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Combination of the insulin sensitizer, pioglitazone, and the long-acting GLP-1 human analog, liraglutide, exerts potent synergistic glucose-lowering efficacy in severely diabetic ZDF rats

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Objective: Severe insulin resistance and impaired pancreatic β-cell function are pathophysiological contributors to type 2 diabetes, and ideally, antihyperglycaemic strategies should address both.
Research Design and Methods: Therapeutic benefits of combining the long-acting human glucagon-like peptide-1 (GLP-1) analog, liraglutide (0.4 mg/kg/day), with insulin sensitizer, pioglitazone (10 mg/kg/day), were assessed in severely diabetic Zucker diabetic fatty rats for 42 days. Impact on glycaemic control was assessed by glycated haemoglobin (HbA_{1C}) at day 28 and by oral glucose tolerance test at day 42.
Results: Liraglutide and pioglitazone synergistically improved glycaemic control as reflected by a marked decrease in HbA_{1C} (liraglutide + pioglitazone: 4.8 ± 0.3%; liraglutide: 8.8 ± 0.6%; pioglitazone: 7.9 ± 0.4%; vehicle: 9.7 ± 0.3%) and improved oral glucose tolerance at day 42 (area under the curve; liraglutide + pioglitazone: 4244 ± 445 mmol/l × min; liraglutide: 7164 ± 187 mmol/l × min; pioglitazone: 7430 ± 446 mmol/l × min; vehicle: 8093 ± 139 mmol/l × min). A 24-h plasma glucose profile at day 38 was significantly decreased only in the liraglutide + pioglitazone group. In addition, 24-h insulin profile was significantly elevated only in the liraglutide + pioglitazone group. Liraglutide significantly decreased food intake alone and in combination with pioglitazone, while pioglitazone alone increased cumulated food intake. As a result, rats on liraglutide alone gained significantly less weight than vehicle-treated rats, whereas rats on pioglitazone alone gained significantly more body weight than vehicle-treated rats. However, combination therapy with liraglutide and pioglitazone caused the largest weight gain, probably reflecting marked improvement of energy balance because of reduction of glucosuria.
Conclusions: Combination therapy with insulinotropic GLP-1 agonist liraglutide and insulin sensitizer, pioglitazone, improves glycaemic control above and beyond what would be expected from additive effects of the two antidiabetic agents.

Keywords: diabetes, peroxisome proliferator-activator receptor (PPAR)γ, Zucker diabetic fatty (ZDF) rat
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Introduction

Throughout the world, the prevalence of type 2 diabetes is on the rise. Formerly, initial treatment of newly diagnosed type 2 diabetes was diet and exercise [1], but as about 95% of successfully weight-reduced people are back to their initial weight few years after initiation of the weight reducing therapy, such therapy is largely futile [2]. Hence, many consider pharmacotherapy the only feasible strategy for treatment of type 2 diabetes. The goal is to both reduce hyperglycaemia and improve peripheral insulin sensitivity. Traditionally, this has been accomplished using either an insulin-sensitizing agent such as metformin or an insulinotropic sulfonylurea compound in monotherapy. Sulfonylurea compounds act at the level of pancreatic β-cells by eliciting insulin release even at normoglycaemia, rendering clinical use associated with frequent episodes of hypoglycaemia [3,4]. In addition, persistent use of sulfonylurea compounds causes β-cell desensitization, resulting in loss of clinical efficacy [5]. The secondary β-cell desensitization accompanying persistent use of sulfonylurea compounds has spurred an extensive search for alternative insulinotropic agents with longer lasting effects. Pharmacotherapy based on glucagon-like peptide-1 (GLP-1) comprises such an alternative, providing efficacious glucose lowering and simultaneous weight loss. Plasma levels of endogenous GLP-1 can be elevated by drugs inhibiting the action of dipeptidyl peptidase IV (DPP-IV), resulting in improved glycaemic control in patients with type 2 diabetes [6]. However, more efficacious glycaemic control is obtained with use of GLP-1 analogs [7,8]. Chronic use of GLP-1 analogs decrease body weight, and experimental evidence exists to support a preserving if not a regenerating proliferative role of GLP-1 on pancreatic β-cells in rodents [9].

Agents acting by activation of nuclear peroxisome proliferator activator receptor-γ (PPARγ) comprise a relatively novel pharmacological route to improve peripheral insulin sensitivity [10]. Recent clinical experience gathered for combination therapy with DPP-IV inhibitors and thiazolidinediones clearly shows that such regimes are more efficacious compared with the use of maximally recommended doses of drugs from either class [11]. As the GLP-1 analogs are more efficacious than DPP-IV inhibitors, it seems evident that combination of these agents with insulin-sensitizing PPARγ agonists could be a particular efficacious therapeutic option for patients with type 2 diabetes.

Using severely insulin-resistant diabetic rats, we have evaluated antihyperglycaemic efficacy of combination therapy with the novel GLP-1 analog, liraglutide, and the PPARγ agonist, pioglitazone.

Materials and Methods

Animals and Dosing

Seventy male Zucker diabetic fatty (ZDF) rats were obtained from Charles River, Belgium. Animals arrived at Rheoscience animal research facilities when 11 weeks of age. On arrival to the animal unit, rats were caged pair wise and allowed to acclimatize for 7 days. Rats were housed under a normal light cycle (light from 06:00 to 18:00 hours) at controlled temperature conditions with ad libitum access to chow (Purina 5008) and water.

All animal experiments were conducted in accordance with Rheoscience bioethical guidelines, which are fully compliant to internationally accepted principles for the care and use of laboratory animals. The described experimets are covered by personal licenses to P. J. L. (2004/561-859) issued by the Danish Committee for Animal Research.

One day before initiation of dosing, rats were stratified into five groups (n = 9–10) according to area under the curve (AUC) for glucose obtained at an oral glucose tolerance test (OGTT) on day 4 (vide infra). One group of animals (n = 9) was sacrificed to serve as baseline at day 0. From this group, whole blood for glycated haemoglobin (HbA_{1C}) and plasma was obtained from cardiac puncture before the pancreas was removed and fixed as described subsequently. Remaining rats received one of four treatments: vehicle 1 b.i.d. + vehicle 2 b.i.d. (n = 10); pioglitazone 5 mg/kg b.i.d. + vehicle 2 b.i.d. (n = 10); vehicle 1 b.i.d. + liraglutide 200 μg/kg b.i.d. (n = 10); and pioglitazone 5 mg/kg b.i.d. + liraglutide 200 μg/kg b.i.d. (n = 10). Pioglitazone suspended in 10% hydroxypropyl beta-cyclodextrin (w/v) was administered in a volume of 0.5 ml by oral gavage b.i.d, whereas liraglutide dissolved in phosphate-buffered saline was administered subcutaneously b.i.d. Drugs were administered between 7:00 and 8:00 hours and between 15:00 and 16:00 hours for 42 days.

Basal Metabolic Parameters

Measurements of 24-h food and water intake were carried out weekly on days 2, 7, 14, 21, 28, 35 and 41. Twenty-four hours' food intake and body weight was measured weekly (from 7:00 to 8:00 hours). After finishing 24-h observation period, a tail blood sample was taken for analysis of plasma glucose and insulin levels (see subsequently). On day 28, a sample for HbA_{1C}

measurement was also taken after termination of the 24-h observation period.

Oral Glucose Tolerance Test

This test was carried out at 8:00 hours 4 days before the first dose was administered (stratification) and on the day before terminating the experiment (day 42). Animals were mildly fasted as they had had access to only 50% of their daily energy requirements in the preceding 20 h (since 12:00 hours the previous day). Blood samples were taken from a tail vein and plasma glucose was measured at time points −30, 0, 15, 30, 60, 90, 120 and 180 min after oral administration of 2 g/kg glucose (glucose 500 mg/ml; Fresenius Kabi, Sweden). Plasma insulin was measured at time points 0, 15, 30, 60, 90, 120, and 180 min using an ultrasensitive enzyme-linked immunosorbent assay (Diamyd, Stockholm, Sweden). A baseline blood sample of 500 μl was taken at time $t = -30$ min from the tail vein. A sample of this size allowed for analysis of both glycaemic and lipid variables (vide infra).

Twenty-four Hours' Glycaemic Profile

On day 38, blood samples for analysis of P-glucose and P-insulin were taken every 4 h at 7:00, 11:00, 15:00, 19:00, 23:00 and 03:00 hours. Glucose and insulin levels were measured as described previously.

Blood Sampling and Plasma Measurements

Terminal blood samples (minimum 600 μl plasma) were collected in heparinized-/LiCl-containing tubes and centrifuged at 2800 *g* for 10 min at 4 °C. Plasma levels of glucose, total cholesterol and triacylglycerol (TG) were measured using standard enzyme assay kits on a fully automated analyser (Vitros DTII, Ortho Diagnostics, Rochester, NY, USA). Plasma non-esterified free fatty acids (NEFA) were determined by a spectrophotometer using acyl-CoA oxidase-based colorimetric kit (NEFA-C; WAKO pure chemicals, Osaka, Japan). Samples taken in serum Vacutainer + 1% NaF were used for free fatty acids (FFA) analyses. HbA_{1C} was measured using a filter photometer (DCA2000; Bayer Health Care, Lyngby, Denmark).

Body Composition and Termination

At termination of the experiments, animals were sacrificed and trunk blood sampled for drug exposure analysis as well as for measurements of aforementioned biochemical variables. White adipose tissue compartments were removed from exsanguinated rats and weighed. Fat depot analysis included retroperitoneal, epididymal and subcutaneous inguinal fat. The pancreas was removed by dissection of the entire mesenterial adipose tissue block, which was transferred to 4% paraformaldehyde and fixed overnight.

The pancreata were isolated, weighed, fractionated by the smooth fractionator method with $F = ¼$ in each of two capsules, dehydrated and embedded in paraffin in a TP1050 tissue preparation machine Leica, Herlev, Denmark [12]. Randomly, microtome sections of 3 μm thickness were used throughout.

Histology

Sections were deparaffinized in xylene, rehydrated and antigen retrieval treatment carried out in 0.01 M citrate buffer pH 6.0 (preheated to 98 °C). Sections were then cooled, rinsed and endogenous peroxidase blocked by 20 min of incubation with 0.5% H_2O_2. Finally, the sections were washed in water, followed by Tris-buffered saline + 0.01% Triton X-100. The remaining immunohistochemical staining reactions for insulin and the sum of glucagon + somatostatin + pancreatic polypeptide were carried out in an Autostainer (Dako Denmark A/S, Glostrup, Denmark). The β- and non–β-cell mass was estimated using stereological point counting on sections immunohistochemically stained for insulin and the non–β-cell hormones, counting two sections, 250 μm apart. The estimated cell volumes were expressed as a percentage of the total pancreatic volume and also converted to milligram cells total by multiplication by the pancreas weight, or as milligram cells per kilogram body weight [12].

Stereology

Sections stained for insulin and the combination of glucagon, somatostatin and pancreatic polypeptide were used for the estimation of the β-cell volume fraction (Vvol-β) and non–β-cell volume fractions (Vvol-non-β). The analysis used an Olympus BX-50 microscope with video camera and monitor, a PC-controlled motorized stage and the CAST-GRID 2.0 software (Olympus, Copenhagen, Denmark).

Initially, the tissue sections were circumscribed using a ×1.25 objective, and within these areas, the counting of β-cell and exocrine structures was carried out. The volume fractions of β-cells (Vvol-β) or non-β-cells (Vvol-non-β) were estimated by point counting stereological techniques at a total on-screen magnification of ×1026 (×20 objective), a grid of 4 × 48 points, and random

systematic scanning of the tissue sections with step lengths of 550 × 410 μm controlled by the CAST-GRID 2.0 software. Two sections were examined from each rat pancreas. The sections were examined, with the origin of the sections blinded to the observer.

Data Management and Statistical Evaluation

Data were presented graphically using GRAPH PAD PRISM software, while statistical analysis was performed using STATVIEW software (GraphPad Software, San Diego, CA, USA). Data were analysed using one-way analysis of variance (ANOVA). Results are presented as mean ± s.e.m. unless otherwise stated. Statistical evaluation of the data was carried out using one-way ANOVA when required and two-way ANOVA when potential interaction of two drugs was assessed. The ANOVA was followed with Fisher's post hoc analysis between control and treatment groups in cases where statistical significance was established ($p < 0.05$).

From stereology, the number of hits recorded for points falling over β-cells, non-β-cells, total pancreas tissue and other tissue (lymph nodes, fat, etc.) was transformed into volume fractions, which were subsequently recalculated into total cell mass in milligrams, and finally into relative cell mass in milligrams per kilogram. Area-weighted mean values were calculated from the two sections. Data accumulation and basic statistics were carried out in MS Excel. Data were presented as mean ± s.e.

Results

Energy Homeostasis

To assess therapeutic impact on energy homeostasis in adult male ZDF rats, food and water intake was measured frequently as was also body weight. As diabetes progresses in male ZDF rats, glucosuria becomes evident and resulting compensatory increase in water intake is noticed. Animals showing marked glucosuria loose large quantities of ingested energy through this route, and hence, their body weight gain progressively dampens as the condition deteriorates [13].

When assessing the drug effect on food intake, no interaction between liraglutide and pioglitazone was observed (two-way ANOVA: $F_{1,36} = 1.49$, $p = 0.2$). Thus, compared with vehicle-treated rats, liraglutide treatment with or without concomitant administration of pioglitazone significantly reduced daily as well as cumulated food intake in ZDF rats (figure 1). In contrast, pioglitazone-treated animals had a significant higher cumulated as well as 24-h food intake at days 14 and 41 when compared vehicle-treated animals. Body weight was measured once a week and expressed as body weight gain in percentage of day 0. Liraglutide and pioglitazone displayed significant interaction on body weight both after 14 and 41 days of dosing (two-way ANOVA: day 41 – $F_{1,36} = 43.95$, $p < 0.0001$). After 14 days of treatment, body weight gain was significantly lower in animals subjected to liraglutide–vehicle treatment ($p < 0.0001$) (figure 1). On day 41, these differences in body weight gain had not changed. After 41 days of treatment, the group of animals that were treated with a combination of liraglutide and pioglitazone had gained significantly more weight than the group treated with only pioglitazone

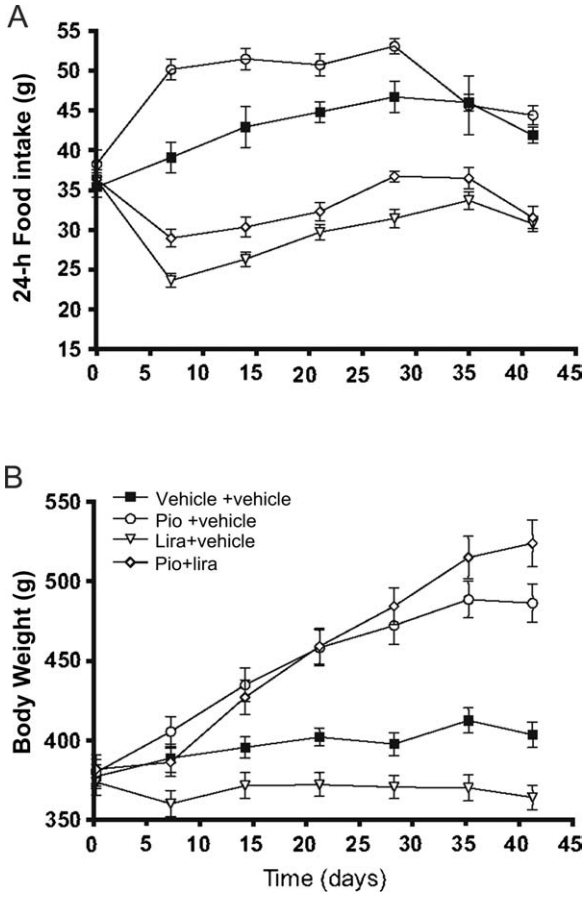

Fig. 1 Cumulated food intake (A) and resulting body weight in adult male Zucker diabetic fatty rats (B) treated with vehicle, liraglutide (Lira), pioglitazone (Pio) or a combination of liraglutide and pioglitazone (n = 10, all groups). Positive energy balance is seen for both pioglitazone monotherapy and combination of liraglutide and pioglitazone. The positive energy balance seen for the combination therapy is seen despite decreased food intake and hence likely to be a result of lessened glucosuria because of significant improvement of glycaemic control.

(137.2 ± 2 vs. 128.1 ± 2%) probably reflecting a marked improvement of metabolic control and hence fall in caloric wasting because of glucosuria.

Fat Depots

On the final day (day 42), animals were sacrificed and subcutaneous inguinal fat, epididymal fat and perirenal fat isolated and weighed. When compared with vehicle-treated rats, all three fat depots were larger in animals treated with pioglitazone ($p < 0.01$ for inguinal fat depot and 0.0001 for epididymal and perirenal fat) or in combination with liraglutide ($p < 0.0001$ for all three fat depots; table 1). Two-way ANOVA test confirmed that the two drugs displayed interaction at all depots (epididymal – $F_{1,36} = 9.3$, $p = 0.004$; inguinal – $F_{1,36} = 5.6$, $p = 0.03$; perirenal – $F_{1,36} = 4.5$, $p = 0.04$), with the two drugs in combination yielding the most prominent weight increase. In liraglutide only–treated rats, the fat depots were slightly smaller than in vehicle-treated rats, but only the decrease in epididymal fat mass reached statistical significance ($p < 0.05$ vs. vehicle).

Temporal Course of Plasma Glucose and Insulin

After 14 days, the combined pioglitazone–liraglutide treatment reduced plasma glucose significantly more than either of the two drugs alone (interaction two-way ANOVA: $F_{1,36} = 37.2$, $p < 0.0001$). A small but significant reduction of plasma glucose was seen in the liraglutide-treated group ($p < 0.05$) at day 14, whereas the pioglitazone alone had no impact on plasma glucose levels when compared with vehicle-treated animals (figure 2). The impressive effect of the pioglitazone–liraglutide combination therapy on morning plasma glucose levels persisted throughout the treatment period, whereas monotherapies neither with liraglutide nor with pioglitazone were efficacious in lowering plasma glucose levels at day 35. Likewise, combination therapy with liraglutide and pioglitazone was the only treatment paradigm that significantly elevated plasma insulin levels at day 35 (two-way ANOVA: $F_{1,36} = 11.1$, $p = 0.002$; figure 2).

Circadian Glycaemic Profile

At day 37, circadian profiles of plasma glucose and insulin were assessed by repeated sampling every 4 h. The resulting profiles are graphically presented in figure 3. Combination therapy with liraglutide and pioglitazone significantly reduced 24-h plasma glucose levels (two-way ANOVA: $F_{1,36} = 12.6$, $p = 0.001$), and also, monotherapy with liraglutide significantly improved circadian plasma glucose ($p < 0.05$ vs. vehicle). Pioglitazone treatment was not able to reduce 24-h plasma glucose profile at day 35. Plasma insulin levels were unaffected by any of the monotherapy regimes, whereas combination of liraglutide with pioglitazone significantly elevated the AUC obtained from the insulin profile ($p < 0.01$ vs. vehicle–vehicle, but without interaction; two-way ANOVA: $F_{1,36} = 3.2$, $p = 0.08$).

Oral Glucose Tolerance Test

On the day of termination of the experiment (day 42), rats were subjected to an OGTT in the semistarved state (figure 4). Combination therapy with liraglutide and pioglitazone significantly and synergistically lowered plasma glucose excursion during the OGTT ($p < 0.001$ vs. vehicle–vehicle; two-way ANOVA: $F_{1,36} = 11.2$, $p = 0.0019$), whereas liraglutide alone marginally improved oral glucose tolerance ($p < 0.05$ vs. vehicle). After 42 days of treatment, pioglitazone alone had no impact on oral glucose tolerance. Insulin release during the OGTT was also measured. Only combination therapy with liraglutide and pioglitazone significantly increased insulin secretion in otherwise relatively hypoinsulinaemic ZDF rats (AUC: 71.7 ± 2.9 vs. 44.5 ± 2.7 nmol/l × min, $p < 0.001$ vs. vehicle; figure 5). This effect was additive as no interaction could be detected (two-way ANOVA: $F_{1,36} = 2.01$, $p = 0.15$). However, sustainability of liraglutide-induced

Table 1 Fat depots at day 42

	Inguinal fat (g)	Epididymal fat (g)	Perirenal fat (g)
Vehicle 1 b.i.d. + vehicle 2 b.i.d.	6.3 ± 0.6	8.9 ± 0.2	17.4 ± 0.5
Pioglitazone 5 mg/kg b.i.d. + vehicle 2 b.i.d.	8.3 ± 0.6**	13.4 ± 0.6***	28.5 ± 1.4***
Liraglutide 200 μg/kg b.i.d. + vehicle 1 b.i.d.	5.0 ± 0.3	7.7 ± 0.3*	15.2 ± 0.7
Pioglitazone 5 mg/kg b.i.d. + liraglutide 200 μg/kg b.i.d.	9.5 ± 0.5***	14.9 ± 0.5***	30.1 ± 0.8***

ANOVA, analysis of variance.
*$p < 0.05$ vs. vehicle, ANOVA followed by Fisher's PLSD (protected least significant difference).
**$p < 0.01$ vs. vehicle, ANOVA followed by Fisher's PLSD.
***$p < 0.001$ vs. vehicle, ANOVA followed by Fisher's PLSD.

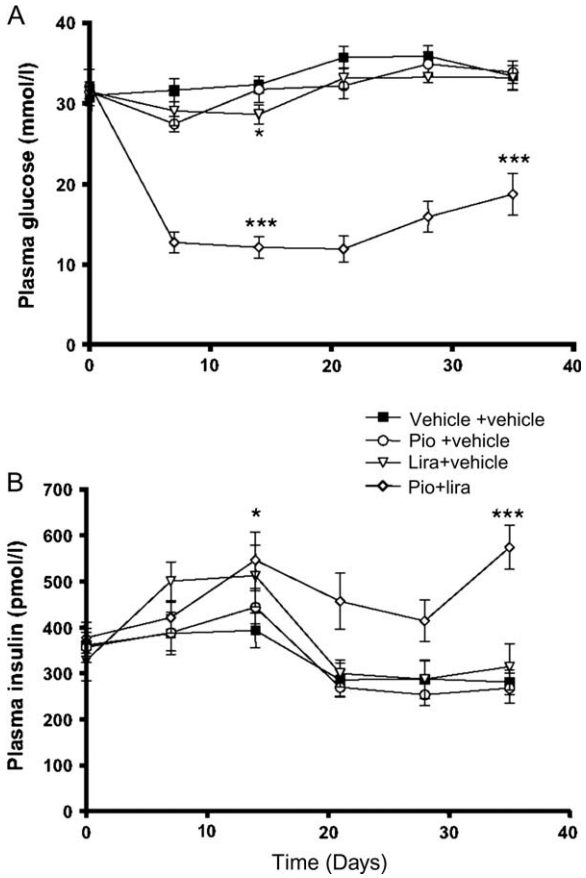

Fig. 2 Temporal course of (A) plasma glucose and (B) plasma insulin levels in adult male Zucker diabetic fatty (ZDF) rats treated for 42 days with vehicle, liraglutide (Lira), pioglitazone (Pio) or a combination of liraglutide and pioglitazone. Statistical analyses were carried out at days 14 and 35. Plasma glucose levels were markedly decreased in ZDF rats treated with a combination of liraglutide and pioglitazone (day 14: 12.1 ± 1.4 vs. 32.3 ± 1.0 mmol/l; day 35: 18.7 ± 2.6 vs. 33.4 ± 1.8 mmol/l, $p < 0.001$ liraglutide + pioglitazone vs. vehicle). On day 14, liraglutide treatment slightly improved glucose profile (28.6 ± 1.2 vs. 32.3 ± 1.0 mmol/l, $p < 0.05$ liraglutide vs. vehicle), whereas monotherapy with either liraglutide or pioglitazone was without impact day 35 plasma glucose profile. An inherent feature of impaired glycaemic control of ZDF rats is a gradual loss of β-cell function. However, combination therapy with liraglutide and pioglitazone improves β-cell secretory capacity as evidenced by maintained high plasma insulin levels (day 14: 393.3 ± 37.7 vs. 546 ± 0 pmol/l; day 35: 280.7 ± 27.3 vs. 573.9 ± 47.9 pmol/l, $p < 0.01$ vehicle vs. liraglutide + pioglitazone). Neither of the monotherapy regimes could halt failing of β-cell function. *$p < 0.05$, ***$p < 0.001$ vs. vehicle + vehicle.

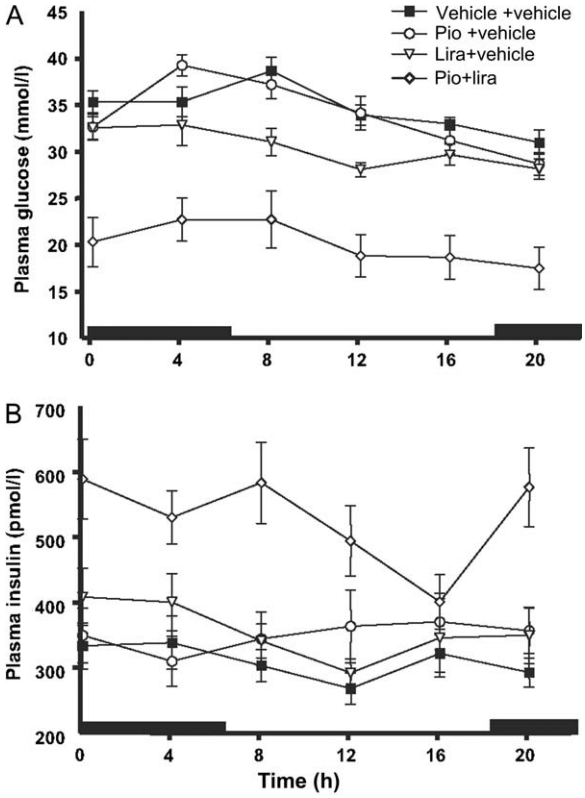

Fig. 3 Circadian profile of (A) plasma glucose and (B) plasma insulin in male Zucker diabetic fatty rats treated for 37 days with vehicle, liraglutide (Lira), pioglitazone (Pio) or a combination of liraglutide and pioglitazone. Measurements were made in freely fed animals. Twenty-four hours' plasma glucose levels were significantly lower in animals treated with a combination of liraglutide and pioglitazone [area under the curve (AUC): 407.3 ± 46.8 vs. 684.4 ± 19.2 mmol/l × min, $p < 0.001$ liraglutide + pioglitazone vs. vehicle]. Monotherapy with liraglutide also reduced 24-h plasma glucose levels (AUC: 605.6 ± 15.9 vs. 684.4 ± 19.2 mmol/l × min, $p < 0.05$ liraglutide vs. vehicle). Plasma insulin levels were significantly elevated in animals treated with a combination of liraglutide and pioglitazone (AUC: 10.3 ± 0.9 vs. 6.3 ± 0.5 pmol/l × min, $p < 0.01$ liraglutide + pioglitazone vs. vehicle), whereas neither of the monotherapies improved 24-h insulin secretion.

insulin secretion in ZDF rats challenged with an oral glucose load was synergistically affected by pioglitazone. Thus, 180 min after application of the glucose load, significant interaction between pioglitazone and liraglutide was observed (two-way ANOVA: $F_{1,36} = 4.4$, $p = 0.04$).

Glycated Haemoglobin

On day 28, HbA_{1C} was measured in all groups and was significantly decreased from 9.7% in vehicle-treated

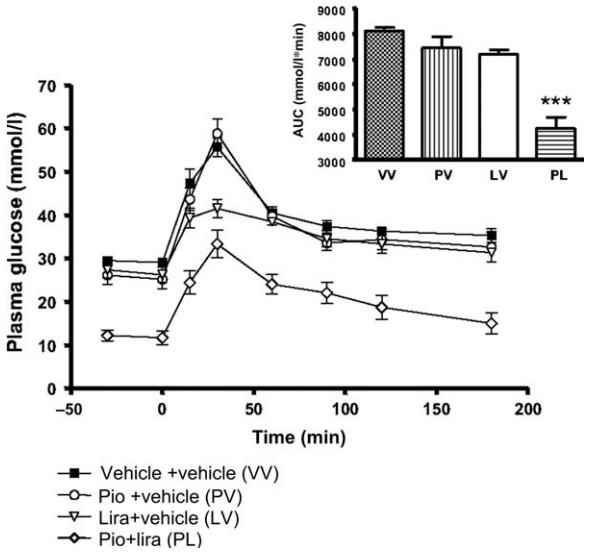

Fig. 4 Oral glucose tolerance test in male Zucker diabetic fatty rats was carried out after 42 days of treatment with vehicle, liraglutide, pioglitazone or a combination of liraglutide (Lira) and pioglitazone (Pio). Plasma glucose excursion was markedly reduced in animals treated with a combination of liraglutide and pioglitazone (area under the curve (AUC): 4244.0 ± 445 vs. 8093 ± 139 mmol/l × min, ***$p < 0.001$ liraglutide + pioglitazone vs. vehicle).

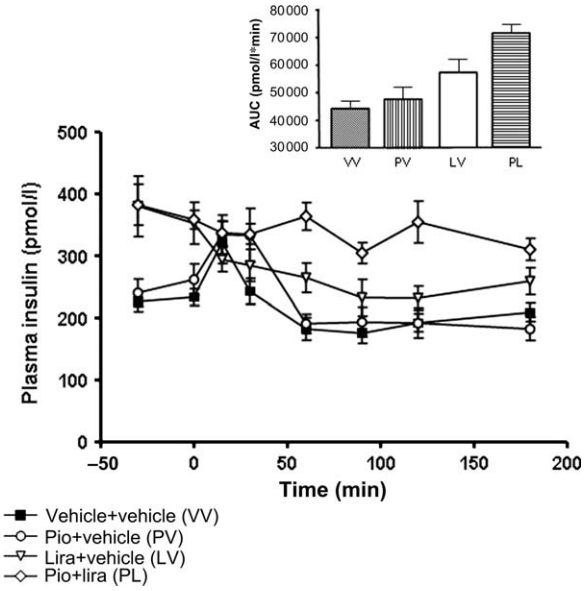

Fig. 5 Oral glucose tolerance test in male Zucker diabetic fatty (ZDF) rats was carried out after 42 days of treatment with vehicle, liraglutide (Lira), pioglitazone (Pio) or a combination of liraglutide and pioglitazone. Plasma insulin levels are shown from 30 min prior to 180 min after administration of the glucose load, and integrated areas under the curve (AUC) are shown in the upper right panel. Plasma insulin levels in ZDF rats treated with liraglutide are significantly higher than in rats treated with subcutaneous saline injections ($p < 0.001$). In ZDF rats treated with a combination of liraglutide and pioglitazone, the higher levels of plasma insulin levels display borderline interaction (two-way analysis of variance: $F_{1,36} = 3.2$, $p = 0.08$).

animals to 7.9% in animals treated with pioglitazone–vehicle ($p < 0.01$) and to 4.8% in animals treated with pioglitazone–liraglutide ($p < 0.001$; table 2), while liraglutide-treated rats displayed an insignificant drop to 8.8% after 28 days of treatment. The combined effect of liraglutide and pioglitazone was synergistic (two-way ANOVA; $F_{1,36} = 6.4$, $p = 0.016$).

Blood Biochemistry

On the day before termination of the experiment (at t_{-30} relative to oral glucose challenge), semifasting plasma levels of glucose, insulin, NEFA, total cholesterol and TG were measured (table 2).

Combination therapy with liraglutide and pioglitazone synergistically lowered plasma glucose levels (two-way ANOVA: $F_{1,36} = 13.7$, $p < 0.001$) compared with vehicle-treated animals.

Compared with vehicle- or pioglitazone-treated animals, baseline plasma insulin concentrations were markedly increased in all groups treated with liraglutide ($p < 0.01$). No synergy was seen between liraglutide and pioglitazone on semifasting plasma insulin levels.

Compared with vehicle-treated animals, plasma levels of TG and FFA were significantly reduced in liraglutide-treated animals ($p < 0.001$ for liraglutide alone as well as for liraglutide in combination with pioglitazone).

Pancreas Histology

In vehicle-treated rats as well as in animals on monotherapy, islets were irregular with fibrotic streaks, and most β-cells stained poorly for insulin, while images of non-β-cells were normal (figure 6). Rats treated with the combination of liraglutide and pioglitazone displayed more compact islets with less fibrosis and higher proportion of well-staining β-cells. However, formal stereological assessment of β-cell mass did not show differences between the groups (liraglutide + pioglitazone: 5.9 ± 0.7 mg; liraglutide: 6.2 ± 0.7 mg; pioglitazone: 5.2 ± 0.5 mg; vehicle: 5.3 ± 0.6 mg) nor did the non–β-cell mass (liraglutide + pioglitazone: 3.0 ± 0.4 mg; liraglutide: 3.0 ± 0.1 mg; pioglitazone: 2.8 ± 0.3 mg; vehicle: 2.8 ± 0.2 mg).

Table 2 Blood biochemistry at day 42 (HbA_{1c} at day 28)

	Vehicle 1 b.i.d. + vehicle 2 b.i.d.	Pioglitazone 5 mg/kg b.i.d. + vehicle 2 b.i.d.	Liraglutide 200 μg/kg b.i.d. + vehicle 1 b.i.d.	Pioglitazone 5 mg/kg b.i.d. + liraglutide 200 μg/kg b.i.d.
P-glucose at t_{-30} (mmol/l)	29.5 ± 0.9	26.2 ± 2.1	27.4 ± 1.8	12.2 ± 1.3***
P-insulin at t_{-30} (mmol/l)	226.3 ± 16.2	240.5 ± 22.1	380.1 ± 49.0**	382.4 ± 33.0**
HbA_{1c} at day 28 (%)	9.7 ± 0.3	7.9 ± 0.4**	8.8 ± 0.6	4.8 ± 0.3***
P-TG (mmol/l)	7.1 ± 0.4	6.7 ± 0.8	4.0 ± 0.6**	2.8 ± 0.4***
P-cholesterol at day 15 (mmol/l)	4.5 ± 0.3	5.2 ± 0.3*	4.1 ± 0.2	4.4 ± 0.1
P-FFA at day 15 (mmol/l)	0.81 ± 0.05	0.76 ± 0.05	0.58 ± 0.05**	0.43 ± 0.06***

ANOVA, analysis of variance; FFA, free fatty acids; HbA_{1c}, glycated haemoglobin; TG, triacylglycerol.
*$p < 0.05$ vs. vehicle, ANOVA followed by Fisher's PLSD.
**$p < 0.01$ vs. vehicle, ANOVA followed by Fisher's PLSD.
***$p < 0.001$ vs. vehicle, ANOVA followed by Fisher's PLSD.

The baseline rats sacrificed at the initiation of the treatment period displayed similar islet morphology and similar β- and non–β-cell mass as the vehicle-treated rats (β-cells: 5.5 ± 0.7 mg; non-β-cells: 2.3 ± 0.3 mg), suggesting that β-cell population did not deteriorate throughout the 42-day course of the study.

Discussion

To further scrutinize the glucose-lowering therapeutic potential combining a GLP-1 receptor agonist with a thiazolidinedione, we have used the once-daily human GLP-1 analog, liraglutide, as an insulinotropic agent and the clinically well-proven PPARγ agonist, pioglitazone, as insulin sensitizer [14,15]. As an animal model for human type 2 diabetes, we have used severely insulin-resistant ZDF rats, which by many investigators are considered an adequate proxy for treatment-resistant human type 2 diabetes [13]. The onset of diabetes in ZDF occurs at 6–7 weeks of age, and when antidiabetic treatment with either GLP-1 analog or PPARγ agonists is initiated at this point in life, progression rate of diabetes

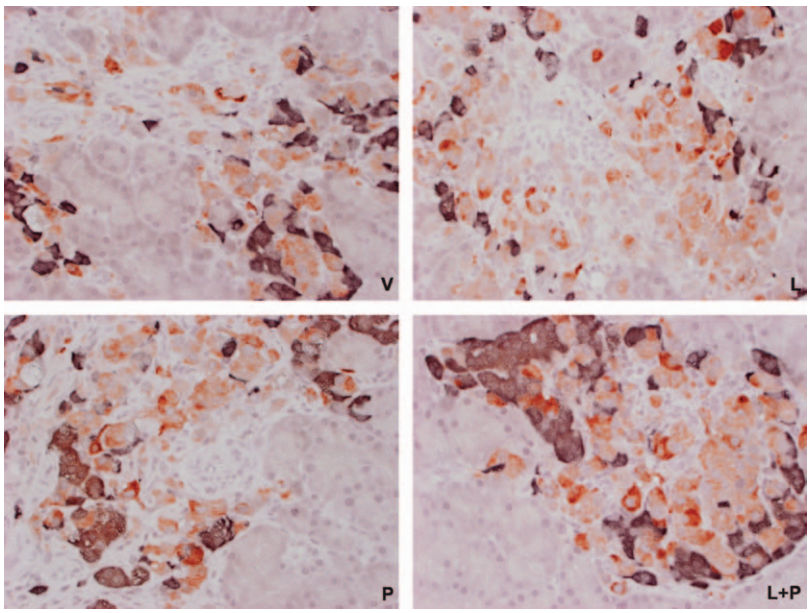

Fig. 6 Microphotographs of pancreatic islets obtained from male Zucker diabetic fatty rats treated for 42 days with vehicle, liraglutide (L), pioglitazone (P) or a combination of liraglutide and pioglitazone (L + P). Immunohistochemical staining of pancreatic islet was immunoreacted with antibodies directed against insulin (brown) or a mixture of glucagon, somatostatin and pancreatic polypeptide (black). In all sections, β-cells are irregular with a degranulated appearance, while non-β-cells display normal morphology. Despite an apparent increase in insulin immunoreactivity in animals treated with liraglutide + pioglitazone, stereological assessment of β-cell mass showed no significant differences between the four groups.

becomes markedly dampened by such early intervention [16–21]. However, earlier experience with the use of older, overtly diabetic ZDF rats in various therapeutic regimes is in line with our current observations, showing that neither of the monotherapy regimes efficaciously improved glycaemic control after 42 days of treatment [18,19,22]. In the present study, all groups had clearly elevated blood glucose (BG) and insulin levels as well as similar β-cell mass. In spite of this, significant lower BG and higher plasma insulin levels were found in the group treated with the combination of liraglutide and pioglitazone compared with groups treated with monotherapy or vehicle. Compared with vehicle and pioglitazone, basal insulin levels were initially slightly elevated in liraglutide-treated ZDF rats, but this effect waned after 2 weeks of treatment, whereafter, only rats in combination therapy displayed elevated insulin levels, suggesting that concomitant improvement of insulin action conserve insulinotropic activity of liraglutide. The underlying mechanism of this particularly advantageous combination is at present unknown, but both liraglutide and pioglitazone have several other beneficial effects in addition to their respective main insulinotropic and insulin-sensitizing effects.

The GLP-1 analog, exenatide, has a half-life of 3.3–4 h after subcutaneous injection in humans, with yielding pharmacodynamic effects lasting for about 12–16 h, when the drug is administered b.i.d. With a twice-daily dosing regime, exenatide is pharmacologically active for approximately 12–16 h. The most striking difference between exenatide and DPP-IV inhibitors is the absence on body weight of the latter most likely explained by their modest impact on plasma GLP-1. Liraglutide is an analog of GLP-1, sharing 97% homology with natural GLP-1. Liraglutide has a subcutaneous half-life of 11–15 h [23], resulting in a lasting 24-h pharmacodynamic profile on once-daily administration [24]. When applying optimally tolerated doses of liraglutide (1.9 mg/day), 14 weeks of therapy lowers HbA_{1C} by 1.74% (12). Similarly, the long-acting formulation of exenatide is significantly more efficacious than the twice-daily injection regime of exenatide, as HbA_{1C} is lowered by 1.7% [25].

Therapeutic strategies addressing both insulin sensitivity and insulin secretion are appealing as a large proportion of subjects with type 2 diabetes are in need of more efficacious therapies if HbA_{1C} is to be reduced to a target of less than 7%. Additional glycaemic control is obtained more efficaciously when combing a long-acting GLP-1 analog with a TZD than seen for the combination of a TZD and a DPP-IV inhibitor [11,26,27]. The maximal antihyperglycaemic efficacy of DPP-IV inhibitors rely on their capability to fully exploit endogenous GLP-1, whereas exogenous GLP-1 analogs can be applied in pharmacological doses and are limited largely by their potential side effects. Compared with normoglycaemic control subjects, meal-induced release of endogenous GLP-1 is diminished in people with type 2 diabetes [28]. People with type 2 diabetes have less than optimal meal-induced GLP-1 secretion to protect against degradation by DPP-IV inhibitors, rendering therapy with GLP-1 analogs, which essentially shortcut the L-cells more likely to exert better efficacy than what can be obtained by full exploitation of body's own GLP-1 source.

The hyperphagia of ZDF rats is a result of dysfunctional leptin signalling as well as caloric wasting because of marked glucosuria [13]. Clearly, liraglutide in monotherapy dampened the hyperphagia resulting from impaired leptin receptor signalling, but it was also capable of reducing the increased feeding response in pioglitazone-treated animals. It is well known that pioglitazone transiently increases food consumption in rats [21,29], and as it occurred together with a slight improvement of glycaemic control, it is likely to cause even larger caloric load of the currently examined ZDF rats. Liraglutide significantly counteracted the pioglitazone-induced hyperphagia, but it is uncertain whether this effect was simply the consequence of improving glycaemic control and hence reducing hyperphagia induced by caloric loss or whether liraglutide can actually neutralize pioglitazone-induced feeding behaviour. A thorough study in animals not affected by caloric wasting diuresis would be required to further scrutinize the impact of liraglutide on pioglitazone-induced feeding, but it is tempting to speculate that people with type 2 diabetes would benefit from such combination therapy as a common side effect of pioglitazone therapy is weight gain [15]. Thus, part of the impressive synergistic effect on glycaemic control seen when combining liraglutide with pioglitazone may be because of the anorectic function of liraglutide. It is well known that pharmacologically induced reduction of caloric consumption in people type 2 diabetes is accompanied by improved glycaemic control [30,31]. Thus, the GLP-1 analogs appear ideally suited for combination therapy with insulin sensitizers as this combination addresses two independent modes of action improving glycaemic control (insulin release and action) and on top of it comes decreased caloric intake, which in its own right improves glycaemic control.

Experience with combination of DPP-IV inhibitors and pioglitazone in rats is limited to short-ranging treatment periods of insulin-resistant, obese Zucker rats [32]. Thus, 10 days of treatment with a combination of vildagliptin

and pioglitazone significantly and synergistically improved plasma clearance of glucose during an OGTT [32]. However, it is impossible to extrapolate what 4 weeks of treatment would have accomplished and also the fact that the study was performed in non-diabetic rats renders a direct comparison with our data impossible. Considering the clinical evidence gathered so far, it seems unlikely that combination of DPP-IV inhibitors and pioglitazone will improve glycaemic control synergistically as seen for liraglutide and TZDs. In subjects with type 2 diabetes, 26 weeks of combination therapy with pioglitazone (30 mg/day) and vildagliptin (100 mg/day) is more efficacious than either drug alone, but the combined effect is less than additive, suggesting that more optimal combinations should be sought [33].

Histological examinations of pancreata obtained from ZDF rats treated with GLP-1 analogs as well as DPP-IV inhibitors have shown that early onset of therapy in a preventive mode can halt the progressive loss of β-cells otherwise characterizing this animal model [12]. In the present study, an impressive improvement of glycaemic control was not accompanied by increased β-cell mass, although circulating plasma insulin levels was markedly elevated in animals on combination therapy. Thus, it was quite obvious that initiation of subchronic antidiabetic treatment with a GLP-1 analog either alone or in combination with pioglitazone was incapable of altering the number of β-cells. However, a significant synergy between liraglutide and pioglitazone was observed with respect to sustainability of insulin secretion, suggesting that β-cells of ZDF rats exposed to this combination therapy maintain a much improved secretory capacity. Although this will have to be verified rigorously by an arginine challenge or by a similar model, there is solid clinical evidence that PPARγ agonists improve β-cell function [34]. Loss of β-cells is a salient feature of ZDF rats, and it remains to be proven that pharmacotherapeutic intervention can actually restore β-cells already lost. A number of *in vitro* studies have shown that GLP-1 treatment directly affects β-cell apoptosis, suggesting lesser decay of secretory β-cell capacity after prolonged GLP-1 receptor activation [35]. However, *in vivo* studies of β-cell protective and/or growth-promoting effects of GLP-1 receptor stimulation have been difficult to interpret as they invariably include improvement of glycaemic control. Hence, it is difficult to ascertain whether improved β-cell function is a direct or an indirect consequence of GLP-1 receptor stimulation. Thus, some scientific reports claim to have observed GLP-1-mediated β-cell regeneration, but as none of the studies has been able to separate indirect impact of improved glycaemic control from direct trophic effects, it is still speculative whether GLP-1 analogs can actually bring more β-cells to a diseased pancreas typically seen at late stages of type 2 diabetes [36]. Circumstantial evidence gathered from few patients suffering severe hypoglycaemic crises subsequent to gastric bypass surgery has linked abnormal GLP-1 secretion to their insulinomas, thereby suggesting a causative link between GLP-1 and pancreatic β-cell growth also in humans [37].

In conclusion, we have shown that combination of otherwise subefficacious doses of a GLP-1 receptor agonist and an insulin sensitizer markedly improves glycaemic control above and beyond what could be expected from monotherapy with either agent. It seems prudent to suggest that this treatment modality offers interesting alternative to insulin, which is otherwise considered last resort in severely insulin-resistant subjects with type 2 diabetes.

References

1 Fonseca V, Bakris GL, Benjamin EM *et al.* Clinical practice recommendations. Diabetes Care 2005; **28** (Suppl. 1). 8–15.

2 Sarlio-Lahteenkorva S, Rissanen A, Kaprio J. A descriptive study of weight loss maintenance: 6 and 15 year follow-up of initially overweight adults. Int J Obes Relat Metab Disord 2000; **24:** 116–125.

3 Groop LC. Sulfonylureas in NIDDM. Diabetes Care 1992; **15:** 737–754.

4 Rorsman P. The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. Diabetologia 1997; **40:** 487–495.

5 Ball AJ, Flatt PR, McClenaghan NH. Desensitization of sulphonylurea- and nutrient-induced insulin secretion following prolonged treatment with glibenclamide. Eur J Pharmacol 2000; **408:** 327–333.

6 Holst JJ. Glucagon-like peptide-1: from extract to agent. The Claude Bernard Lecture, 2005. Diabetologia 2006; **49:** 253–260.

7 Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 2006; **368:** 1696–1705.

8 Green BD, Flatt PR, Bailey CJ. Dipeptidyl peptidase IV (DPP IV) inhibitors: a newly emerging drug class for the treatment of type 2 diabetes. Diab Vasc Dis Res 2006; **3:** 159–165.

9 Bonner-Weir S, Weir GC. New sources of pancreatic beta-cells. Nat Biotechnol 2005; **23:** 857–861.

10 Berger JP, Akiyama TE, Meinke PT. PPARs: therapeutic targets for metabolic disease. Trends Pharmacol Sci 2005; **26:** 244–251.

11 Garber AJ, Schweizer A, Baron MA, Rochotte E, Dejager S. Vildagliptin in combination with pioglitazone improves

glycaemic control in patients with type 2 diabetes failing thiazolidinedione monotherapy: a randomized, placebo-controlled study*. Diabetes Obes Metab 2007; **9:** 166–174.
12 Vilsbøll T, Zdravkovic M, Le-Thi T *et al.* Liraglutide significantly improves glycemic control, and lowers body weight without risk of either major or minor hypoglycemic episodes in subjects with type 2 diabetes. Diabetes 2006; **55** (Suppl. 1): A27.
13 Etgen GJ, Oldham BA. Profiling of Zucker diabetic fatty rats in their progression to the overt diabetic state. Metabolism 2000; **49:** 684–688.
14 Joy SV, Rodgers PT, Scates AC. Incretin mimetics as emerging treatments for type 2 diabetes. Ann Pharmacother 2005; **39:** 110–118.
15 Waugh J, Keating GM, Plosker GL, Easthope S, Robinson DM. Pioglitazone: a review of its use in type 2 diabetes mellitus. Drugs 2006; **66:** 85–109.
16 Young AA, Gedulin BR, Bhavsar S *et al.* Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). Diabetes 1999; **48:** 1026–1034.
17 Smith SA, Lister CA, Toseland CD, Buckingham RE. Rosiglitazone prevents the onset of hyperglycaemia and proteinuria in the Zucker diabetic fatty rat. Diabetes Obes Metab 2000; **2:** 363–372.
18 Sturis J, Gotfredsen CF, Romer J *et al.* GLP-1 derivative liraglutide in rats with beta-cell deficiencies: influence of metabolic state on beta-cell mass dynamics. Br J Pharmacol 2003; **140:** 123–132.
19 Brand CL, Sturis J, Gotfredsen CF *et al.* Dual PPARalpha/gamma activation provides enhanced improvement of insulin sensitivity and glycemic control in ZDF rats. Am J Physiol Endocrinol Metab 2003; **284:** E841–E854.
20 Gedulin BR, Smith P, Prickett KS *et al.* Dose-response for glycaemic and metabolic changes 28 days after single injection of long-acting release exenatide in diabetic fatty Zucker rats. Diabetologia 2005; **48:** 1380–1385.
21 Pickavance LC, Brand CL, Wassermann K, Wilding JP. The dual PPARalpha/gamma agonist, ragaglitazar, improves insulin sensitivity and metabolic profile equally with pioglitazone in diabetic and dietary obese ZDF rats. Br J Pharmacol 2005; **144:** 308–316.
22 Wargent E, Stocker C, Augstein P *et al.* Improvement of glucose tolerance in Zucker diabetic fatty rats by long-term treatment with the dipeptidyl peptidase inhibitor P32/98: comparison with and combination with rosiglitazone. Diabetes Obes Metab 2005; **7:** 170–181.
23 Agersø H, Jensen LB, Elbrond B, Rolan P, Zdravkovic M. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. Diabetologia 2002; **45:** 195–202.
24 Degn KB, Juhl CB, Sturis J *et al.* One week's treatment with the long-acting glucagon-like peptide 1 derivative liraglutide (NN2211) markedly improves 24-h glycemia and alpha- and beta-cell function and reduces endogenous glucose release in patients with type 2 diabetes. Diabetes 2004; **53:** 1187–1194.
25 Kim D, MacConell L, Zhuang D *et al.* Effects of once-weekly dosing of a long-acting release formulation of exenatide on glucose control and body weight in subjects with type 2 diabetes. Diabetes Care 2007; **30:** 1487–1493.
26 Rosenstock J, Brazg R, Andryuk PJ, McCrary Sisk C, Lu K, Stein P. Addition of sitagliptin to pioglitazone improved glycaemic control with neutral weight effect over 24 weeks in inadequately controlled type 2 diabetes (T2DM). Diabetes 2006; **55:** A132.
27 Zinman B, Hoogwerf B, Garcia SD *et al.* Safety and efficacy of exenatide in patients with type 2 diabetes mellitus using thiazolidinediones with or without metformin. Diabetes 2006; **55:** A28.
28 Vilsboll T, Krarup T, Sonne J *et al.* Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. J Clin Endocrinol Metab 2003; **88:** 2706–2713.
29 Larsen PJ, Jensen PB, Sorensen RV *et al.* Differential influences of peroxisome proliferator-activated receptors{gamma} and -{alpha} on food intake and energy homeostasis. Diabetes 2003; **52:** 2249–2259.
30 Scheen AJ, Finer N, Hollander P, Jensen MD, Van Gaal LF. Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. Lancet 2006; **368:** 1660–1672.
31 Rowe R, Cowx M, Poole C, McEwan P, Morgan C, Walker M. The effects of orlistat in patients with diabetes: improvement in glycaemic control and weight loss. Curr Med Res Opin 2005; **21:** 1885–1890.
32 Burkey BF, Li X, Bolognese L *et al.* Combination treatment of a DPP-IV inhibitor NVP-LAF237 with pioglitazone completely normalized glucose tolerance in adult obese Zucker rats. Diabetes 2002; **51:** A338.
33 Rosenstock J, Baron MA, Camisasca RP, Cressier F, Couturier A, Dejager S. Efficacy and tolerability of initial combination therapy with vildagliptin and pioglitazone compared with component monotherapy in patients with type 2 diabetes. Diabetes Obes Metab 2007; **9:** 175–185.
34 Wajchenberg BL. Beta-cell failure in diabetes and preservation by clinical treatment. Endocr Rev 2007; **28:** 187–218.
35 Li L, El-Kholy W, Rhodes CJ, Brubaker PL. Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. Diabetologia 2005; **48:** 1339–1349.
36 Brubaker PL, Drucker DJ. Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. Endocrinology 2004; **145:** 2653–2659.
37 Patti ME, McMahon G, Mun EC *et al.* Severe hypoglycaemia post-gastric bypass requiring partial pancreatectomy: evidence for inappropriate insulin secretion and pancreatic islet hyperplasia. Diabetologia 2005; **48:** 2236–2240.