Glucagon-Like Peptide 1 Receptor Agonist ZP10A Increases Insulin mRNA Expression and Prevents Diabetic Progression in db/db Mice

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Received March 24, 2003; accepted July 29, 2003

ABSTRACT
We characterized the novel, rationally designed peptide glucagon-like peptide 1 (GLP-1) receptor agonist H-HGEGTFTSDL-SKQMEEEEAVRLFIEWLKNGGPGSSGAPPSSK KKKKK-NH₂ (ZP10A). Receptor binding studies demonstrated that the affinity of ZP10A for the human GLP-1 receptor was 4-fold greater than the affinity of GLP-1 (7–36) amide. ZP10A demonstrated dose-dependent improvement of glucose tolerance with an ED₅₀ value of 0.02 nmol/kg i.p. in an oral glucose tolerance test (OGTT) in diabetic db/db mice. After 42 days of treatment, ZP10A dose-dependently (0, 1, 10, or 100 nmol/kg b.i.d.; n = 10/group), decreased glycosylated hemoglobin (HbA₁C) from 8.4 ± 0.4% (vehicle) to a minimum of 6.2 ± 0.3% (100 nmol/kg b.i.d.; p < 0.05 versus vehicle) in db/db mice. Fasting blood glucose (FBG), glucose tolerance after an OGTT, and HbA₁C levels were significantly improved in mice treated with ZP10A for 90 days compared with vehicle-treated controls. Interestingly, these effects were preserved 40 days after drug cessation in db/db mice treated with ZP10A only during the first 50 days of the study. Real-time polymerase chain reaction measurements demonstrated that the antidiabetic effect of early therapy with ZP10A was associated with an increased pancreatic insulin mRNA expression relative to vehicle-treated mice. In conclusion, long-term treatment of diabetic db/db mice with ZP10A resulted in a dose-dependent improvement of FBG, glucose tolerance, and blood glucose control. Our data suggest that ZP10A preserves β-cell function. ZP10A is considered one of the most promising new drug candidates for preventive and therapeutic intervention in type 2 diabetes.

Glucagon-like peptide 1 (GLP-1) is a physiological incretin hormone that is released from the L-cells in the intestine and serves to augment the insulin response after an oral intake of glucose or fat (Orskov, 1992; Holst, 1999). GLP-1 lowers glucagon concentrations, stimulates [pro]insulin biosynthesis, enhances insulin sensitivity, stimulates the insulin-independent glycogen synthesis, slows gastric emptying, and reduces appetite (Wettengel et al., 1993; Nauck et al., 1997; Lopez-Delgado et al., 1998; Weir et al., 1989; Flint et al., 2001). Infusion of GLP-1 has been shown to normalize the level of HbA₁C and enhance the ability of β-cells to sense and respond to increased glucose levels in humans with impaired glucose tolerance (Byrne et al., 1998; Nathan et al., 1992; Nauck et al., 1993; Rachman et al., 1997; Zander et al., 2002). The antidiabetic effect of GLP-1 agonists is only observed during conditions with elevated blood glucose, and therefore, the risk of drug-induced hypoglycemia is low with this new class of agents (Rolin et al., 2002; Vella et al., 2002). These pharmacological features of GLP-1 agonists make the GLP-1 receptor one of the most attractive antidiabetic drug targets.

The low half-life of native GLP-1 (90–120 s) (Deacon et al., 1995; Egan et al., 2003) has led to extensive research to find new compounds with pharmacokinetic properties suitable for development of a drug candidate. Exendin-4 was first isolated from the salivary gland of the Gila monster (Heloderma suspectum), and characterization showed that the peptide was structurally related to, but distinct from GLP-1 for development of a drug candidate. Exendin-4 was first isolated from the salivary gland of the Gila monster (Heloderma suspectum), and characterization showed that the peptide was structurally related to, but distinct from GLP-1 with a sequence homology of only 52%. Further characterization of exendin-4 showed that the peptide is a potent agonist for the mammalian GLP-1 receptor with a longer in vivo half-life and prolonged duration of action compared with GLP-1 (Raufman et al., 1992; Young et al., 1999). Recent studies have shown that administration of exendin-4 induces pancreatic endocrine differentiation, islet proliferation and an increase in β-cell mass (Edvell and Lindström, 1999; Xu et al., 1999), indicating that exendin-4 may exert insulinotropic effects on the β-cells (Greig et al., 1999; Parkes et al., 2001).

In the present article, we present work that describes the in vivo pharmacological properties of the novel exendin-4

ABBRIVIATIONS: GLP-1, glucagon-like peptide 1; CHO, Chinese hamster ovary; OGTT, oral glucose tolerance test; BG, blood glucose; AUC, area under the curve; HbA₁C, glycosylated hemoglobin; PCR, polymerase chain reaction; LSD, least significant difference; FBG, fasting blood glucose; ANOVA, analysis of variance.
analog ZP10A in diabetic db/db mice. These mice are characterized by a leptin receptor mutation leading to severe obesity, early onset of type 2 diabetes manifesting as hyperglycaemia with hyperinsulinaemia and marked peripheral insulin resistance (Chen et al., 1996). Ultimately these animals develop β-cell exhaustion with loss of most of the insulin-producing capacities (Coleman, 1973). Thus, the progression in db/db mice from insulin resistance with hyperinsulinaemia in the earlier stages to hypoinsulinaemia in the advanced stage shares clinical characteristics with the clinical development in human type 2 diabetes.

**Materials and Methods**

**Drugs.** ZP10A (H-HGEGTFTSDLKQMEEEAVRFLFIEWLKNG-GPSSGAPKSKKKK-NH2, batch ZP15.65-3A) was synthesized at Zealand Pharma A/S (Glostrup, Denmark) using the Merifield standard solid phase methodology (Larsen and Holm, 1998).

**GLP-1 Receptor Binding Studies.** The studies were carried out at MDS Panlabs, Panlabs Taiwan Ltd. (Taipei 106 Taiwan). In short, CHO-K1 cells harboring the human recombinant GLP-1 receptor were harvested. The membrane fraction containing the receptor was purified and used for binding assays. ZP10A and GLP-1 were dissolved in 0.4% dimethyl sulfoxide. Membranes were incubated with different concentrations of test compounds covering three decades of concentrations in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 20 mM NaCl, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride (protease inhibitor) and 2% bovine serum albumin for 90 min at 37°C in the presence of 0.03 nM [125I]GLP-1 (7-36) amide. Radioactivity was measured in a gamma counter, and IC50 values were determined as the concentrations diminishing the specific binding (total binding minus nonspecific binding in the presence of 100 nM GLP-1 (7-36) amide) by 50%.

**Animals.** Male db/db mice C57BLKS/J-Leprdb/Leprdb were used in all studies, and each animal was only allocated to one experiment. The mice were housed (3 mice/cage) under controlled conditions (20°C, 55–85% humidity) with a 12:12-h light/dark cycle with light on at 6:00 AM. The animals were fed ad libitum with a standard Altromin 1324 diet (Chr. Petersen, Ringsted, Denmark) and had free access to domestic quality tap water. At the time of inclusion, all mice had overnight fasting (17 h) blood glucose levels below 10 mM. No animals included in the study displayed blood glucose levels above 33 mM when subjected to an oral glucose load (see below). All injections with vehicle and ZP10A were given i.p. at doses of 0.01, 0.1, 1, 10, and 100 nmol/kg (n = 4–7/group), and 15 min later the animals were subjected to the oral glucose load and the test performed as described above. Based on the dose-response relationship an ED50 dose was estimated by linear interpolation between the two doses that produced responses around the EP0 response. Data did not allow nonlinear fitting for estimations of ED50.

**Effect of ZP10A on Glucose-Induced Insulin Release in db/db Mice.** To examine the effect of ZP10A on physiological insulin release during hyperglycaemia, insulin levels were determined after an oral glucose load (1 g/kg). Thirty-nine animals (11–15 weeks old) were subjected to an OGTT as described above and stratified into two groups displaying similar glucose tolerances. One week later, the overnight fasted animals entered the experiment. Fifteen minutes before the animals were given an oral glucose load, each animal received vehicle (phosphate-buffered saline, pH 7.4) or 100 nmol/kg ZP10A i.p. Thirty minutes after the glucose administration, the animals were bled by left ventricular puncture during carbon dioxide anesthesia. The blood was collected using a syringe mounted with a needle preflushed with heparin (5000 IU/ml). The blood samples were quickly transferred to prechilled test tubes that contained 5 µl of 0.5 M EDTA and 5 µl of Trasylol (aprotinin, 20 × 10−6 IU/ml) and centrifuged at 3,000g for 10 min at 2 to 4°C. Plasma was kept cold during harvest, frozen, on dry ice and stored at −80°C for later analysis of insulin. The BG (millimolar) concentration was analyzed as described above.

Plasma concentrations of insulin were measured in samples of 5 µl of plasma using a commercial available enzyme immunoassay kit (catalog no. ELISA 7537; Peninsula Laboratories, Merseyside, UK). This kit uses an enzyme-linked immunosorbent assay monoclonal anti-body-sandwich method to measure the concentration of the insulin in the samples. Anti-insulin was used as capture antibody and biotin-conjugated anti-insulin as detection antibody. Bound biotin-conjugated anti-insulin was quantified by reaction with horseradish peroxidase-conjugated streptavidin followed by peroxidase-catalyzed color development. The absorbance was measured at 450 nm, and the insulin concentrations in the samples were calculated from the standard curve.

**The 42-Day Study.** Animals included in this study were db/db mice between 6 and 10 weeks old at the beginning of the study. Four days before the first dosing, the animals were subjected to an OGTT and stratified into four groups (n = 15/group) exhibiting similar glucose tolerances. The animals were subjected to two daily i.p. doses of ZP10A at 8:00 AM and 4:00 PM, respectively, for 42 days. Doses were given either without vehicle or with 100 nmol/kg ZP10A. The injection anti-body and biotin-conjugated anti-insulin as detection antibody. Bound biotin-conjugated anti-insulin was quantified by reaction with horseradish peroxidase-conjugated streptavidin followed by peroxidase-catalyzed color development. The absorbance was measured at 450 nm, and the insulin concentrations in the samples were calculated from the standard curve.

**The 90-Day Crossover Study.** At the time of inclusion, the db/db mice were 6 to 10 weeks old. Three days before the first dosing, the animals were weighed and subjected to an overnight fasting. The fasted animals were subjected to an OGTT and stratified into two groups (21 animals/group) exhibiting similar glucose tolerances. ZP10A, 100 nmol/kg, or vehicle was injected once daily for a period of 50 days. The dosing was performed between 3:00 and 4:00 PM to ensure pharmacological efficacy during the period with maximal food intake, i.e., during night. After 50 days of dosing another OGTT was performed and based on glucose tolerances each group (vehicle or ZP10A) was restratified into two groups (10–11 animals/group) displaying similar glucose tolerances on day 50. Group 1, which initially
received vehicle, continued receiving vehicle (V + V). Group 2, which initially received vehicle, was switched to ZP10A treatment (100 nmol/kg i.p.) (V + ZP10A); Group 3, which initially received ZP10A, was changed to vehicle treatment (ZP10A + V), and group 4, which initially received ZP10A, was continued on ZP10A treatment (100 nmol/kg i.p.), (ZP10A + ZP10A). The treatment regimen is outlined in Table 1. This dosing regimen continued for 40 days.

During the 90-day dosing period, body weight and water consumption were recorded daily. The animals fasting BG levels were measured after 6 h of fasting (8:00 AM–2:00 PM) on days 44, 58, 65, 72, 86, and 91. This regimen was used because fasting beyond 6 h affected the clinical condition of db/db mice as indicated by decreased physical activity and cold skin. To evaluate the diabetic progression, an OGTT was performed on days 1, 50, 67, 78, and 90 of the treatment period in connection with the daily dosing as described above. On day 91, the animals were sacrificed, blood samples collected for measuring HbA1C, and pancreas removed for insulin mRNA measurements.

**Isolation of Total RNA from Mouse Pancreas.** The frozen pancreatic glands were weighed and minced in a mortar under liquid nitrogen. The extraction of total RNA was conducted according to the kit manufacturer’s manual (QIAGEN RNeasy kit; VWR, West Chester, PA). In short, the frozen tissue was homogenized, lysated, and RNA purified by chromatography.

**First Strand Synthesis.** Total RNA (1.0 µg) was used for first strand synthesis. RNA was incubated for 10 min at 70°C and quenched on ice. The RNA was equilibrated to 42°C and mixed with 10 mM dNTP, 1 µl of Superscript II (Invitrogen, Carlsbad, CA) in a final volume of 20 µl and incubated for an additional hour at 42°C.

**Quantitative PCR.** One microgram of sample was subjected to first strand synthesis as described above and subjected to quantitative PCR using the following probe (mouse insulin Taqman probe, 110–138): 5′-FAM-AGGCTCTCTACCTGGTGTGTGGGGAGCGT-Tamra-3′ and the primers 5′-AACCCACCCAGGCTTTTGTCA-3′ and 5′-CTTCTCCAGGTCCAGTTGTC-3′. All PCR reactions were done in duplicate. The Ct (threshold cycles) were measured, and the initial amount of pancreatic insulin mRNA in ZP10A treated relative to vehicle-treated animals was calculated.

**Statistics.** All statistical calculations were performed using GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA). One-way ANOVA and Fisher’s LSD test were used for post hoc comparison. Unpaired data were also analyzed using a two-way ANOVA and Fisher’s LSD test for post hoc comparison. Unpaired data were analyzed using the Student’s t test for unpaired data. Results were considered statistically significant at the p = 0.05 level.

**Results**

**Binding of ZP10A to Human GLP-1 Receptor.** The concentration resulting in half-maximal inhibition (IC_{50}) of binding to the human GLP-1 receptor expressed in CHO-K1 cells was 5.5 ± 1.3 nM for GLP-1 (7–36) amide, a value within the range of those reported for GLP-1 binding to the endogenous receptor found in islet cell lines and to the recombinant receptor expressed in COS-7 cells (Goke and Conlon, 1988; Goke et al., 1989; Fehmann and Habener, 1991; Thorens, 1992; Wheeler et al., 1993). The IC_{50} value of ZP10A for the human GLP-1 receptor was 1.4 ± 0.2 nM, which was approximately 4 times higher than the affinity of GLP-1 (7–36) amide.

**Acute Dose-Response Effect of ZP10A in the Oral Glucose Tolerance Test.** The dose-response relationship after acute i.p. administration of ZP10A demonstrated an ED_{50} value of 0.021 nmol/kg (Fig. 1). Doses above 1 nmol/kg i.p. produced maximal responses, and the antidiabetic effect of 100 nmol/kg i.p. lasted 18 h (data not shown).

**Acute Effect of ZP10A on Plasma Insulin Levels.** In animals pretreated with ZP10A, 100 nmol/kg i.p., the oral glucose load produced an increase in plasma insulin levels that was about twice as high as the responses observed in vehicle-treated animals (p = 0.002) (Fig. 2).

**Dose-Response Effect of ZP10A in the 42-Day Study.** The body weight of the animals increased between 22 and 27% during the experiment (Table 2). There was no statistically significant difference in weight gain between the ZP10A- and the vehicle-treated group.

The recorded water consumption revealed an extensive water intake in the vehicle-treated animals, suggesting that these animals had diabetes-induced polydipsia. Moreover, the daily water intake was reduced significantly and dose dependently in the mice treated with ZP10A (Table 2).

Diabetic progression was also examined by performing an OGTT before (day −3), and on days 1, 14, and 41 of the treatment period (Fig. 3). Already after the first dose of ZP10A, glucose tolerance was markedly improved in ZP10A-treated animals relative to vehicle-treated control animals. In vehicle-treated animals, the glucose tolerance was progressively impaired, and at the end of the study, this group showed a 7-fold increase in the blood glucose AUC_{0–240 min} after an oral glucose load. In contrast, ZP10A produced a clear and sustained improvement in glucose tolerance in all treated groups (Fig. 3).

As an indicator of long-term blood glucose control, HbA_{1C}
was measured at the end of the study (Fig. 4). The level of HbA1C was expressed as a percentage of the total hemoglobin concentration. These data showed that long-term treatment with ZP10A produced a significant and dose-dependent decrease in the concentration of HbA1C.

Effect of ZP10A in the 90-Day Crossover Study. The body weight of the animals was monitored throughout the study. During the first 50 days of the treatment period, no significant difference between the two initial groups could be detected (data not shown). However, after 90 days the body weight of groups 3 and 4 (ZP10A from days 1–50) was significantly higher than that of groups 1 and 2 (vehicle from days 1–50) (Table 2). This suggests that the general condition of the mice treated initially with ZP10A was better than in the vehicle-treated group.

Similar to findings in the 42 days study, daily water consumption was highest in the vehicle-treated groups. Interestingly, even 40 days after cessation of ZP10A therapy, the group treated with ZP10A during the first 50 days, still had lower water consumption than animals treated with vehicle throughout.

Fasting blood glucose (FBG) was measured after 6 h of fasting (Fig. 5). During the initial period from day 1 to 50, the FBG level was significantly higher in the vehicle-treated animals than in the ZP10A-treated animals. During the second treatment period (days 51–90), FBG levels continued to be higher in mice treated with vehicle throughout than in any other group. In mice treated with ZP10A only during the first 50 days, FBG increased slightly after cessation of treatment. Conversely, in animals that were treated with vehicle during the first 50 days, ZP10A produced a rapid and sustained improvement in FBG. Animals treated with ZP10A throughout showed constantly low FBG levels from day 1 to day 90.

Oral glucose tolerance was measured five times during the study (Fig. 6). After the first 50 days, vehicle-treated animals showed an impaired response to an oral glucose load. In animals that were only treated with ZP10A from day 51 and

### TABLE 2

| Changes in body weight and water consumption at the end of the study period |
|---------------------------------|-----------|-----------------|
| **Data are mean ± S.E.M.**               |           |                 |
| **42-Day Study**                  | Δ Body Weight | Water Consumption on Days 32–42 |
| Vehicle                          | 7.5 ± 1.2  | 36.2 ± 0.78     |
| 1 nmol/kg                        | 8.9 ± 1.1  | 21.7 ± 0.40*    |
| 10 nmol/kg                       | 8.6 ± 1.8  | 16.2 ± 0.35*    |
| 100 nmol/kg                      | 9.3 ± 1.1  | 14.0 ± 0.32*    |
| 90-Day study                     | Δ Body Weight | Water Consumption on Days 80–90 |
| Vehicle-vehicle                  | 7.8 ± 1.4  | 31.7 ± 0.22     |
| Vehicle-ZP10                     | 8.5 ± 1.2  | 14.7 ± 0.13*    |
| ZP10-vehicle                     | 9.2 ± 1.6* | 21.6 ± 0.14*    |
| ZP10-ZP10                        | 9.0 ± 0.4* | 12.2 ± 0.09*    |

* p < 0.05 versus vehicle.
throughout, glucose tolerance was significantly improved during the last period of the study. In fact, during the last 40 days, glucose tolerances were similar in all three groups that had received treatment with ZP10A.

The sustained effect on diabetic status after termination of ZP10A treatment indicates an improved β-cell function. Therefore, to examine the β-cell function, we determined the expression of insulin mRNA at the end of the study (Fig. 7). In animals treated with ZP10A throughout, the expression of insulin mRNA was significantly higher than in vehicle-treated mice. Intriguingly, the expression of insulin mRNA was similar in animals treated with ZP10A only during the first 50 days and in mice treated for 90 days. Animals in which treatment with ZP10A was not initiated until day 50 showed an expression of insulin that was similar to the expression found in vehicle-treated mice.

The HbA1C levels were measured at the end of the study (Fig. 8). All three groups that received ZP10A treatment had lower levels of HbA1C than animals receiving vehicle; however, there was no overall significant difference among groups.

The HbA1C levels were measured at the end of the study. One-way ANOVA showed no overall significant difference among groups (p = 0.22). However, Fisher’s LSD test for post hoc comparisons showed that HbA1C was significantly lower in mice treated with ZP10A (100 nmol/kg i.p. once daily) throughout (group 4, 6.65 ± 0.22%) relative to vehicle-treated mice (group 1, 7.99 ± 0.51%).
Discussion

This study demonstrates that ZP10A is a potent GLP-1 receptor agonist with antidiabetic effects in db/db mice. It binds to the human GLP-1 receptor with an affinity 4 times higher than GLP-1 itself, it potentiates the secretion of insulin in response to an oral glucose load, and normalizes glucose intolerance in diabetic db/db mice at doses in the low nanomole per kilogram range. Furthermore, prolonged treatment with ZP10A reduced Hba_1C levels dose-dependently, and insulin mRNA levels were higher in db/db mice treated with ZP10A during early diabetic development. Our long-term studies indicate that prolonged treatment with ZP10A reduces the progression of diabetes in db/db mice.

In the 42-day study, all three doses of ZP10A produced a similar improvement of glucose tolerance and a dose-dependent decrease of Hba_1C, suggesting that long-term administration of ZP10A improves glucose control in the dose range 1 to 100 nmol/kg i.p. The improvement of glucose tolerance was closely related to a decrease in daily water intake in ZP10A-treated mice. Daily water intake was significantly lower in the animals that received 100 nmol/kg than in both vehicle-treated mice and in animals treated with only 1 nmol/kg ZP10A. These results are consistent with the clinical finding that thirst and polydipsia are closely related to blood glucose levels in diabetic subjects.

To examine whether an early treatment with ZP10A could prevent diabetes development, the 90-day crossover study was conducted. The major finding of this study was that 3 months of continuous treatment with ZP10A, 100 nmol/kg i.p., once daily completely prevented the progression of diabetes in db/db mice. After 90-day treatment with ZP10A treatment, db/db mice had an improved glucose tolerance, lower FBG level, lower Hba_1C, lower water intake, and a higher expression of insulin mRNA in pancreatic β-cells relative to vehicle-treated control mice. The increased expression of pancreatic insulin mRNA suggests that the improved glucose tolerance in ZP10A-treated db/db mice was related to an improved β-cell function, which results in ability to release more insulin in response to an oral glucose load. The finding that glucose-induced plasma insulin levels were doubled in ZP10A-treated mice relative to untreated control animals supports this interpretation.

Interestingly, in mice where treatment with ZP10A was discontinued after 50 days, there was a sustained improvement of glucose tolerance and FBG levels, lower water intake, and an elevated expression of insulin mRNA, indicating that the beneficial effects of ZP10A are preserved even 40 days after cessation of treatment. These results demonstrate that once daily i.p. administration of ZP10A effectively prevents the progression of diabetes in db/db mice. The sustained effect on glucose metabolism and pancreatic expression of insulin mRNA indicates that ZP10A effectively preserves β-cell function and/or induces β-cell neogenesis in diabetic db/db mice. However, there was no significant difference in insulin mRNA levels in animals treated with ZP10A for 50 or 90 days, respectively. This suggests that early treatment with ZP10A prevents β-cell exhaustion and significantly delays the progression of diabetes in these animals. When starting treatment with ZP10A at a more advanced stage of the diabetic progression (i.e., day 51), the compound still effectively improved glucose tolerance, FBG, and water intake. However, late introduction of therapy with ZP10A did not improve the expression of insulin mRNA in pancreatic β-cells.

It is generally believed that the late decompensated stage of diabetes in db/db mice is related to exhaustion of β-cell insulin production (Leiter et al., 1983), and the present data concur with the notion that pancreatic islets in db/db mice gradually disintegrate and that these animals eventually develop insulin-dependent diabetes mellitus. Thus, the preventive effect of ZP10A on the progression of diabetes observed in this study is in agreement with other studies indicating that exendin-4 and GLP-1 analogs exert insulinoergic activity (Parkes et al., 2001). The nature of this effect is not fully understood, but recent data suggest that GLP-1 agonists are able to induce differentiation of PDX-1-positive cells into insulin-secreting cells and to reduce apoptosis (Hui et al., 2001; Tourrel et al., 2001). Intriguingly, the protective action of ZP10A on diabetic β-cell exhaustion was most effective when therapy was initiated early in the diabetes development. When therapy with ZP10A was initiated at more advanced stages of diabetes, ZP10A improved glucose tolerance in absence of changes in insulin expression. However, the presence of detectable insulin mRNA in vehicle-treated mice suggests that even these severely ill animals were able to produce insulin.

In conclusion, these studies demonstrate that ZP10A is an effective antidiabetic compound that effectively improves FBG and glucose tolerance, resulting in a long-term improvement of total glucose control. Furthermore, the sustained effect on glucose metabolism, and pancreatic expression of insulin even after discontinuation of ZP10A treatment indicates that ZP10A preserves β-cell function in diabetic db/db mice. Therefore, it is concluded that ZP10A is not only a promising candidate for the treatment of human type 2 diabetes but also it has the potential to prevent the progression of the disease.

Acknowledgments

We thank Marianne Valla Nielsen, Peter Jess Hansen, Christina Hansen, and Jesper Rasmussen for excellent technical assistance and Drs. Thomas Hartig Braunstein and Michael Schandorff Sørens for kind assistance with interpretation of quantitative PCR data.

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