The Novel GLP-1–Gastrin Dual Agonist ZP3022 Improves Glucose Homeostasis and Increases β-Cell Mass without Affecting Islet Number in *db/db* Mice

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ABSTRACT

Antidiabetic treatments aiming to preserve or even to increase β-cell mass are currently gaining increased interest. Here we investigated the effect of chronic treatment with the novel glucagon-like peptide-1 (GLP-1)–gastrin dual agonist ZP3022 (HGEGTFTSDLSKQMEEEAVRLFIEWLKN-8Ado-8Ado-YGWLDF-NH2) on glycemic control, β-cell mass and proliferation, and islet number. Male *db/db* mice were treated with ZP3022, liraglutide, or vehicle for 2, 4, or 8 weeks, with terminal assessment of hemoglobin A1c, basal blood glucose, and plasma insulin concentrations. Pancreata were removed for immunohistochemical staining and stereological quantification of β-cell mass, islet numbers, proliferation, and apoptosis. Treatment with ZP3022 or liraglutide led to a significant improvement in glycemic control. ZP3022 treatment resulted in a sustained increase in β-cell mass after 4 and 8 weeks of treatment, whereas the effect of liraglutide was transient. The expansion in β-cell mass observed in the ZP3022-treated mice appeared to be driven by increased β-cell proliferation in existing islets rather than by formation of new islets, as mean islet mass increased but the number of islets remained constant. Our data demonstrate that the GLP-1–gastrin dual agonist ZP3022 causes a sustained improvement in glycemic control accompanied by an increase in β-cell mass, increased proliferation, and increased mean islet mass. The results highlight that the GLP-1–gastrin dual agonist increases β-cell mass more than liraglutide and that dual agonists could potentially be developed into a new class of antidiabetic treatments.

Introduction

Pathogenesis of type 2 diabetes (T2D) is characterized by insulin resistance and β-cell dysfunction. In human studies, β-cell mass declines in patients with T2D compared with healthy individuals (Butler et al., 2003; Rahier et al., 2008; Meier and Bonadonna, 2013). Consequently, potential antidiabetic treatments increasing β-cell mass or preventing β-cell loss are gaining increased interest (Meier, 2008). Glucagon-like peptide-1 (GLP-1) analogs are emerging as an important class of drugs for T2D. Physiologically, GLP-1 is traditionally believed to be released from the intestinal endocrine L cells in response to a meal and serves as an important incretin hormone (Baggio and Drucker, 2007; Barrera et al., 2011). In addition to its effect on postprandial glycemia, GLP-1 also inhibits gastric emptying, inhibits glucagon secretion from pancreatic α cells, and suppresses appetite (Baggio and Drucker, 2007; Barrera et al., 2011). Pharmacologically improved GLP-1

receptor agonists, like exendin-4 and liraglutide, improve glucose tolerance and cause weight loss (Astrup et al., 2009). Furthermore, some studies indicate that GLP-1 receptor agonists increase β-cell proliferation and prevent apoptosis in rodent models of T2D (Xu et al., 1999; Rolin et al., 2002; Kwon et al., 2009). Whether this is a consequence of the improvements in glucose homeostasis or a direct trophic effect on the pancreatic β cell remains to be determined (Vrang et al., 2012).

Gastrin is a gastrointestinal peptide secreted from antral G cells and released into systemic circulation during meal ingestion, where it stimulates gastric acid secretion via activation of cholecystokinin B (CCK-B) receptors (Rehfeld et al., 2007). Gastrin exists in multiple forms, the most predominant being gastrin-17 and gastrin-34 (Rehfeld et al., 2007). Gastrin and the CCK-B receptor have been suggested to be involved in fetal pancreas development (Saillan-Barreau et al., 1999; Rehfeld et al., 2007). Furthermore, gastrin has been reported to increase β-cell mass in rodent models of pancreatic regeneration, suggesting a potential role of gastrin in the treatment of diabetes (Rooman et al., 2002; Téllez et al., 2011).

Interestingly, multifunctional peptides with agonistic properties toward not only the GLP-1 receptor but also other gastrointestinal hormone receptors such as glucagon or glucose-dependent insulinotropic polypeptide have been developed recently (Day et al., 2009;

ABBREVIATIONS: CCK-B, cholecystokinin B; GLP-1, glucagon-like peptide-1; HbA1c, hemoglobin A1c; NOD, nonobese diabetic; PBS, phosphate-buffered saline; T2D, type 2 diabetes; ZP3022, HGEGTFTSDLSKQMEEEAVRLFIEWLKN-8Ado-8Ado-YGWLDF-NH2.

Pocai et al., 2009; Finan et al., 2013). These novel dual-acting peptides have highlighted possible synergistic effects of combining GLP-1 agonism with other gastrointestinal hormones as an effective treatment of T2D and/or obesity.

Administration of gastrin in combination with epidermal growth factor, GLP-1, or exendin-4 has been shown to improve glucose homeostasis and increase β-cell mass in a rodent model of diabetes potentially via islet neogenesis (Suarez-Pinzon et al., 2005, 2008b; Tamaki et al., 2010). In addition, combination therapy with exendin-4 and a proton pump inhibitor, which raise endogenous gastrin levels, has been shown to improve glucose homeostasis and increase pancreatic insulin content in db/db mice (Patel et al., 2013). Finally, we have recently shown that short-term treatment with the novel GLP-1–gastrin receptor dual agonist ZP3022 (HGEGTFTSDLSKQMEEEAVRLFIEWLKN-8Ado-8Ado-YGWLDF-NH2) improves glycemic control and increases β-cell mass in male diabetic db/db mice (Fosgerau et al., 2013). Collectively, these data suggest that a GLP-1–gastrin receptor dual agonist might prove beneficial in treating diabetes by increasing β-cell mass. The above-mentioned studies showing a beneficial effect on β-cell mass after GLP-1 and gastrin cotreatment are, however, based on short-term efficacy studies (2–3 weeks), and it is therefore not clear if the beneficial effect on β-cell mass is persistent or transient. Therefore, we investigated the effect of 2, 4, and 8 weeks of treatment with ZP3022 on β-cell mass in male db/db mice. Gastrin and GLP-1 receptor coagonism has previously been reported to increase β-cell mass by stimulating β-cell neogenesis, increasing β-cell proliferation, or preventing β-cell apoptosis (Xu et al., 1999; Rooman et al., 2002; Wang and Brubaker, 2002; Téllez et al., 2011). Consequently, we applied stereological methods to investigate if a potential increase in β-cell mass was caused by a direct proliferative effect of ZP3022 on existing β cells, by an increase in the number of islets, or via an antiapoptotic protective effect of ZP3022.

Materials and Methods

Compounds. ZP3022 and liraglutide were synthesized at Zealand Pharma A/S (Glostrup, Denmark) using solid-phase peptide synthesis as previously described (Knudsen, 2004; Fosgerau et al., 2013). Compounds were first dissolved in 0.1% aqueous ammonia to a concentration of 2 mg/ml and then further diluted in phosphate-buffered saline (PBS) (Gibco 10010; Invitrogen Corporation, Grand Island, NY) to final concentration.

Animal Study. All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals, and covered by an institutional license issued to Zealand Pharma A/S (approved by the Danish Committee for Animal Research; permit no. 2009/561-1633).

Male db/db (BKS.Cg-m +/+ $Lepr^{db}$/J) mice aged 5 weeks at the time of arrival were obtained from Charles River Labs (Calco, Italy) and acclimatized to their new environment. Animals were housed in groups of n = 3–4 in a light-, temperature-, and humidity-controlled room (a 12-hour light/dark cycle, with lights on at 6:00 AM; 23 ± 1°C; 50% relative humidity). All animals had free access to standard chow (Altromin 1324; Brogaarden A/S, Lynge, Denmark) and domestic-quality tap water with added citric acid to achieve a pH ~3.6.

The study was initiated in mice aged 10 weeks. Five days before first dosing, a blood sample from 4-hours-fasted mice was collected for determination of baseline blood glucose and hemoglobin A1c (HbA1c) levels. Based on these baseline HbA1c and glucose levels as well as body weight, 120 mice were stratified into nine treatment groups (n = 11)

receiving vehicle, liraglutide (2 × 50 nmol·kg^{-1}·day^{-1}), or ZP3022 (2 × 50 nmol·kg^{-1}·day^{-1}) for 2, 4, or 8 weeks.

All mice were administered PBS vehicle (see above) subcutaneously for 3 days before treatment to acclimatize the animals to handling and injections. Throughout the experimental period, mice were treated subcutaneously twice daily between 8:00 and 9:00 AM and 2:00 and 3:00 PM. Body weight and food and water intake (cage averages, three cages/group) were measured daily in the morning. Animals were terminated after a total of 2, 4, or 8 weeks of dosing. Animals received the last dose the evening before termination. Prior to the termination, animals were fasted for 4 hours from 8:00 AM to 12:00 PM before a blood sample was obtained from the tail vein for measurement of terminal fasted blood glucose. Then the animals were anesthetized using CO_2, and terminal blood samples were collected from the orbital plexus vein for measurements of blood HbA1c and plasma insulin levels as previously described (Fosgerau et al., 2013). Subsequently, animals were killed by cervical dislocation and pancreata were removed en bloc, immersion-fixed in 4% formaldehyde (4% formaldehyde in 0.1 M phosphate buffer; PBS pH 7.4), and stored at 4°C until further processing.

Pancreas Immunohistochemistry: Embedding and Sectioning. Dissected pancreata were processed as previously described (Fosgerau et al., 2013). Briefly, the pancreas was rolled into a cylinder, infiltrated with paraffin overnight, and cut into four or five systematic uniform random tissue slabs with a razor blade fractionator. The slabs were embedded on their cut surface in one paraffin block. Subsequently, the blocks were trimmed before two 5-μm sections (300 μm apart) were cut from each block on a Microm HM340E (Thermo Scientific, Walldorf, Germany) and arranged on one object slide representing in total a systematic uniform random sample of the whole pancreas. One series was sampled from each animal for β- and non–β-cell analysis. From the 8-weeks-treated groups three additional series were collected for estimation of islet-cell apoptosis, β-cell proliferation, and islet number. For quantification of islet apoptosis, the sections were collected as described above. For quantification of β-cell proliferation, a series of 4-μm-thin neighboring sections were cut (as reference and lookup sections). For islet number estimation, the distance between the two neighboring sections was increased to 12 μm.

Immunohistochemistry. The paraffin-embedded sections were subjected to double immunohistochemistry using an antibody against insulin and an antibody cocktail against pancreatic polypeptide, somatostatin, and glucagon as markers of β cells and non–β cells, respectively. A double labeling against Ki-67 (1:200) (catalog no. M7249; Dako, Glostrup, Denmark) and insulin was used as a measure of β-cell proliferation. All sections were immunostained manually using optimized staining protocols with diaminobenzidine and diaminobenzidine-nickel as chromogens as previously described (Dalbøge et al., 2013). The double labeling against cleaved caspase-3 and insulin as a measure of islet-cell apoptosis was performed using a similar approach with a rabbit anti-mouse caspase-3 antibody (1:25) (#9661; Cell Signaling Technology, Danvers, MA) detecting the cleaved (activated) form of caspase-3 used as a substitute for the non–β-cell antibody cocktail.

Stereological Estimation of Pancreatic β-Cell Mass, Number of Islets, Proliferation, and Apoptosis. Stereological estimations were performed using the newCAST system (Visiopharm, Hørsholm, Denmark) on virtual images obtained using an Aperio ScanScope scanner with a 20× objective. β- and non–β-cell masses were estimated using the Cavalieri principle (Bock et al., 2003). In short, a point grid was used to estimate the area fraction of the specific cell types. The number of points hitting the structure of interest was then converted into mass by taking the grid ratio into consideration (Bock et al., 2005). Caspase-3 mass was estimated using a similar approach.

Quantitative estimates of the number of Ki-67–positive β cells and total number of islets were performed using the physical disector as previously described (Dalbøge et al., 2013). A cell was counted only if it appeared on the reference and not the lookup section. The numerical density was estimated by counting cells within a reference

volume defined by the area of an unbiased counting frame and the distance between the two neighboring sections. Lastly, the total number of Ki-67–positive cells was obtained by multiplying the numerical density with the total reference (β-cell) volume. Islet numbers were likewise estimated using the physical disector principle where an islet was defined as a cluster of at least three endocrine cells similar to the procedure described by Bock et al. (2003). The mean islet mass was calculated as the total mass of islets (β-cell + non–β-cell mass) divided by the total number of islets.

Statistics. Graphical presentations, calculations, and statistical analyses were carried out using GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, CA). Statistical analysis was performed using a one-way analysis of variance followed by Newman–Keuls post hoc test within the respective treatment period. $P < 0.05$ was considered significant. A two-way analysis of variance with Bonferroni's post-test was used to analyze body weight and food and water intake data. Results are presented as mean $\pm$ S.E.M.

Results

Body Weight and Food and Water Intake. Both ZP3022 and liraglutide led to a significant decrease in cumulative food and water intake after 2, 4, and 8 weeks of treatment (Table 1). In accordance with a reduction in food intake, a significant decrease in body weight was observed after 2 weeks of treatment in the ZP3022-treated group and after 2 and 8 weeks of treatment in the liraglutide-treated group as compared with vehicle control (Table 1).

Glycemic Control. Both compound regimens resulted in a significant decrease in the terminal fasted blood glucose as compared with baseline and the vehicle control following 2, 4, and 8 weeks of treatment (Fig. 1A). The reductions in blood glucose were reflected in the HbA1c levels, which were significantly lowered relative to the vehicle control for both the ZP3022- and liraglutide-treated groups following 2, 4, and 8 weeks of treatment (Fig. 1B).

A significant increase in plasma insulin concentration (Fig. 1C) was observed in the liraglutide- and ZP3022-treated groups after 2, 4, and 8 weeks of treatment compared with vehicle. Also, plasma insulin concentration (Fig. 1C) was significantly increased in the ZP3022-treated group as compared with the liraglutide-treated group after 2 weeks ($P < 0.001$), 4 weeks ($P < 0.001$), and 8 weeks of treatment ($P < 0.01$).

Stereological Estimation of β-Cell Mass, Islet Mass, Islet Number, and Mean Islet Mass. Treatment with ZP3022 resulted in a significant and sustained increase in β-cell mass as compared with vehicle after 4 weeks of treatment (5.8 $\pm$ 0.4 mg versus 4.4 $\pm$ 0.3 mg; $P < 0.05$) and as compared with vehicle and liraglutide after 8 weeks of treatment [7.3 $\pm$ 0.8 mg versus 3.7 $\pm$ 0.4 mg (vehicle) ($P < 0.001$); and versus 5.1 $\pm$ 0.5 mg (liraglutide) ($P < 0.05$)] (Fig. 2A). In contrast, the effect of liraglutide appeared to be transient and displayed a higher β-cell mass versus vehicle following 4 weeks of treatment only ($P < 0.05$). In general, ZP3022 appeared to increase the β-cell mass throughout the study period. On the other hand, the vehicle groups displayed a decrease in β-cell mass during the 8-week study period as diabetes progressed (Fig. 2A). A similar picture was observed for islet mass (Fig. 2B). Non–β-cell mass (cells positive for glucagon, pancreatic polypeptide, and somatostatin) was similar in vehicle-treated animals across all time groups (0.87 $\pm$ 0.05 mg) but significantly reduced in mice treated for 2 weeks with liraglutide (0.68 $\pm$ 0.07 mg; $P < 0.05$ versus vehicle) and increased in the ZP3022 group following 8 weeks of treatment (1.21 $\pm$ 0.14 mg; $P < 0.01$ versus vehicle). Representative images of pancreatic islet morphology after 8 weeks' treatment are shown in Fig. 2, C–E. The stereological estimation of the total number of islets and mean islet mass following 8 weeks of treatment is shown in Fig. 3, A and B, respectively. Although visually there appears to be an increase in the number of islets in the ZP3022-treated group (Fig. 2E), the stereological analysis revealed that the total number of islets was unchanged between the groups. In contrast, a significant increase in mean islet mass was observed in the ZP3022-treated group ($P < 0.001$ versus vehicle and $P < 0.05$ versus liraglutide).

β-Cell Proliferation and Apoptosis. Treatment with ZP3022 and liraglutide for 8 weeks significantly increased the total number of proliferating Ki-67–immunoreactive β cells (Fig. 4A) as compared with vehicle control [12,057 $\pm$ 1578 (vehicle) versus 40,365 $\pm$ 4921 (ZP3022) ($P < 0.001$); and versus 32,056 $\pm$ 6306 (liraglutide) ($P < 0.01$)]. Also, the density (Fig. 4B) was significantly higher after 8 weeks of treatment in both the ZP3022- and liraglutide-treated animals as compared with the vehicle control [5258 $\pm$ 535 n·mm^{-3} β cells (vehicle) versus 9013 $\pm$ 933 n·mm^{-3} β cells (ZP3022) ($P < 0.05$); and versus 8498 $\pm$ 1427 n·mm^{-3} β cells (liraglutide) ($P < 0.05$)]. No apparent
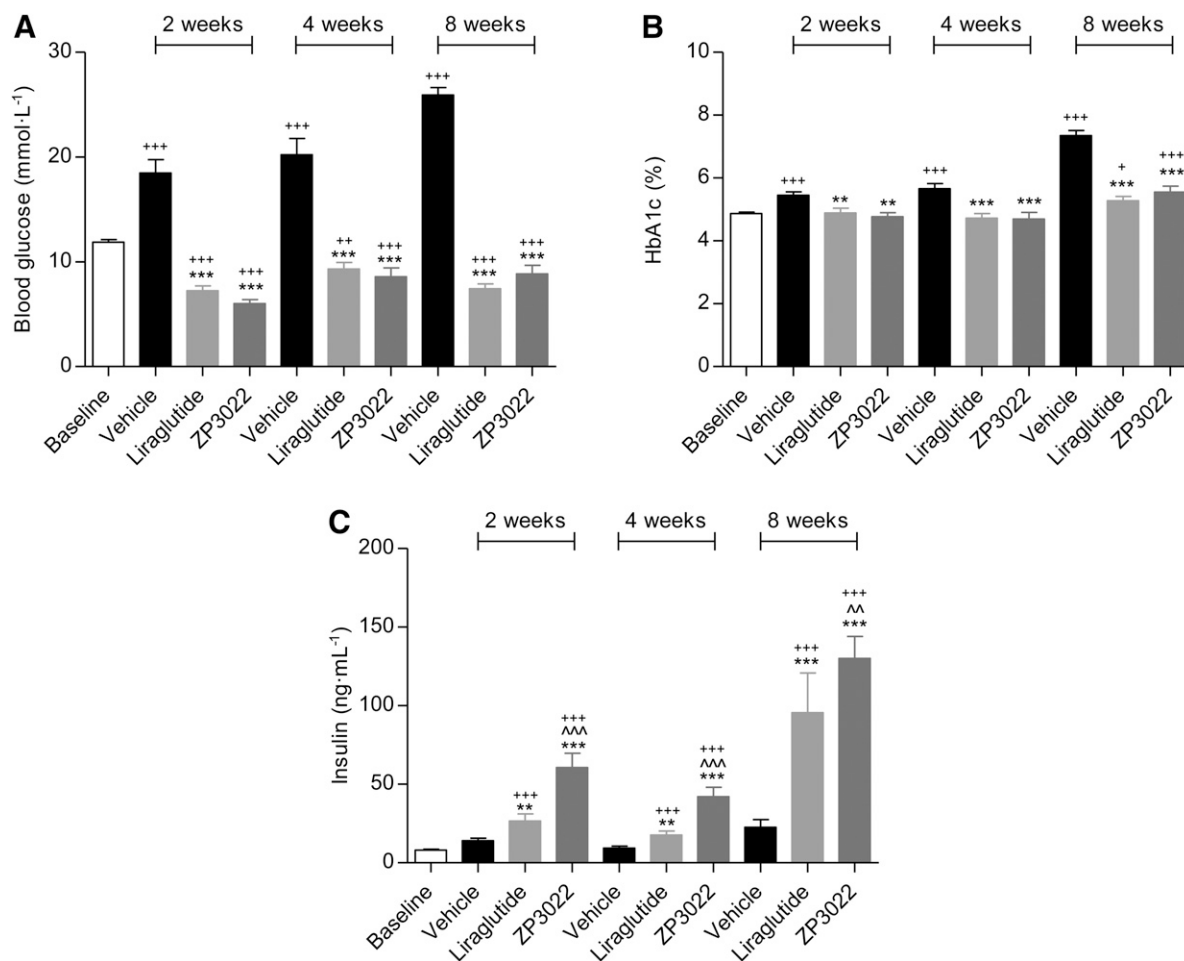
TABLE 1

Effects of treatment with liraglutide and ZP3022 on cumulative food intake, water intake, and body weight in male *db/db* mice

Effect of subcutaneous administration of liraglutide (2 $\times$ 50 nmol·kg^{-1}·day^{-1}), ZP3022 (2 $\times$ 50 nmol·kg^{-1}·day^{-1}), or vehicle. Data are given as mean $\pm$ S.E.M. with n = 11/group; see *Materials and Methods* for further details. Statistical analysis: one-way analysis of variance with Newman–Keuls post hoc test within respective treatment periods.

	Vehicle	Liraglutide	ZP3022
Body weight (g)			
0 weeks	47.7 $\pm$ 0.4	47.4 $\pm$ 0.6	47.2 $\pm$ 0.4
2 weeks	50.3 $\pm$ 0.8	46.9 $\pm$ 0.6**	46.9 $\pm$ 0.6**
4 weeks	52.3 $\pm$ 0.7	51.0 $\pm$ 0.8	50.9 $\pm$ 0.7
8 weeks	55.0 $\pm$ 1.2	51.3 $\pm$ 0.9*	53.3 $\pm$ 0.6
Cumulative food intake (g)			
2 weeks	89.1 $\pm$ 3.3	60.3 $\pm$ 2.5***	64.9 $\pm$ 2.8***
4 weeks	151.5 $\pm$ 6.4	120.9 $\pm$ 6.5*	126.0 $\pm$ 3.2*
8 weeks	311.3 $\pm$ 3.9	208.7 $\pm$ 11.9***	243.2 $\pm$ 6.1***,^
Cumulative water intake (g)			
2 weeks	107.0 $\pm$ 3.9	49.7 $\pm$ 5.2***	55.3 $\pm$ 2.6***
4 weeks	198.9 $\pm$ 13.3	95.6 $\pm$ 10.3**	133.5 $\pm$ 11.0**
8 weeks	454.0 $\pm$ 47.1	203.4 $\pm$ 13.8**	250.1 $\pm$ 8.8**

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle; ^$P < 0.05$ versus liraglutide.

Fig. 1. Effects of 2, 4, and 8 weeks of treatment with liraglutide and ZP3022 on glucose homeostasis in male db/db mice. Effect of 2, 4, and 8 weeks' subcutaneous administration of liraglutide (2×50 nmol·kg^{-1}·day^{-1}), ZP3022 (2×50 nmol·kg^{-1}·day^{-1}), or vehicle control on fasted blood glucose (A), HbA1c (B), and insulin (C) levels. Data are given as mean $\pm$ S.E.M. with $n = 11$/group; see *Materials and Methods* for further details. Statistical analysis: one-way analysis of variance with Newman–Keuls post hoc test within baseline and the respective treatment periods. **$P < 0.01$; ***$P < 0.001$ versus vehicle; $^+P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ versus baseline; $^{\wedge\wedge}P < 0.01$; $^{\wedge\wedge\wedge}P < 0.001$ versus liraglutide.

differences in the mass of active caspase-3–immunoreactive apoptotic islet cells were observed when comparing the groups after 8 weeks of treatment (Fig. 5A). In contrast, a tendency toward a reduction in the caspase-3 fraction (caspase-3 mass in percent of islet mass) was observed in the ZP3022-treated group ($P = 0.06$) (Fig. 5B).

Discussion

In the present study, we show that chronic treatment with ZP3022, a GLP-1–gastrin dual agonist acting on both the GLP-1 and CCK-B receptors, for up to 8 weeks not only improves glucose homeostasis in the diabetic male db/db mouse but also leads to a sustained increase in pancreatic β-cell mass. This increase appears to be caused by increased β-cell proliferation rather than an increase in islet numbers.

Interestingly, the present stereological analysis showed that ZP3022 treatment, unlike liraglutide treatment, leads to an improved β-cell mass during the whole treatment period. Our results support earlier findings that GLP-1 receptor and gastrin (CCK-B) receptor coactivation induces a more profound increase in β-cell mass as compared with GLP-1 receptor agonism alone in animal models of diabetes (Suarez-Pinzon

et al., 2008b; Tamaki et al., 2010; Patel et al., 2013). Studies have indicated that there is an age-dependent decline in β-cell mass in diabetic animal models, such as the Zucker diabetic fatty rat and the db/db mouse, with a strong coupling between blood glucose and β-cell mass (Paulsen et al., 2010; Dalbøge et al., 2013). Glucolipotoxicity is believed to contribute to the age-dependent decrease in β-cell mass, whereas treatment with liraglutide and the accompanying improvement in glycemic control has been suggested to postpone the age-dependent decline in β-cell mass but not prevent it (Sturis et al., 2003; Prentki and Nolan, 2006; Vrang et al., 2012). In agreement with this, the liraglutide-induced effect on β-cell mass was transient in the present study. Importantly, ZP3022 treatment led to a sustained increase in β-cell mass during the whole treatment period, suggesting that a mechanism in addition to a direct β-cell protective effect might be involved in mediating the ZP3022-induced effect on β-cell mass. Notably, the increase in β-cell mass observed in the ZP3022-treated groups was accompanied by a pronounced increase in insulin levels, suggesting that the additional β cells were functionally active. This is in line with the report of Suarez-Pinzon et al. (2008b) of increased pancreatic insulin content along with an increase in β-cell mass after 3 weeks of treatment with GLP-1
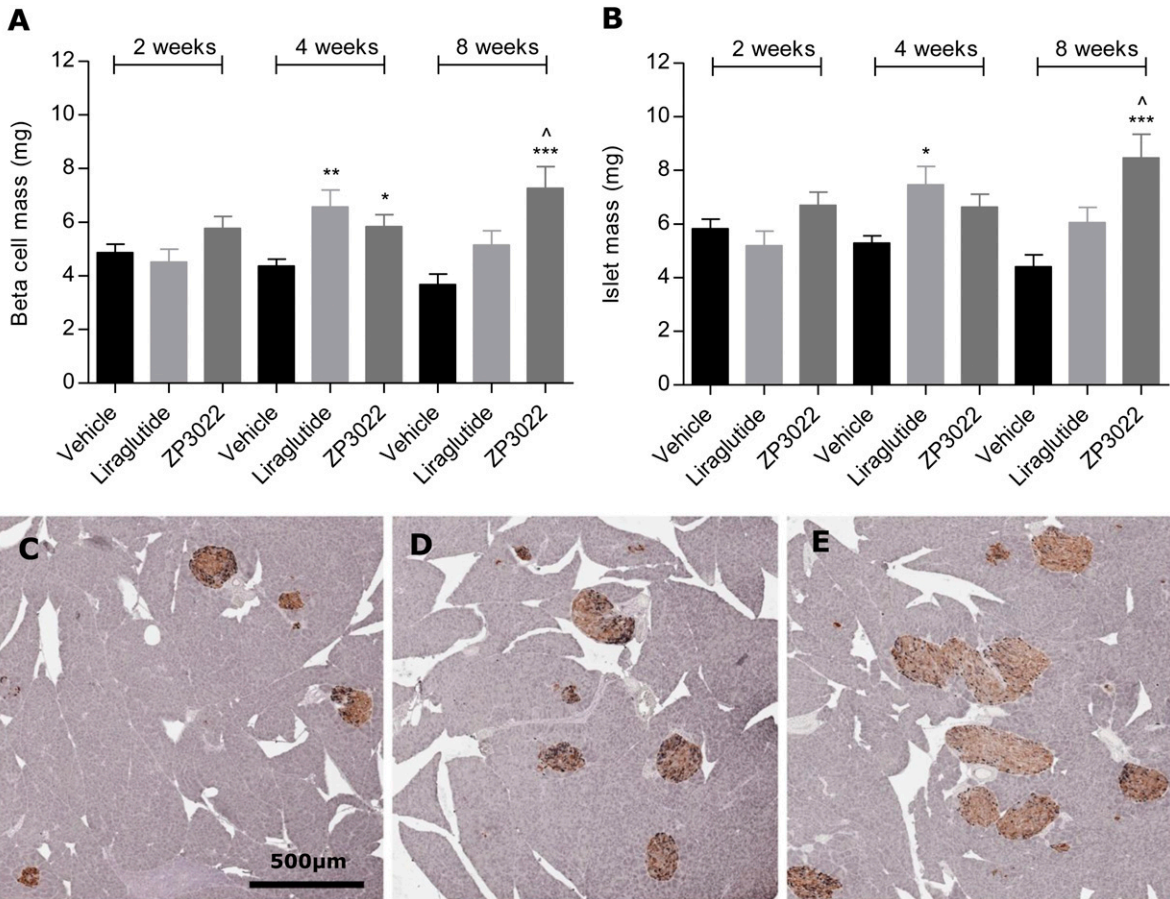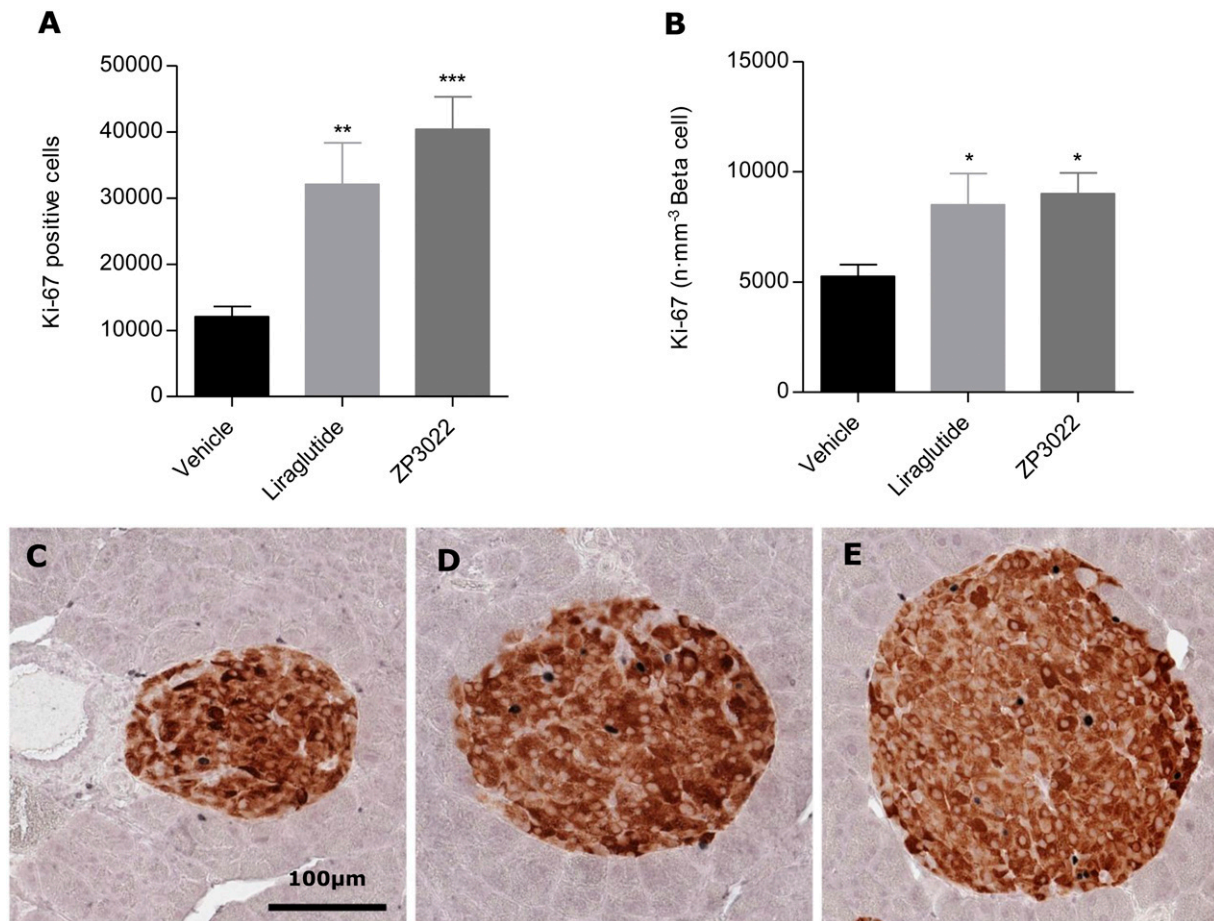
Fig. 2. Effects of 2, 4, and 8 weeks of treatment with liraglutide and ZP3022 on β-cell and islet mass in male *db/db* mice. Stereological estimation of β-cell mass (A) and islet mass (B) following 2, 4, and 8 weeks' s.c. treatment with liraglutide (2×50 nmol·kg^{-1}·day^{-1}), ZP3022 (2×50 nmol·kg^{-1}·day^{-1}), or vehicle control. Representative images of islet morphology after 8 weeks' treatment with vehicle control (C), liraglutide (D), and ZP3022 (E). Sections were immunohistochemically stained for insulin to detect β cells (brown) and pancreatic polypeptide, somatostatin, and glucagon (antibody cocktail) to detect non–β cells (black). Data are given as mean $\pm$ S.E.M. with $n = 11$/group; see *Materials and Methods* for further details. Statistical analysis: one-way ANOVA with Newman–Keuls post hoc test within baseline and the respective treatment periods. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle; ^$P < 0.05$ versus liraglutide.

and a gastrin-17 analog in acutely diabetic nonobese diabetic (NOD) mice. Similarly, it has been shown that addition of the proton pump inhibitor omeprazole to exendin-4 treatment significantly improves glucose homeostasis and increases pancreatic insulin content in *db/db* mice after 14 days of

treatment compared with exendin-4 treatment alone (Patel et al., 2013). Collectively, these data underline the additional beneficial effects of GLP-1–gastrin receptor coagonism. ZP3022 led to an increase in non–β-cell mass following 8 weeks of treatment. Gastrin has been reported to increase non–β-cell
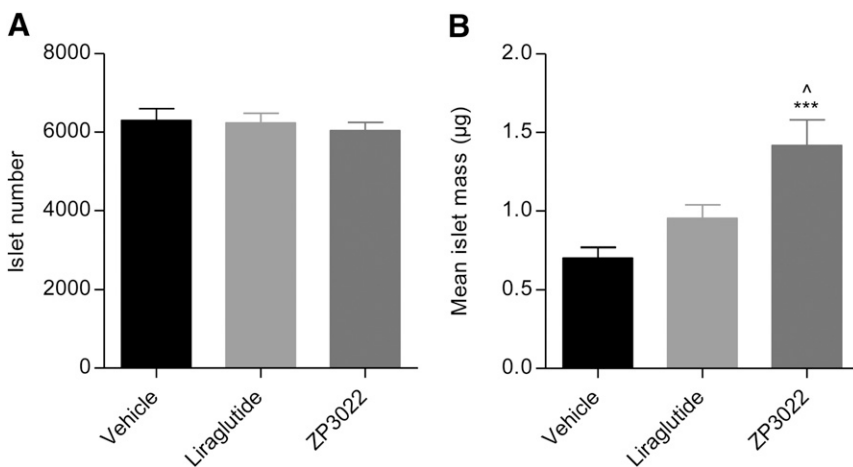
Fig. 3. Effects of 8 weeks of treatment with liraglutide and ZP3022 on islet number and mean islet mass in male *db/db* mice. Stereological estimation of total number of islets (A) and mean islet mass (B). Data are given as mean $\pm$ S.E.M. with $n = 11$/group; see *Materials and Methods* for further details. Statistical analysis: one-way ANOVA with Newman–Keuls post hoc test within respective treatment periods. ***$P < 0.001$ versus vehicle; ^$P < 0.05$ versus liraglutide.

Fig. 4. Effects of 8 weeks of treatment with vehicle, liraglutide, and ZP3022 on β-cell proliferation in male db/db mice. Stereological estimation of total number (A) and density (B) (number of Ki-67–positive nuclei per mm^3 β cells) of Ki-67–positive β cells following subcutaneous treatment with liraglutide (2×50 nmol·kg^{-1}·day^{-1}), ZP3022 (2×50 nmol·kg^{-1}·day^{-1}), or vehicle control. Representative images of β cells (brown) with Ki-67–positive nuclei (black) after 8 weeks' treatment with vehicle (C), liraglutide (D), and ZP3022 (E). Sections were immunohistochemically stained for insulin and Ki-67 as a measure of proliferating β cells. Data are given as mean $\pm$ S.E.M. with $n = 11$/group; see *Materials and Methods* for further details. Statistical analysis: one-way ANOVA with Newman–Keuls post hoc test within respective treatment periods. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle.

mass in a rodent model of pancreatic regeneration (Téllez et al., 2011). Similarly, gastrin in combination with epidermal growth factor has been shown to increase α-cell number in vivo (Suarez-Pinzon et al., 2005). However, an increase in non–β-cell mass following GLP-1 and gastrin cotreatment has not been previously reported. Liraglutide treatment led to a transient
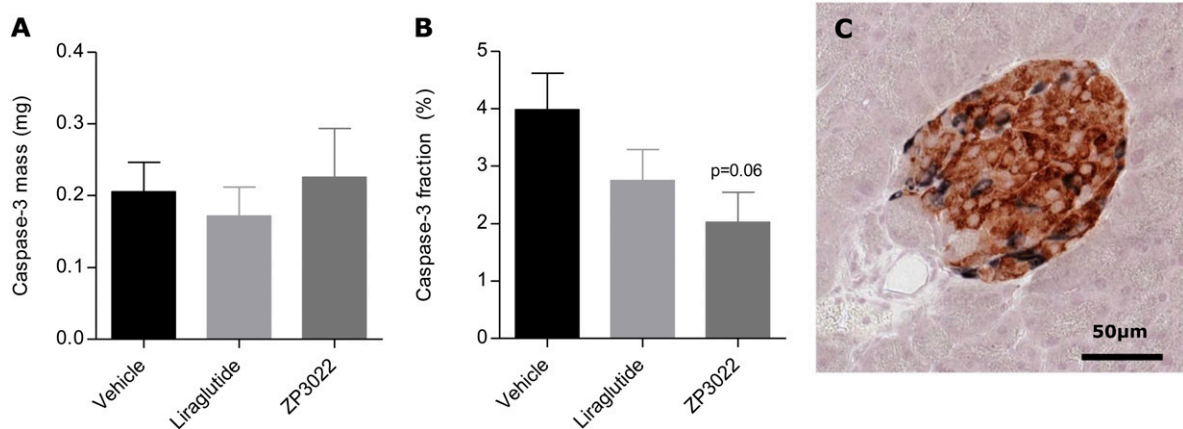
Fig. 5. Effects of 8 weeks of treatment with vehicle, liraglutide, and ZP3022 on β-cell apoptosis in male db/db mice. Stereological estimation of active caspase-3–positive islet cell mass (A) and caspase-3 fraction (caspase-3 mass in percent of islet mass) (B) following 8 weeks' subcutaneous treatment with liraglutide (2×50 nmol·kg^{-1}·day^{-1}), ZP3022 (2×50 nmol·kg^{-1}·day^{-1}), or vehicle. Sections were immunohistochemically stained for insulin (brown) and cleaved caspase-3 (black) as a measure of apoptosis (C). Data are given as mean $\pm$ S.E.M. with $n = 11$/group; see *Materials and Methods* for further details. Statistical analysis: one-way analysis of variance with Newman–Keuls post hoc test.

effect on non–β-cell mass following 2 weeks of treatment, partly in line with previous reports showing decreased α-cell mass after liraglutide treatment in Zucker diabetic fatty rats and normoglycemic mice (Ellenbroek et al., 2013; Schwasinger-Schmidt et al., 2013).

Both ZP3022 and the commercially available GLP-1 analog liraglutide improved glucose homeostasis and reduced food intake and body weight. Different pharmacokinetic properties of the two compounds (ZP3022 having a shorter half-life) may account for the observed differences in food intake and body weight (Rolin et al., 2002; Fosgerau et al., 2013). As both ZP3022 and liraglutide are full agonists at the GLP-1 receptors, with ZP3022 being 10 times more potent (EC$_{50}$ values of 0.02 and 0.19 nM, respectively), the more pronounced decrease in food intake and body weight observed in the liraglutide-treated group is not a result of dissimilarities in in vitro activity (Fosgerau et al., 2013), although it can be speculated that differences in receptor-ligand interaction as well as internalization and recycling might play a role (Roed et al., 2014). However, although liraglutide caused a more profound decrease in food intake and body weight than ZP3022, the dual agonist was equipotent with respect to glycemic control, which may suggest an improvement of glycemic control that is not derived from body weight loss following GLP-1–gastrin dual agonism.

To further investigate the mechanism behind the increased β-cell mass following GLP-1–gastrin coagonism, we investigated proliferation by a stereological quantification of Ki-67–positive β cells as well as a stereological determination of total islet apoptotic mass by assessing cleaved activated caspase-3–positive cells after 8 weeks of treatment. We found an increase in both the number and density of Ki-67–positive β cells, demonstrating that increased proliferation contributes to the increased β-cell mass after ZP3022 treatment. These data are in accordance with previously reported results based on exendin-4 and gastrin cotreatment in *db/db* mice (Tamaki et al., 2010). No significant differences were observed in total islet apoptotic mass between groups, although a tendency toward a decreased fraction was observed in the ZP3022-treated group compared with liraglutide- and vehicle-treated groups. These data are somewhat contradictory to recent data obtained in NOD mice from Suarez-Pinzon et al. (2008b), demonstrating decreased apoptosis but no differences in β-cell proliferation. These differences may, however, be related to the specific animal model used, with NOD mice experiencing rapid autoimmune β-cell loss (apoptosis) (Sreenan et al., 1999). The molecular mechanism by which ZP3022 increases β-cell mass is not fully understood, and the interaction between gastrin (CCK-B) and GLP-1 receptor agonists remains to be elucidated. It is conceivable that the GLP-1 part of the molecule might activate GLP-1 receptors on β cells and induce proliferation via increased expression of pancreatic duodenal homeobox gene-1 and cyclin D1, as well as a variety of other transcription factors and signaling molecules (Perfetti et al., 2000; Friedrichsen et al., 2006). In contrast, the CCK-B receptor has been reported to be located on pancreatic α and δ cells but not on β cells (Saillan-Barreau et al., 1999; Morisset et al., 2003). Alternatively, it can be speculated that the gastrin part of the molecule affects β-cell proliferation and mass expansion via an indirect mechanism, possibly by increasing pancreatic NeuroD1, MafA, or Ngn3 expression, as shown by Tamaki et al. (2010). Further studies

are required to clarify the exact molecular mechanism of action resulting in the observed GLP-1– and gastrin-induced effect on β-cell mass.

Gastrin-induced effects on β-cell mass have previously been coupled to islet neogenesis (Larsson et al., 1973; Rooman et al., 2002; Téllez et al., 2011). Consequently, we estimated the total number of islets to investigate whether the increase in β-cell mass was a consequence of an increased number of islets or an increased mean islet mass. A visual analysis of the number of islet profiles per area of pancreas gives the impression of an increase in the number of islets in the ZP3022-treated group. However, an increase in the size of the individual islets will result in an increase in the number of islet profiles per area of pancreas. Therefore, a conclusion on islet number based on the number of islet profiles per area of pancreas would in fact be erroneous, as discussed in detail by Bock et al. (2003). Accordingly, we applied unbiased stereology and found that the total number of islets was unchanged between the groups, providing strong support for the interpretation that the expansion of β-cell mass is driven by an increase in the size of existing islets rather than formation of new islets. In opposition to our data, Rooman et al. (2002) found that gastrin infusion increased β-cell mass by neogenesis in the ligated part of the pancreas undergoing regeneration after partial duct ligation. Similarly, Téllez et al. (2011) showed that gastrin induced β-cell neogenesis from pancreatic duct cells in 95% pancreatectomized rats. Correspondingly, Inada et al. (2008) proved by lineage tracing that islet cells can be regenerated from ductal progenitor cells in injured adult mouse pancreas, suggesting that a potent stimulus/stressor may induce islet neogenesis. In the present study, we did not investigate specific cell markers associated with islet neogenesis and thus cannot definitely exclude that islet neogenesis may contribute to the β-cell mass expansion observed after ZP3022 treatment. In agreement with our data, however, Dor et al. (2004) showed by genetic lineage tracing that under normal physiologic conditions the primary source of new β cells was related to islet proliferation rather than stem cell formation. A similar pattern was demonstrated during regeneration after 70% pancreatectomy (Dor et al., 2004). Also, our data are supported by previous reports in *ob/ob* mice (Bock et al., 2003) and by recent data published by our group from *db/db* mice (Dalbøge et al., 2013), reporting that β-cell proliferation is the major mechanism for increasing β-cell mass, rather than the formation of new islets.

It is important to note that we do not yet know to what extent treatment with ZP3022 may translate to β-cell mass and proliferation in nonhuman primates or humans. The capacity for β-cell proliferation is thought to be rather modest in humans under normoglycemic conditions (Perl et al., 2010). However, during periods of increased insulin demand (pregnancy and obesity), β-cell mass has been reported to increase (Van Assche et al., 1978; Saisho et al., 2013). Additionally, Rabinovitch and colleagues showed that GLP-1 and gastrin cotreatment increased β-cell mass of human isolated pancreatic material transplanted into streptozotocin-diabetic immunodeficient mice (Suarez-Pinzon et al., 2008a). Furthermore, treatment with proton pump inhibitors (which increase endogenous gastrin levels) has been associated with improved glucose control and β-cell function in patients with T2D. Collectively, these data indicate a potential for β-cell effects of gastrin also in humans (Singh et al., 2012).

In conclusion, we have demonstrated that treatment with the GLP-1–gastrin dual agonist ZP3022, but not liraglutide, causes a sustained improvement in glycemic control in *db/db* mice, accompanied by an increase in β-cell mass. The increase in β-cell mass was driven by an increase in β-cell proliferation and an increased mean islet mass rather than by the formation of new islets. Our data demonstrate that dual GLP-1–gastrin agonists could potentially be developed into a new class of antidiabetic treatment with preventive and curative aspects.

Authorship Contributions

Participated in research design: Almholt, Neerup, Fosgerau, Dalbøge, Vrang, Jelsing.

Conducted experiments: Almholt, Dalbøge.

Performed data analysis: Almholt, Dalbøge.

Wrote or contributed to the writing of the manuscript: Dalbøge, Jelsing, Fosgerau.

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