GLP-1 receptor agonist ZP10A increases insulin mRNA

expression and prevents diabetic progression in *db/db* mice

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DMSO	Dimethylsulphoxide
GLP-1	Glucagon-like peptide 1
HbA_{1C}	Glycosylated hemoglobin
I.p.	Intraperitoneal
OGTT	Oral Glucose Tolerance Test
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Abstract

We characterized the novel, rationally designed peptide GLP-1 receptor agonist, 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 ED_{50} 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 HbA_{1c} from 8.4±0.4% (vehicle) to a minimum of 6.2±0.3% (100 nmol/kg b.i.d.; $p<0.05$ *vs.* vehicle) in *db/db* mice. Fasting blood glucose (FBG), glucose tolerance after an OGTT, and HbA_{1c} levels were significantly improved in mice treated with ZP10A for 90 days compared to 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 PCR 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.

Introduction

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 (Holst, 1999;Orskov, 1992). GLP-1 lowers glucagon concentrations, stimulates (pro)insulin biosynthesis, enhances insulin sensitivity, stimulates the insulin-independent glycogen synthesis, slows gastric emptying and reduces appetite (Nauck et al., 1997;Lopez-Delgado et al., 1998;Flint et al., 2001;Nauck, Holst, Willms, and Schmiegel, 1997;Lopez-Delgado, Morales, Villanueva-Penacarrillo, Malaisse, and Valverde, 1998;Weir et al., 1989;Wettergren et al., 1993). Infusion of GLP-1 has been shown to normalize the level of HbA_{1c} 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 seconds), (Deacon et al., 1995;Egan et al., 2002) has led to extensive research to find new compounds with pharmakokinetic properties suitable for development of a drug candidate. Exendin-4 was first isolated from the salivary gland of the Gila monster lizard (*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 when compared to GLP-1 (Raufman et al., 1992;Young et al., 1999;Young, Gedulin, Bhavsar, Bodkin, Jodka, Hansen, and Denaro, 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;Xu, Stoffers, Habener, and Bonner-Weir, 1999) indicating that Exendin-4 may exert insulinotropic effects on the β-cells (Greig et al., 1999;Parkes et al., 2001;Parkes, Pittner, Jodka, Smith, and Young, 2001).

In the present paper, we present work that describes the *in vivo* pharmacological properties of the novel Exendin-4 analogue 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 hypoinsulineamia in the advanced stage shares clinical characteristics with the clinical development in human type 2 diabetes.

Materials and methods

Drugs: ZP10A (H-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPSK

KKKKK-NH$_2$, Batch: ZP15.65-3A) was synthesized at Zealand Pharma A/S

using the Merifield standard solid phase methodology (Due and Holm, 1998).

GLP-1 receptor binding studies: The studies were carried out at MDS

Panlabs, Panlabs Taiwan Ltd. 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% DMSO. Membranes were incubated with

different concentrations of test compounds covering 3 decades of

concentrations in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 20 mM NaCl, 1 mM

leupeptin, 1 mM PMSF (protease inhibitor) and 2% BSA for 90 min at 37°C in

the presence of 0.03 nM ^{125}I-GLP-1 (7-36) amide. Radioactivity was measured

in a γ-counter and IC$_{50}$-values were determined as the concentrations

diminishing the specific binding (total binding minus non-specific 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)

following a 12:12-h light/dark cycle with light on at 6 am. The animals were fed

ad libitum with a standard Altromin No. 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 hrs) blood glucose levels below

10 mmol/l. 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.

Acute effects of ZP10A on glucose tolerance.

Db/db mice (11-15 weeks old) were subjected to an oral glucose tolerance test (OGTT). Before the OGTT was performed, animals were subjected to an overnight fasting (17 hours). Blood samples were taken from the tip of the tail and blood glucose (BG) measured. The whole blood glucose (mM) concentration was analysed by the immobilized glucose oxidase method (Elite Glycometer Autoanalyser, Bayer, Denmark) using a drop of blood (3 µl) applied to a disposable stick inserted in the instrument following the manufacturer's manual. Blood samples containing glucose concentrations outside the measuring range of the Elite Autoanalyser was measured by an enzymatic/photometrical method at Nova Medical Medi-Lab A/S; Denmark. After initial blood sampling an oral dose of glucose was administered (1 g/kg, 4 ml/kg, Sigma, St. Louis, MO, U.S.A.) dissolved in a phosphate buffer (pH = 7.40). BG levels were measured at t=30 min, t=60 min, t=120 min and t=240 min and the area under the curve obtained over a 240-minute period (AUC_{0-240}; unit: mM·min) was calculated. Prior to the study, an OGTT was performed and the AUC_{0-240} was used to stratify animals into five groups exhibiting similar glucose tolerances. During the test the animals were administered ZP10A or vehicle immediately after the initial blood sampling, which reflected the fasting BG level. ZP10A was administered i.p. at doses of: 0.01, 0.1, 1, 10 and 100 nmol/kg (n=4-7/group) and fifteen minutes later the animals were

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subjected to the oral glucose load and the test performed as described above. Based on the dose-response relationship an ED_{50} dose was estimated by linear interpolation between the two doses that produced responses around the ED_{50} response. Data did not allow non-linear fitting for estimations of ED_{50}.

Effect of ZP10A on glucose-induced insulin release in db/db mice.

To examine the effect of ZP10A on physiological insulin release during hyperglycemia, 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 (PBS)) or 100 nmol/kg ZP10A i.p. Thirty minutes after the glucose administration, the animals were bled by left ventricular puncture during carbon dioxide anaesthesia. The blood was collected using a syringe mounted with a needle pre-flushed with heparine (5000 i.u./ml). The blood samples were quickly transferred to pre-chilled test tubes that contained 5 μl 0.5 M EDTA and 5 μl Trasylol® (aprotinin, 20 x 10^{-6} IU/ml) and centrifuged at 3000 g for 10 minutes at 2-4 °C. Plasma was kept cold during harvest, frozen on dry ice and stored at -80°C for later analysis of insulin. The BG (mM) concentration was analysed as described above.

Plasma concentrations of insulin were measured in samples of 5 μl plasma using a commercial available enzyme immunoassay kit (Peninsula

Laboratories Europe, Ltd.; cat.no. ELIS 7537). This kit employs an ELISA monoclonal anti-body-sandwich method to measure the concentration of the insulin in a sample. Anti-insulin was used as capture 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 catalysed colour development. The absorbance was measured at 450 nm and the insulin concentrations in the samples calculated from the standard curve.

The 42 days study.

Animals included in this study were *db/db* mice between 6 and 10 weeks old at the beginning of the study. Four days prior to 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 am and 4 pm, respectively, for 42 days. Doses were 0 (vehicle), 1, 10 or 100 nmol/kg. The injection volume was 5 ml/kg in all groups**.** During the 42 days dosing period, body weight, food and water consumption were recorded daily. OGTT was performed on days –3, 1, 14 and 41 of the treatment period. On day 43 the animals were sacrificed and blood samples collected for measuring HbA_{1c}. Briefly, HbA_{1C} and total Hb were determined from hemolysate, prepared from whole blood. HbA_{1C} was measured from the hemolysate by a latex enhanced turbidimetric immunoassay. Total Hb was measured from the same hemolysate by a colorimetric cyanide free alkaline hematin method. Both tests were performed

at 550 nm from hemolysed blood in which Hb was proteolytically degraded. The final HbA_{1C} test result was determined from the HbA_{1C}/Hb ratio.

The 90 days cross-over study.

At the time of inclusion, the *db/db* mice were 6-10 weeks old. Three days prior to the first dosing, the animals were weighed and subjected to an overnight fast. 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 and 4 p.m. in order 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) were re-stratified 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 days dosing period, body weight and water consumption were recorded daily. The animals fasting BG levels were measured after 6 hours of fasting (8 am – 2 pm) on days 44, 58, 65, 72, 86 and 91. This regimen was used because fasting beyond 6 hours 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 HbA_{1C}, 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 manufacturers manual (Qiagen Rneasy kit, VWR International). In short, the frozen tissue was homogenised, lyzated and RNA purified by chromatography.

First strand synthesis: 1.0 μg total RNA 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 10mM dNTP, 1ul Superscript II (Life Technologies) in a final volume of 20 μl and incubated for an additional hour at 42°C. The reaction was terminated by incubation for 5 min at 94°C.

Quantitative PCR: One μg of sample were 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'

5'-CTTCCTCCCAGCTCCAGTTGTTC-3'

All PCR reactions were done in triplicates. The C_t (threshold cycles) were measured and the initial amount of pancreatic insulin mRNA in ZP10A treated relative to vehicle treated animals were calculated.

Statistics: All statistical calculations were performed using Statistica® software version 6.0 (Statsoft, Tulsa, OK). One-way classified data were analysed using one-way ANOVA and Fisher's LSD test for post-hoc comparison. Two-way classified data were analysed using a two-way ANOVA and Fisher's LSD test for post-hoc comparison. Unpaired data were analysed 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 $\pm$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) (Fehmann and Habener, 1991;Goke and Conlon, 1988;Goke et al., 1989;Thorens, 1992;Wheeler et al., 1993). The IC_{50} of ZP10A for the human GLP-1 receptor was 1.4 $\pm$ 0.2 nM, which was approximately 4 times larger 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 (Figure 1). Doses above 1 nmol/kg i.p. produced maximal responses and the antidiabetic effect of 100 nmol/kg i.p. lasted 18 hours (data not shown).

Acute effect of ZP10A on plasma insulin levels.

In animals pre-treated with ZP10A, 100 nmol/kg i.p., the oral glucose load produced an increase in plasma insulin levels that were about twice as high as the responses observed in vehicle-treated animals (P = 0.002), (Figure 2).

Dose-response effect of ZP10A in the 42 days study.

The body weight of the animals increased between 22-27% 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 (Figure 3). Already after the very 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 seven-fold increase in the blood glucose $AUC_{0-240\ min}$ after an oral glucose load. In contrast, in all ZP10A-treated mice OGTT responses were not statistically different on day 41 and on the day of stratification (day –3) meaning that glucose tolerance did not deteriorate over time. Thus, ZP10A produced a clear and sustained improvement in glucose tolerance in all treated groups (Figure 3).

As an indicator of long-term blood glucose control, HbA_{1c} was measured at the end of the study (Figure 4). The level of HbA_{1c} 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 HbA_{1c}.

Effect of ZP10A in the 90 days crossover study.

The body weight of the animals was monitored throughout the study. During the first 50 days treatment period, no significant difference between the two initial groups could be detected (data not shown). However, after 90 days the body weight of group 3 and 4 (ZP10A from day 1-50) was significantly higher than group 1 and 2 (vehicle from day 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.

The fasting blood glucose (FBG) was measured after six hours of fasting (Figure 5). During the initial period from day 1-50, the FBG level was significantly higher in the vehicle-treated animals than in the ZP10A-treated animals. During the second treatment period (day 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 (Figure 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 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, in order to examine the β-cell function, we determined the expression of insulin mRNA at the end of the study (Figure 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 only treated with ZP10A on days 1-50 and in animals treated for the entire 90 days study period.

The HbA_{1c} levels were measured at the end of the study (Figure 8). All three groups that received ZP10A treatment had lower levels of HbA_{1c} than animals receiving vehicle, however, there was no overall significant difference among groups.

Discussion

This study demonstrates that ZP10A is a potent GLP-1 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 nmol/kg range. Furthermore, prolonged treatment with ZP10A reduced HbA$_{1c}$ levels dose-dependently and insulin mRNA level 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 days 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 1-100 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 of ZP10A. These results are consistent with the clinical finding that thirst and polydipsia are closely related to blood glucose levels in diabetic subjects.

In order to examine if an early treatment with ZP10A could prevent diabetes development, the 90 days crossover study was conducted. The major finding of this study was that three months continuous treatment with ZP10A, 100 nmol/kg i.p. once daily completely prevented the progression of

diabetes in *db/db* mice. After 90 days 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 ß-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 support 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 between insulin mRNA level 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 IDDM. 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 insulinotropic activity (Parkes, Pittner, Jodka, Smith, and Young, 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;Tourrel, Bailbe, Meile, Kergoat, and Portha, 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 anti-diabetic 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 it also has the potential to prevent the progression of the disease.

Acknowledgements

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Figure legends

Figure 1. Acute effect of ZP10A on oral glucose tolerance in *db/db* mice. Animals fasted overnight were given an oral glucose load of 1 g/kg 15 min before i.p. administration of either vehicle (n=4) or ZP10A, 0.01, 0.1, 1, 10 and 100 nmol/kg (n=6-7). Area under the blood glucose concentration curve was measured over the following 240 min ($AUC_{0-240\ min}$). *: $p<0.05$ *vs*. vehicle.

Figure 2. Effect of ZP10A, 100 nmol/kg i.p. on release of insulin in *db/db* mice. Animals fasted overnight were given an oral glucose load of 1 g/kg 15 min before i.p. administration of either vehicle (n = 20) or ZP10A, 100 nmol/kg (n = 19). Animals were decapitated and bled after 30 min and concentrations of plasma insulin measured. (**: P = 0.002 vs. control animals).

Figure 3. Oral Glucose Tolerance Test (OGTT) before treatment (day -3) and on day 1, 14 and 41 of i.p. treatment with vehicle or 1, 10, or 100 nmol/kg ZP10A twice daily. Mean ± SEM. *: $p<0.05$ vs. $AUC_{0-240\ min}$ on day -3 within group. §: $p<0.05$ vs. $AUC_{0-240\ min}$ in all three ZP10-treated groups.

Figure 4. HbA_{1c} expressed as percent of total Hgb after 42 days of i.p. treatment with vehicle or 1, 10, or 100 nmol/kg ZP10A twice daily. Data are mean ± SEM. *: $p<0.01$ vs. vehicle.

Figure 5. FBG levels after eight hours of fasting. During Day 0-50, FBG was significantly lower in animals treated with ZP10A (100 nmol/kg i.p. once daily)

compared with vehicle. Moreover, during the second treatment period (Days 51-90), FBG was significantly higher in mice treated with vehicle throughout relative to the other three groups. However, mice that were changed from ZP10A to vehicle had a significant higher FBG level than mice treated with ZP10A throughout.

Figure 6. Oral glucose tolerance test performed on Days 0, 50, 67, 78 and 90. Vehicle-treated *db/db* mice displayed progressively impaired glucose tolerance during the study. On Days 67-90, glucose tolerances were similar in the three groups of animals that were treated i.p. with ZP10A, 100 nmol/kg once daily either during Day 1-50 (Group 2), Day 51-90 (Group 3), or throughout the entire study period (Group 4).

Figure 7. Level of pancreatic insulin mRNA after the respective treatments. Mice treated with ZP10A (100 nmol/kg i.p. once daily) throughout had an increased expression of insulin mRNA after 90 days of administration (Group 4) relative to vehicle-treated mice (Group 1). Interestingly, the expression of insulin mRNA was similar in mice 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.

Figure 8. HbA$_{1c}$ levels (% of total hemoglobin concentration) measured on the day of termination. One-way ANOVA showed no overall significant difference among groups (p=0.22). However, Fisher's LSD test for posthoc comparisons

showed that HbA$_{1c}$ 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%).

**Figure 1**

## Acute Effect in Oral Glucose Tolerance Test (OGTT)

Figure 2

Plasma insulin

Figure 3

Oral Glucose Tolerance Test (OGTT)

Figure 4

HbA$_{1C}$

Figure 5

Fasting Blood Glucose

Figure 6

Oral Glucose Tolerance Test (OGTT)

Figure 8

Pancreatic Insulin mRNA

Figure 7

HbA$_{1C}$
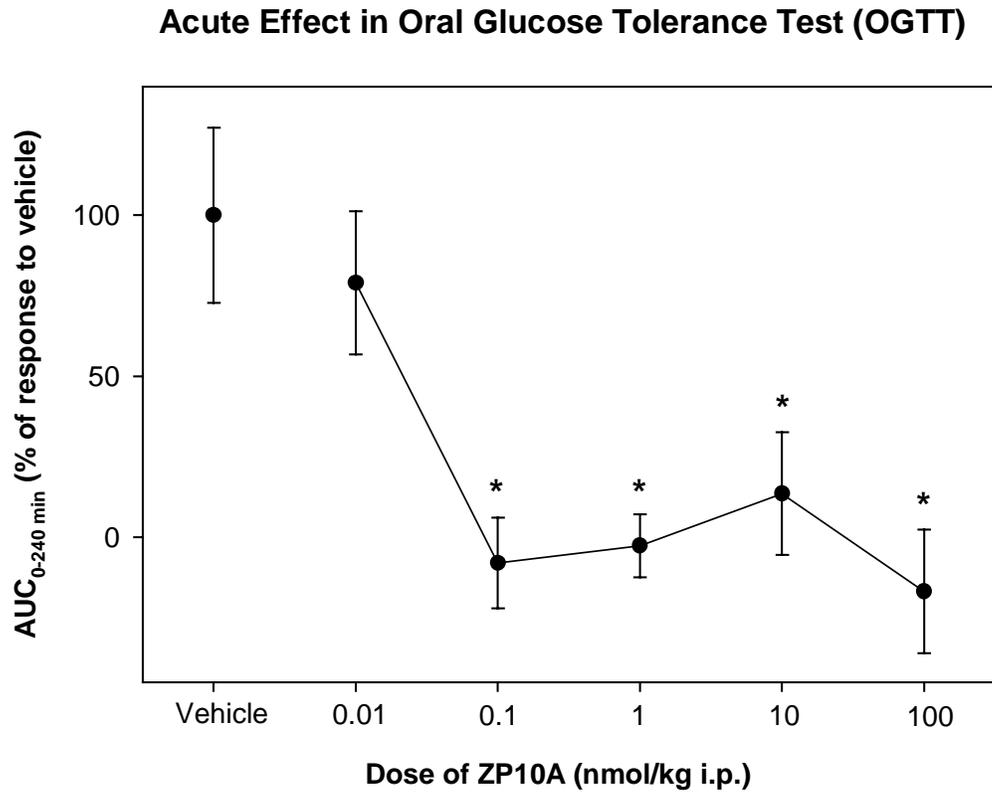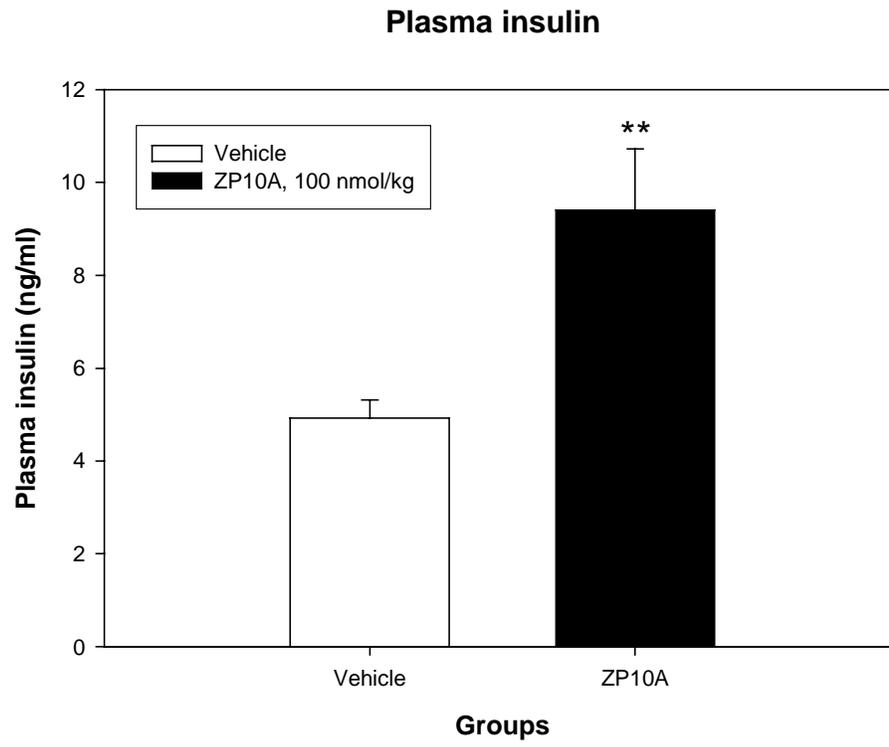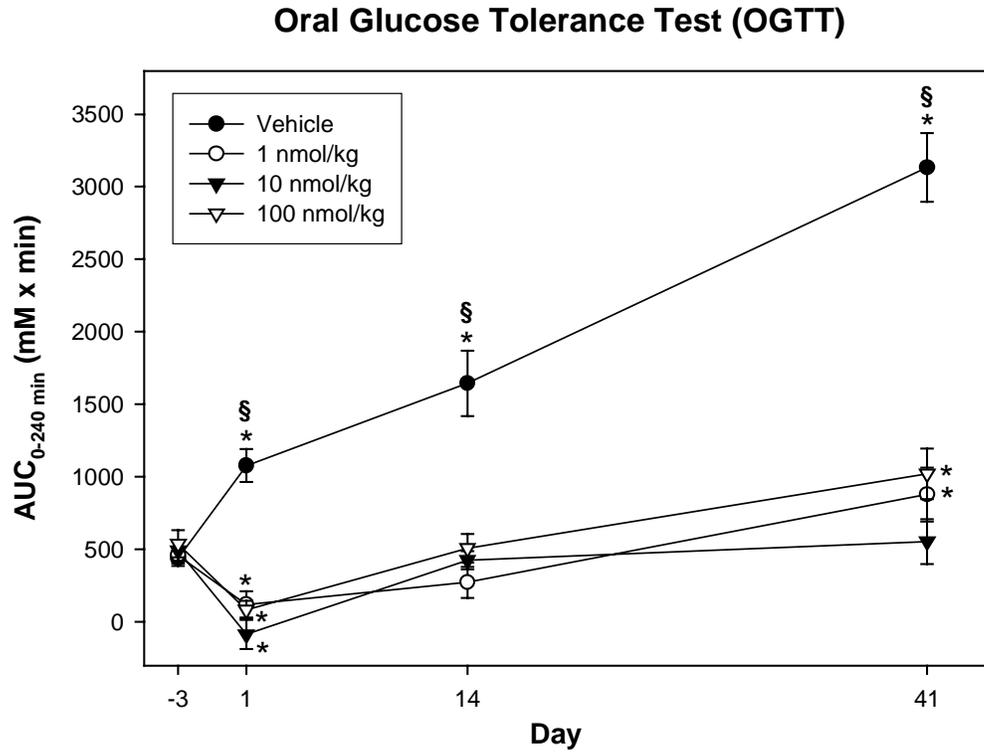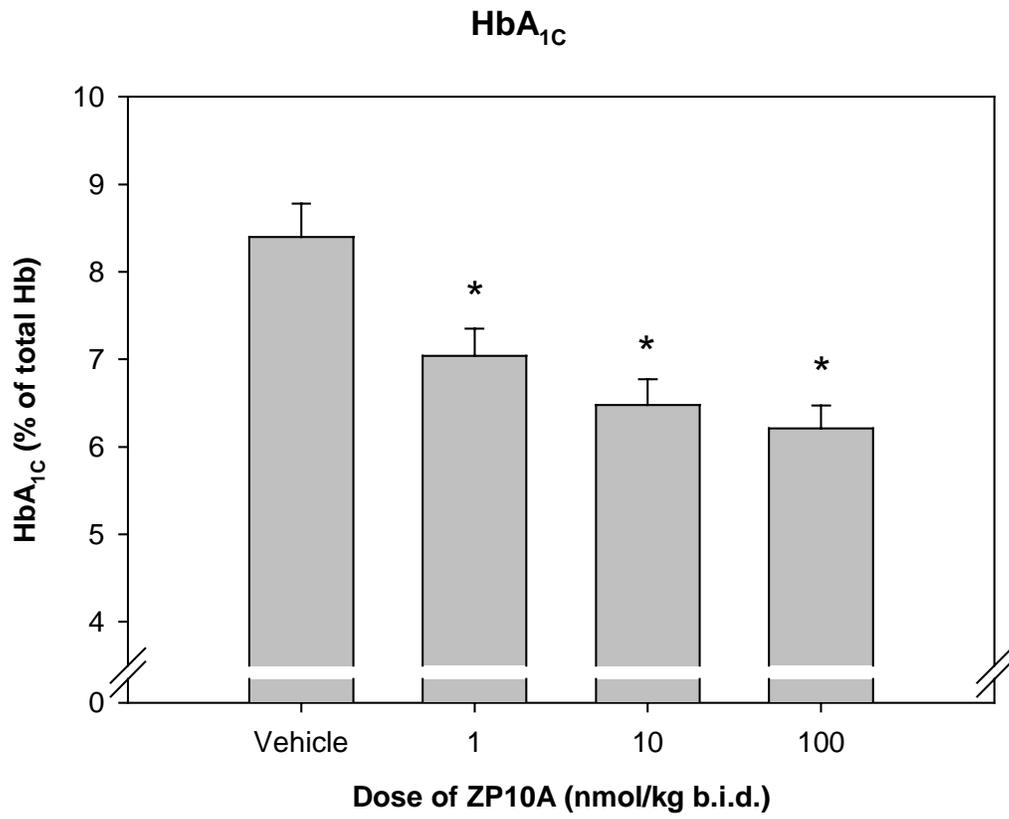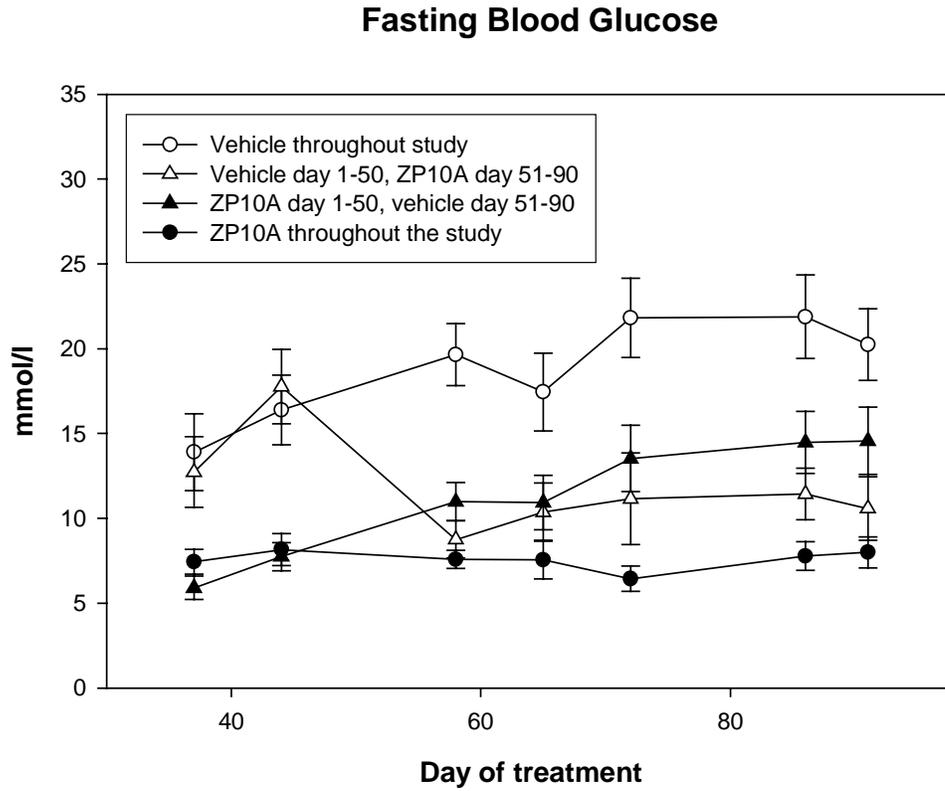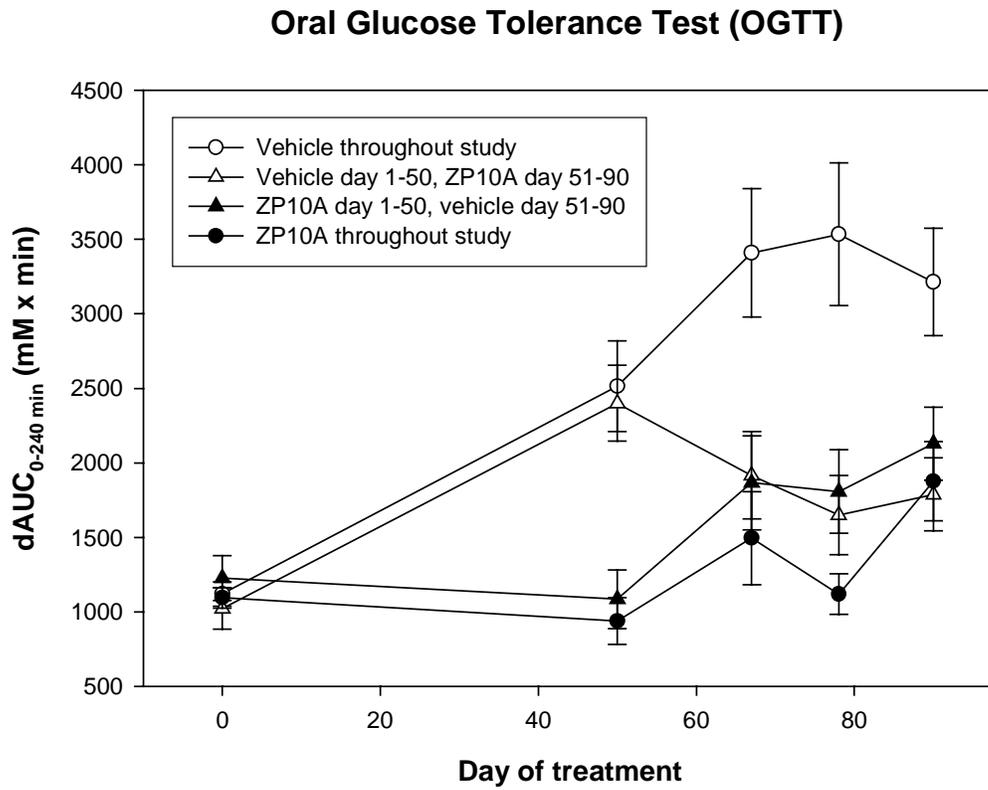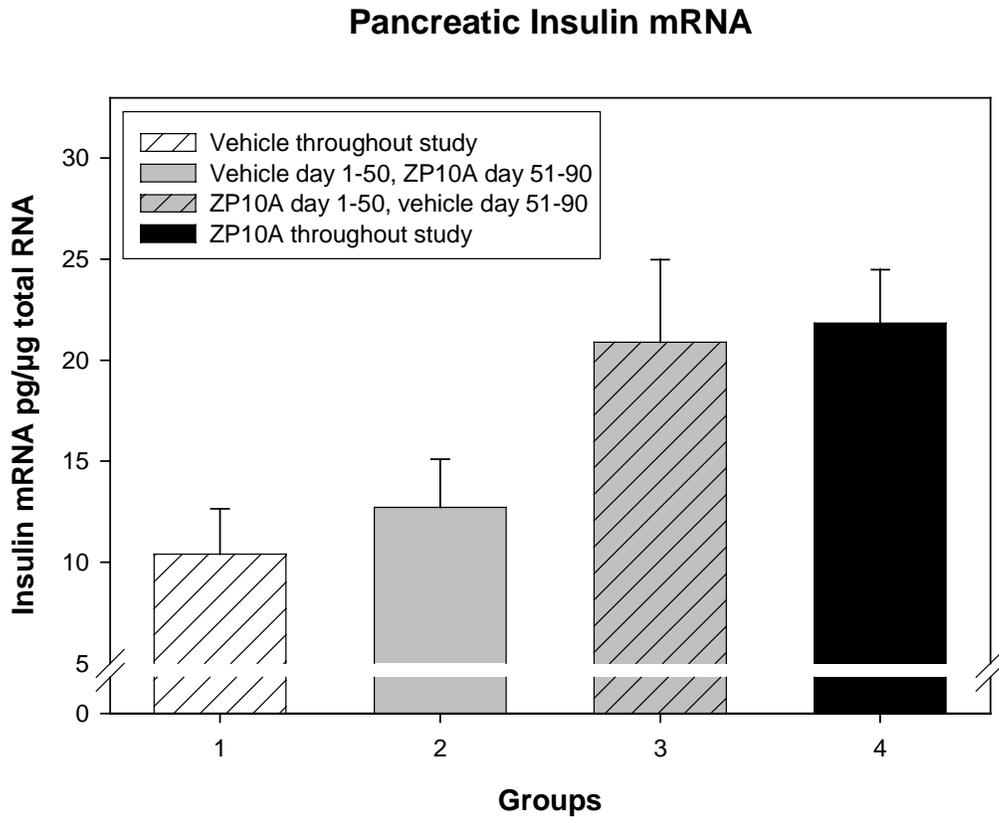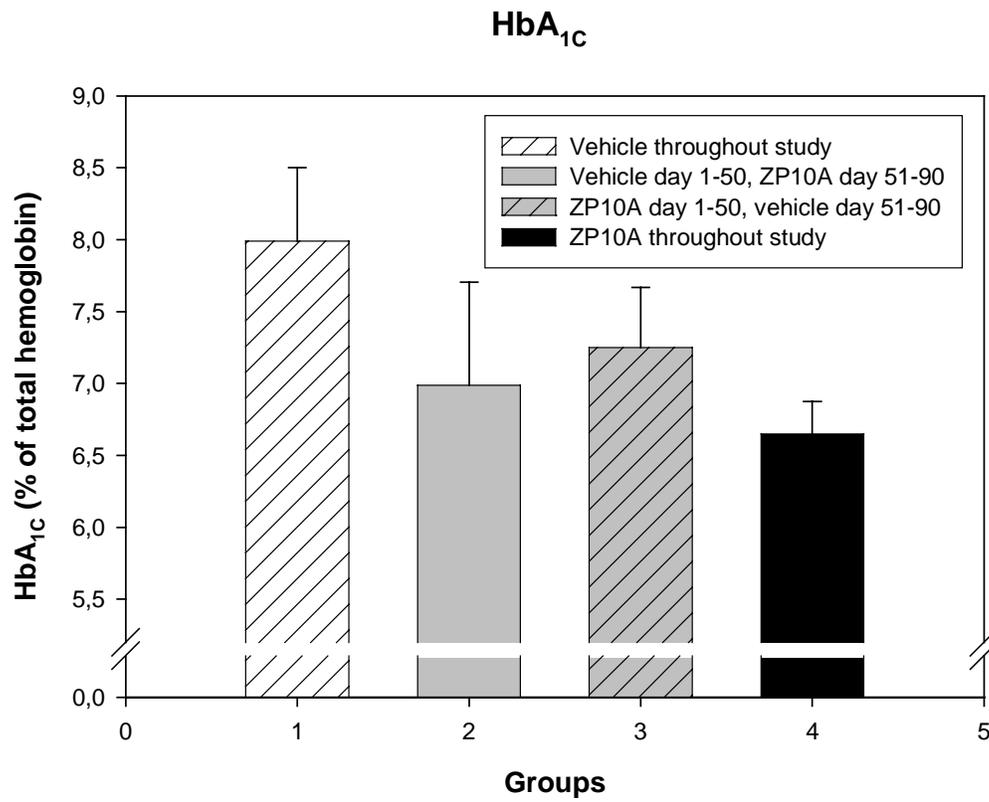
Table 1: Groups of treatment in the 90 days study

Group	Days 1-50	Days 51-90
1	Vehicle (n=21)	Vehicle (n=11)
2		ZP10A (n=9*)
3	ZP10A (n=21)	Vehicle (n=11)
4		ZP10A (n=10)

* One animal died on Day 71

Table 2. Changes in body weight and water consumption at the end of the study period.

42 days study	Δ Body weight (Mean ± SEM)	Water consumption on day 32-42 (Mean ± SEM)
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*

*: $p < 0.05$ vs. vehicle

90 days study	Δ Body weight (Mean ± SEM)	Water consumption on days 80-90 (Mean ± SEM)
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*

*: $p < 0.05$ vs. vehicle