downers Grove, IL). For each OGTT, glucose time-course data were converted to AUC values by trapezoidal summation using GraphPad Prism v4.03.

**SGLT2 and SGLT1 Cell Lines.** The full-length coding sequence of mouse SGLT2 and SGLT1, containing an HA-tag 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 mouse HA-SGLT2-pIRESpuro2 or mouse HA-SGLT1-pIRESpuro2 vectors and bulk stable cell lines were selected in the presence of 0.5 µg/mL of puromycin. Mouse HA-SGLT2 and HA-SGLT1 cells were maintained in DMEM media containing 10% FBS, 1% GPS and 0.5 µg/mL of puromycin. These HEK293 cell lines were used in experiments.
to determine the IC$_{50}$ (concentration causing half-maximal inhibition) of LX4211 against mouse SGLT2 and SGLT1.

**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 (Wright and Turk, 2004). The inhibition of SGLT2 and SGLT1 by LX4211 was determined by measuring SGLT2 and SGLT1-mediated $^{14}$C-AMG uptake in the presence of increasing compound concentration. Phlorizin, a well-characterized, non-selective inhibitor of SGLT1 and SGLT2, was used as reference compound (Ehrenkranz et al, 2005). To compute the IC$_{50}$, the percent inhibition of SGLT-mediated $^{14}$C-AMG uptake at different compound concentrations was calculated as follows:

$$\% \text{ Inhibition} = \left( \frac{B-X}{B-A} \right) \times 100.$$  

Where A is the uptake in the presence of 10 µM phlorizin (baseline response; no SGLT-mediated uptake); B is the uptake in the absence of SGLT inhibitor (maximum response, total uptake); and X is the $^{14}$C-AMG uptake at a given compound concentration. Standard sigmoidal dose-response model curves were fitted, and the IC$_{50}$ value was computed as the compound concentration that inhibited the $^{14}$C-AMG uptake by 50% between baseline and maximum uptake.

**Statistics.** Data are presented as mean ± standard error of the mean (SEM). Comparisons between two groups were analyzed by unpaired Student’s T-test, and comparisons among three or more groups were analyzed by one-way ANOVA with post-hoc analysis performed by the
Bonferroni method, using PRISM 4.03 (GraphPad) software. Values were considered statistically significant when P < 0.05.

Results

Because LX4211 was developed based on its ability to inhibit glucose uptake by human SGLT1 (IC50 = 36 nM) and human SGLT2 (IC50 = 1.8 nM) (Goodwin et al., 2009; Zambrowicz et al., 2012), we wanted to establish the extent to which LX4211 inhibits mouse SGLT1 and SGLT2 before performing studies in mice. We found that LX4211 inhibited 14C-AMG uptake in a dose-dependent manner with an IC50 of 62.0 ± 26 nM for mouse SGLT1 (n=8) and IC50 of 0.6 ± 0.2 nM for mouse SGLT2 (n=8). These results indicate that LX4211 is a potent dual inhibitor of both mouse SGLT1 and SGLT2, and support the relevance of using mouse models to investigate the pharmacologic activity of LX4211.

To determine if LX4211 treatment increases levels of GLP-1 and PYY in mice as in humans, we gave mice increasing doses of LX4211, followed in 30 minutes by a meal challenge. Three hours after the meal challenge, LX4211-treated mice showed a dose-dependent increase in circulating levels of tGLP-1, aGLP-1 and PYY, with the greatest increases following a dose of 60 mg/kg (Figure 1A-C). To determine if the increased GLP-1 and PYY levels were associated with increased intestinal glucose levels, which would suggest inhibition of intestinal SGLT1, we measured the amount of glucose in the intestinal contents of these same mice 3 hours after meal challenge. As shown in Figure 1D-F, LX4211 increased the amount of glucose in SI, cecum and colon in a dose-dependent manner, with the greatest increases again following a dose of 60 mg/kg. To determine if LX4211 also increased UGE in mice as in humans, we measured 24-hour
UGE in a separate cohort of mice treated with 60 mg/kg of LX4211, and found that their 24-hour UGE was 696 ± 103 mg/day compared to 1.1 ± 0.3 mg/day measured on the day prior to LX4211 treatment. Based on these results, LX4211 was dosed at 60 mg/kg in all remaining studies.

We next studied these LX4211-mediated effects over time. LX4211 treatment was associated with significant increases in the circulating levels of tGLP-1, aGLP-1 and PYY between 30 minutes and 6 hours after meal challenge (Figure 2A-C). The increased GLP-1 and PYY levels were associated with significant increases in total glucose present in the contents of the SI, cecum and colon after meal challenge; these increases were highest in SI and lowest in colon (Figure 2D-F). The increase in GI glucose noted in LX4211-treated mice was associated with a decrease in pH of cecal contents (Figure 2G), but not of SI or colon contents (data not shown). The increase in GI glucose after a glucose-containing meal in LX4211-treated mice is consistent with the dose-dependent decrease in blood glucose excursions, and lack of significant increase in circulating insulin levels, during OGTTs performed on an independent cohort of LX4211-treated mice (Figure 3A-B).

Next, we studied SGLT1 -/- and SGLT2 -/- mice. As shown in Figure 4, circulating levels of tGLP-1, aGLP-1 and PYY were significantly elevated between 1 and 6 hours after a meal challenge in SGLT1 -/-, but not SGLT2, -/- mice. In the same mice, we found a significant increase in total glucose present in contents of the SI, cecum and colon after meal challenge in SGLT1 -/-, but not SGLT2, -/- mice (Figure 5A-F). The increased GI glucose levels found in SGLT1 -/- mice were associated with a decrease in pH of cecal contents (Figure 5G), but not of SI or colon contents (data not shown). These data were confirmed in an independent study (data not shown). The increase in GI glucose after a glucose-containing meal in SGLT1 -/- mice is
consistent with the lack of increase in circulating glucose or insulin levels during OGTTs performed on an independent cohort of SGLT1 -/- mice (Figure 3C-D).

To determine if a glucose challenge alone was sufficient to increase GLP-1 levels in mice lacking functional SGLT1, we first took LX4211-treated mice and measured their tGLP-1 levels after challenging them with increasing doses of glucose. We found that tGLP-1 AUCs measured between 5 minutes and 6 hours after glucose challenge increased in the LX4211-treated mice as their dose of glucose increased (Figure 6A). We then examined these data for the effect of LX4211 treatment on tGLP-1 levels at different time points after glucose challenge, and found that tGLP-1 levels were higher in LX4211-treated mice, relative to vehicle controls, at all time points later than 5 minutes for each glucose dose (data not shown). However, tGLP-1 levels appeared lower in LX4211-treated mice 5 minutes after some of the glucose doses (Figure 6B); when data from all 4 glucose doses at the 5 minute time point were pooled, tGLP-1 levels of 123 ± 6 pM in the LX4211-treated mice (N = 20) were slightly, but not significantly, lower than levels of 134 ± 7 pM in the vehicle control mice (N = 20). Similar to LX4211-treated mice, SGLT1 -/- mice showed an increase in tGLP-1 AUCs measured between 5 minutes and 6 hours after glucose challenge (Figure 6C). However, the decrease in tGLP-1 levels 5 minutes after glucose challenge was consistently observed at all glucose doses given to the -/- mice (Figure 6D); when data from all 4 glucose doses at the 5 minute time point were pooled, tGLP-1 levels in the 20 SGLT1 -/- mice (65 ± 2 pM) were significantly lower (p<0.0001) than levels in their 20 wild type (+/+) littermates (103 ± 5 pM).

To determine if glucose challenge has the same effect on tGIP as on tGLP-1 levels, we first took mice treated with LX4211 and measured their tGIP and tGLP-1 levels between 5 minutes and 6 hours after a challenge with 6 g/kg glucose. We found that tGIP AUCs were
significantly decreased while tGLP-1 AUCs were significantly increased in LX4211-treated mice relative to vehicle controls during this time interval, with no difference in levels at 5 minutes after glucose challenge (Figure 7A-B). As is shown in Figure 7C-D, we obtained similar results when studying SGLT1 -/- mice, except at the time point 5 minutes after glucose challenge when SGLT1 -/- mice showed a significant decrease relative to +/- littermates for both tGIP levels (778 ± 93 vs 2306 ± 119 pg/ml, respectively, p<0.0001) and tGLP-1 levels (58 ± 4 vs 92 ± 6 pM, respectively, p<0.0005). The decrease in tGIP levels between 5 minutes and 6 hours after glucose challenge was confirmed in an independent cohort of SGLT1 -/- mice (data not shown).

Discussion

Data presented here show that LX4211, developed as a dual inhibitor of SGLT1 and SGLT2, raises postprandial plasma GLP1 and PYY levels in mice as in humans (Zambrowicz et al, 2012). This was accompanied by increased GI glucose levels, suggesting that glucose absorption from the GI tract, which is primarily SGLT1-mediated in mice as in humans (Gorboulev et al, 2012; Wright et al, 2004), was inhibited by LX4211. Increased postprandial plasma GLP1 and PYY levels, and GI glucose levels, were observed after meal challenge in SGLT1 -/- mice but not SGLT2 -/- mice, which suggests that LX4211 mediates these effects by inhibiting intestinal SGLT1, not intestinal SGLT2. Increased tGLP-1 after oral glucose alone was observed both in LX4211-treated mice and in SGLT1 -/- mice, indicating that glucose is responsible for this effect. Finally, LX4211 dose-response data presented here are consistent with data showing progressively higher postprandial levels of plasma tGLP-1 and GI glucose in SGLT1 +/+ vs SGLT1 +/- vs SGLT1 -/- littermates (Powell et al, 2013); together, these data
suggest that partial inhibition of intestinal SGLT1 can increase levels of plasma GLP-1 and GI glucose.

GLP-1 and GIP, secreted from the GI tract in response to ingested glucose, act on pancreatic β-cells to increase insulin release – the incretin effect. Also, GLP-1 decreases pancreatic glucagon secretion and appetite, and may improve cardiac function while increasing β-cell mass, among other effects that should benefit patients with T2DM (Baggio and Drucker, 2007; Flint et al, 1998; Reimann, 2010; Vilsbøll et al, 2003). In contrast, GIP secretion does not decrease postprandial glucose excursions in patients with T2DM despite stimulating insulin release by β-cells, probably due to concomitant glucagon release from pancreatic α-cells; also, increased GIP activity may be obesogenic, suggesting that lower GIP levels may be advantageous in patients with T2DM (Asmar et al, 2010; Baggio and Drucker, 2007; Chia et al, 2009; Flint et al, 1998). GIP is secreted by K-cells present in the proximal SI, more abundant in the mid-SI and rare after the distal SI, while GLP-1 is secreted, along with PYY, by L-cells present in comparable numbers to K-cells in the proximal SI, more abundant in the distal SI and also abundant in cecum and colon (Eissele et al, 1992; Mortensen et al, 2003; Theodorakis et al, 2006; Wu et al, 2012). We used PYY as a biomarker for hormone release by L-cells, but it is an anorexigenic peptide and increased levels may improve glycemic control in obese patients with T2DM by inducing weight loss (Batterham et al, 2002). Based on the above observations, it appears that patients with T2DM may benefit from increased L-cell activity and decreased or unchanged K-cell activity (Reimann, 2010).

Oral glucose rapidly increases plasma GLP-1 and GIP, probably through direct stimulation of L- and K-cells, respectively, by luminal glucose in the proximal SI (Elliott et al,
1993; Herrmann et al, 1995; Kim et al, 2005; Parker et al, 2009; Reimann et al, 2008; Reimann, 2010; Wu et al, 2012; Yoder et al, 2010). Studies of the signal transduction pathway through which glucose stimulates GLP-1 and GIP release from these cells provide evidence that SGLT1 is directly involved. First, primary murine L- and K-cells in tissue culture express SGLT1 and respond to glucose and AMG by secreting GLP-1 and GIP, respectively, into the culture medium; AMG responsiveness suggests SGLT1 involvement (Parker et al, 2009; Reimann et al, 2008). Second, SGLT1 is highly expressed in the proximal SI, and mice treated with the SGLT1 inhibitor phlorizin had lower plasma GLP-1 and GIP levels than vehicle-treated mice 5 minutes after glucose or AMG were introduced into their proximal SI (Moriya et al, 2009; Wright et al, 2011). Third, plasma GLP-1 and GIP levels were both lower 5 minutes post oral glucose challenge in SGLT1 -/- mice relative to +/- littermates (Gorboulev et al, 2012). Our data support these findings; 5 minutes after oral glucose challenge, GLP-1 and GIP levels were significantly lower in SGLT1 -/- mice than +/- littermates. A similar trend was observed in LX4211-treated mice relative to vehicle-treated controls, but the effect was not observed in all studies, perhaps due to incomplete inhibition of SGLT1 by 60 mg/kg of LX4211.

GLP-1 release appears to be biphasic, with the early phase described above followed by a delayed phase that results, in part, from local stimulation of L-cells in the distal SI, cecum and colon by luminal contents (Herrmann et al, 1995). After glucose challenge in LX4211-treated mice and SGLT1 -/- mice, we observed a delayed phase of increased GLP-1 release that was present from 30 minutes to 6 hours after oral glucose, dwarfed the transient GLP-1 lowering noted at 5 minutes, and was accompanied by elevated PYY levels. Consistent with these findings, a recent study showed increased portal GLP-1 levels in postprandial diabetic rats fed diet containing the selective SGLT1 inhibitor KGA-2727 for 48 days (Shibazaki et al, 2012). If
this delayed phase of GLP-1 release is directly stimulated by luminal glucose in the distal SI and colon, it requires a mechanism different from the SGLT1-mediated pathway that participates in the early phase. A more likely possibility is that luminal glucose is indirectly involved, through bacterial fermentation to SCFAs. Studies have shown that: i) delayed digestion of dietary-resistant starch leads to exaggerated and prolonged increases in circulating GLP-1 and PYY, and to increased production of luminal SCFAs by fermentation of glucose in cecum and colon; and ii) these increased SCFAs are associated with increased proglucagon and PYY gene expression in the distal GI tract (Zhou et al, 2006; Zhou et al, 2008). Additional studies have shown that: i) orally administered SCFAs increase GLP-1 and PYY release; ii) the effects of SCFAs are conferred through GPCRs including GPR41 and GPR43; iii) expression of these GPCRs is increased in GLP-1-secreting L-cells; and iv) SCFA-triggered GLP-1 secretion is attenuated in vitro and in vivo by L-cells of GPR41 -/- and GPR43 -/- mice (Cherbut et al, 1998; Dumoulin et al, 1998; Lin et al, 2012; Tolhurst et al, 2012). Consistent with these findings, we observed lower pH of cecal contents after glucose challenge both in LX4211-treated mice and SGLT1 -/- mice relative to controls, suggesting that SCFAs were generated by fermentation of cecal glucose and that these SCFAs might be triggering the increased GLP-1 levels. In addition to the potential role for SCFAs, it is possible that additional SGLT-1 independent pathways exist through which glucose directly or indirectly stimulates increased GLP-1 and PYY release from intestinal L-cells.

Blood glucose excursions after oral glucose challenge were markedly decreased both in LX4211-treated mice and in SGLT1 -/- mice. The possibility that LX4211-mediated SGLT1 inhibition might contribute to this process is supported by the observation that blood glucose excursions were blunted more when patients with T2DM were treated with the dual SGLT1 and
SGLT2 inhibitor LX4211 (Zambrowicz et al, 2012) than with selective SGLT2 inhibitors dapagliflozin or canagliflozin (Komoroski et al, 2009; Devineni et al, 2012). In fact, canagliflozin at doses < 300 mg did not decrease postprandial glucose excursions in healthy subjects, but doses >300 mg, which are higher than those proposed for human use, did decrease postprandial glucose excursions despite no further increase in UGE (Sha et al, 2011). This suggests that inhibiting SGLT1-mediated intestinal glucose uptake might directly decrease blood glucose excursions. Also, the increased GLP-1 release that indirectly results from inhibiting intestinal SGLT1 might further contribute to blunting of blood glucose excursions (DeFronzo et al, 2008). In contrast, the decreased GIP levels that accompany LX4211 treatment suggest that GIP does not participate in this process.

Our working hypothesis is that LX4211 reduces glucose absorption by inhibiting intestinal SGLT1. In the proximal SI, this inhibition interferes with glucose-mediated early release of GLP-1 and GIP by L- and K-cells, respectively, a process that requires SGLT1-mediated glucose transport in the signal transduction pathway. The reduced glucose absorption also leads to a second and quantitatively much greater release of GLP-1 and PYY by L-cells, a prolonged phase not requiring SGLT1. This late release of GLP-1 and PYY may be mediated by SCFA produced by cecal fermentation of unabsorbed glucose. Our data also suggest that the GIP increase after oral glucose requires the presence of functional SGLT1. Although oral delivery of SCFAs transiently increases GIP release, presumably by stimulating K-cells in the proximal SI (Lin et al, 2012), lack of a delayed GIP response to oral glucose after LX4211 pretreatment is explained by lack of K-cells in the cecum and colon, where SCFA are generated during glucose fermentation. Ultimately, the delayed glucose absorption and resulting increase in GLP-1 levels lead to a decrease in blood glucose excursions. In summary, our data suggest that
LX4211 has a net stimulatory effect on L-cell release of GLP-1 and PYY, and a net inhibitory effect on K-cell release of GIP, which, when combined with an associated decrease in blood glucose excursions, should prove beneficial to patients with T2DM.

Authorship Contributions

Participated in research design: Powell, Smith, DaCosta, Shadoan, Mseeh, Zambrowicz, Ding

Conducted experiments: Smith, Greer, Harris, Zhao, DaCosta, Mseeh, Shadoan

Contributed new reagents or analytic tools: Mseeh

Performed data analysis: Powell, Smith, DaCosta, Shadoan, Mseeh, Ding

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References


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Figure Legends

Figure 1. Mice treated with increasing doses of LX4211 respond to a glucose-containing meal challenge with dose-dependent increases in plasma GLP-1 and PYY levels and GI glucose levels. Mice were treated either with vehicle or one of several doses of LX4211 by oral gavage; 30 minutes later the mice received, by oral gavage at Time 0, a glucose-containing meal challenge. Three hours after meal challenge, plasma samples were obtained and assayed for A) tGLP-1, B) aGLP-1 and C) PYY; in addition, total glucose was measured in contents taken from D) small intestine, E) cecum and F) colon. Data were analyzed by ANOVA. P values with a statistically significant (P<0.05) difference between an LX4211-treated group and their vehicle-treated controls are shown; n signifies the number of mice/group.

Figure 2. LX4211-treated mice respond to a glucose-containing meal challenge with increased plasma GLP-1 and PYY levels and GI glucose levels, and decreased cecal pH. Mice were dosed with LX4211 (60 mg/kg) or vehicle (n = 5 for each group at each time point) by oral gavage; 30 minutes later the mice received, by oral gavage at Time 0, a glucose-containing meal challenge. The 30 minute to 6 hour time-course data for A) tGLP-1, B) aGLP-1, C) PYY, D) small intestinal glucose, E) cecal glucose, F) colon glucose and G) cecal pH were converted to AUC values. P values are shown for each AUC comparison.
Figure 3. Decreased blood glucose excursions observed during OGTTs performed in LX4211-treated mice and SGLT1 -/- mice. OGTTs were performed on unanesthetized male mice as described in Methods. Five groups of 7 mice each received, by oral gavage at Time 0, a glucose bolus along with either vehicle or one of four LX4211 doses. A) Blood glucose levels measured immediately before, and at multiple time points after, the glucose bolus were used to calculate glucose AUC values for each group. AUC different from vehicle AUC, * p< 0.05; ** p< 0.01. B) Change in serum insulin levels between samples drawn immediately before, and 30 minutes after, the glucose bolus in the same mice studied in Figure 3A. In addition, 10 week-old SGLT1 -/- mice (n=6) and +/- littermates (n=4) received glucose by oral gavage at Time 0. C) Blood glucose levels measured immediately before, and 30 and 60 minutes after, the glucose bolus. The P value represents a comparison of glucose AUC values for each group. D) Change in serum insulin levels measured immediately before and 30 minutes after the glucose bolus in the same mice studied in Figure 3C.

Figure 4. SGLT1 -/- mice, but not in SGLT2 -/- mice, respond to a glucose-containing meal challenge with increased plasma levels of GLP-1 and PYY. At Time 0, SGLT1 -/- and SGLT2 -/- mice and their +/- littermates (n = 5 for each group at each time point) received a glucose-containing meal challenge by oral gavage. The 30 minute to 6 hour time-course data from SGLT1 -/- mice for levels of A) tGLP-1, B) aGLP-1 and C) PYY, and from SGLT2 -/- mice for levels of D) tGLP-1, E) aGLP-1 and F) PYY, were converted to AUC values. P values are shown for each AUC comparison with a statistically significant (P<0.05) difference.
Figure 5. SGLT1 -/- mice, but not in SGLT2 -/- mice, respond to a glucose-containing meal challenge with increased GI glucose levels and decreased cecal pH. The mice studied in Figure 4 also had their intestinal contents analyzed. The 30 minute to 6 hour time-course data from SGLT1 mice for levels of A) SI glucose, B) cecal glucose, C) colon glucose and G) cecal pH, and from SGLT2 mice for levels of D) SI glucose, E) cecal glucose and F) colon glucose, were converted to AUC values. P values are shown for each AUC comparison with a statistically significant (P<0.05) difference.

Figure 6. Increasing oral glucose doses increase plasma GLP-1 levels in LX4211-treated mice and in SGLT1 -/- mice in a dose-dependent manner. Mice treated with LX4211, and SGLT1 -/- mice, were challenged with increasing oral doses of glucose. Blood samples obtained between 5 minutes and 6 hours after glucose challenge were assayed for tGLP-1; these time-course data were converted to AUC values and analyzed by ANOVA. A) Glucose dose-response effects on tGLP-1 levels in LX4211 pre-treated mice; P values with a statistically significant (P<0.05) difference are shown for comparisons, at each glucose dose, of tGLP-1 AUC values measured in LX4211-treated mice and vehicle-treated mice. B) Levels of tGLP-1 measured in samples taken 5 minutes after each oral glucose dose from mice treated with LX4211 or vehicle. C) Glucose dose-response effects on tGLP-1 levels in SGLT1 -/- mice; P values with a statistically significant (P<0.05) difference are shown for comparisons, at each glucose dose, of tGLP-1 AUC values measured in SGLT1 -/- mice and +/- littermates. D) Levels of tGLP-1 measured in samples taken 5 minutes after each oral glucose dose from SGLT1 -/- mice and +/- littermates.
Figure 7. Affect of an oral glucose challenge on plasma levels of tGIP and tGLP-1 in LX4211-treated mice and in SGLT1 -/- mice. Mice treated with LX4211, and SGLT1 -/- mice, were challenged with oral glucose (6 g glucose/kg body weight). Blood samples obtained between 5 minutes and 6 hours after glucose challenge were assayed for tGIP and tGLP-1; these time-course data were converted to AUC values. P values are shown for each AUC comparison.

A) Levels of tGIP and B) levels of tGLP-1 measured in 10 LX4211-treated mice and 10 vehicle-treated mice. C) Levels of tGIP and D) levels of tGLP-1 measured in 10 SGLT1 -/- mice and 10 +/- littermates.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7