Effects of the dual PPAR-α/γ agonist aleglitazar on glycaemic control and organ protection in the Zucker diabetic fatty rat
A. Bénardeau¹, P. Verry¹, E.-A. Atzpodien², J. M. Funk², M. Meyer¹, J. Mizrahi¹, M. Winter², M. B. Wright¹, S. Uhles¹ & E. Sebokova¹

¹pRED, Pharma Research & Early Development, DTA Cardiovascular & Metabolism, F. Hoffmann-La Roche AG, Basel, Switzerland
²pRED, Non-Clinical Safety, F. Hoffmann-La Roche AG, Basel, Switzerland

Aims: To evaluate the effects of aleglitazar, a dual peroxisome proliferator-activated receptor-α/γ agonist, on the development of diabetes-related organ dysfunction, in relation to glycaemic and lipid changes, in Zucker diabetic fatty (ZDF) rats.

Methods: Six-week-old, male ZDF rats received aleglitazar 0.3mg/kg/day or vehicle as food admix for 13 weeks (n = 10 per group). Age-matched male Zucker lean rats served as non-diabetic controls. Plasma and renal markers were measured at several time points. Histopathology and quantitative immunohistochemistry were performed at 13 weeks.

Results: Glycated haemoglobin (5.4 vs. 9.2%) and blood glucose (8.3 ± 0.3 vs. 26.1 ± 1.0 mmol/l) were significantly reduced at 12 weeks with aleglitazar versus vehicle-treated ZDF rats (both p < 0.01), while aleglitazar preserved near-normal plasma insulin levels. Aleglitazar prevented the development of hypertriglyceridaemia (1.4 ± 0.1 vs. 8.5 ± 0.9 mmol/l) and reduced plasma non-esterified fatty acids (0.09 ± 0.02 vs. 0.26 ± 0.04 mmol/l) relative to vehicle-treated animals (both p < 0.01). Urinary glucose and protein concentrations were significantly reduced at 13 weeks with aleglitazar versus vehicle-treated rats (both p < 0.01). Consistent with its effect on glycaemic control, aleglitazar protected β-cell morphology, as evidenced by preservation of islet integrity, and reduction of β-cell apoptosis and islet fibrosis. Aleglitazar prevented renal glomerular hypertrophy, podocyte degeneration, glomerulosclerosis, tubulo-interstitial lesions and development of cataracts.

Conclusions: Aleglitazar strongly improved glycaemic and lipid parameters while protecting key tissues, including the pancreas, kidneys and eyes, against diabetes-associated structural and functional changes in the ZDF rat.

Keywords: β cell, diabetes complications, dual PPAR-α and -γ agonist, Zucker diabetic fatty rat

Introduction
Landmark studies emphasize the need for comprehensive management of multiple cardiovascular (CV) risk factors in patients with type 2 diabetes mellitus (T2DM) to reduce CV morbidity and mortality and microvascular complications [1]. While agonists of peroxisome proliferator-activated receptor (PPAR)-γ (thiazolidinediones) provide effective glycaemic control, and thus may reduce microvascular complications, only pioglitazone in the PROactive trial showed a reduction in CV events, as reflected by a reduction in the composite (secondary) end point of all-cause mortality, non-fatal myocardial infarction and stroke [2]. PPAR-α agonists (fibrates) lower triglycerides (TG) and increase high-density lipoprotein cholesterol (HDL-C) [3], although their effects to reduce risk for CV events have been modest [4].

Dual-selective agonists of PPAR-α and -γ are of interest as they may combine the glucose-lowering and lipid-modifying properties of both agents. Aleglitazar is a balanced dual PPAR-α/γ agonist designed to optimize effects on glucose and lipids. In diabetic rodent models, aleglitazar significantly improved insulin sensitivity (Zucker fa/fa rats and db/db mice) and improved plasma lipids (human apolipoprotein A1 transgenic mice) [5]. In a Rhesus monkey model of T2DM, aleglitazar more than doubled levels of HDL-C and significantly reduced both low-density lipoprotein cholesterol (LDL-C) and TG, while lowering glucose and glycated haemoglobin (HbA1c) [6]. In a phase II dose-range-finding study (SYNCHRONY) in T2DM patients, aleglitazar dose-dependently improved the lipid profile (TG, LDL-C and HDL-C) and decreased glucose (HbA1c and fasting plasma glucose), as well as reduced other markers of CV risk, such as plasminogen activator inhibitor-1, fibrinogen and C-reactive protein [7].

Zucker diabetic fatty (ZDF) rat is a useful animal model of T2DM disease progression and end-organ dysfunction [8–10]. Insulin resistance and slightly elevated plasma glucose are typically evident by 7 weeks of age, with hyperinsulinaemia occurring between weeks 7 and 10. Insulin levels eventually decline, due to β-cell loss [11], with overt diabetes developing by 12 weeks. The disease course in the ZDF rat has similarities...
to T2DM in humans, including declining renal function (glomerular and tubulo-interstitial alterations with proteinuria and glucosuria) [10], presence of neuropathy (decreases in peripheral nerve conduction and alterations in sensory parameters) [12] and retinopathy (retinal vascular changes and cataracts) [13].

The therapeutic effects of thiazolidinediones and fibrates have been previously studied in the ZDF rat. Rosiglitazone was shown to improve insulin sensitivity and glucose control, leading to preservation of pancreatic β-cell mass and insulin content [14,15]. Rosiglitazone had protective effects on kidney function, resulting in significantly reduced proteinuria and improvements in glomerular and tubular morphology – effects that were superior to the angiotensin-converting enzyme inhibitor cilazapril [16]. Pioglitazone has also been shown to reduce proteinuria in ZDF rats because of glucose-dependent and -independent mechanisms. Pioglitazone reduced glomerulosclerosis by reducing podocyte activation and endothelial injury [17,18]. Effects were evident despite minor reductions in glucose, suggesting they may occur via anti-fibrotic PPAR-dependent mechanisms directly in the kidney, where PPAR-γ is highly expressed [19].

Fibrate PPAR-α agonists have also been evaluated in ZDF rats. Fenofibrate reduced systolic blood pressure, and attenuated glomerular hypertrophy and fibrosis [20]. Fenofibrate also reduced tubular injury, possibly by reducing interstitial macrophages in the kidney, leading to suppression of B and transforming growth factor-β1/Smad3 signalling [21].

In this study, we investigated effects of the dual PPAR-α/γ agonist aleglitazar on glycaemic control and prevention of diabetes-related organ dysfunction in ZDF rats, employing quantitative immunohistochemistry with accepted and emerging structural and functional markers.

Materials and Methods

Animals

Five-week-old male ZDF rats (ZDF fa/fa or ZDF) and lean, aged-matched male Zucker rats (ZDF +/fa or lean [ZL]) from Charles River Laboratories (Wilmington, MA, USA) were fed a diabetogenic diet (KLIBA 2437; Provimi KLIBA AG, Kaiseraugst, Switzerland) immediately after weaning. The animal study was performed under an animal research protocol (ARP) authorization to F. Hoffmann-La Roche by the Swiss Cantonal Veterinary Office Basel-Stadt. Animals were housed individually under standard hygienic conditions in an AAALACi-accredited facility with controlled temperature and humidity, a 12 h/12 h light/dark cycle, and full access to food and water.

Study Design and Blood/Urine Sampling

Rats were 6 weeks old when treatment commenced. Animals received aleglitazar (0.3 mg/kg/day; n = 10; Roche, Basel, Switzerland) as food admix or vehicle (vehicle; n = 10) for 13 weeks. For each treatment group, 50 kg of food (KLIBA 2437) was homogenized in 101 of vehicle buffer solution (0.89% di-sodium hydrogenphosphate-dihydrate; 0.4% polysorbate 80, 0.29% 1 N HCl in sterile water), with or without aleglitazar, and the fluid phase was carefully evaporated. Final food was prepared in pellets by KLIBA. Average daily food consumption was 100 g/kg body weight during treatment (confirmed by recording of daily food intake), delivering aleglitazar at a daily dose of 0.3 mg/kg/day. The potential food aversion risk was explored prior to starting chronic treatment by monitoring food and water intake and body weight on diet over a 1-week period. No food aversion behaviour was observed. Pharmacokinetic analysis confirmed that the expected plasma exposure of aleglitazar was achieved (data not shown). Age-matched male ZL rats (n = 10) received vehicle and served as non-diabetic controls.

Retro-orbital blood (200 μl of blood with EDTA) was taken from anaesthetized rats (isoflurane) into tubes coated with EDTA, containing 1% aprotinin (to inhibit protein degradation), and kept on ice. Animals were carefully monitored both before and after blood sampling and no signs of distress or complications were observed. Tubes were then centrifuged (9279 g for 15 min at 4 °C) and plasma was aliquoted in different tubes or 96-well plates for analysis. Blood samples were taken under postprandial conditions in the morning 2 h following switch from dark to light cycle to assess glucose, HbA1c, insulin, TG, non-esterified fatty acids (NEFA), total cholesterol (TC) and adiponectin at several time points under non-fasting conditions. Glucose and HbA1c were measured in whole blood, while plasma was used for analysis of insulin, TG, NEFA, TC and adiponectin. Urine samples were taken after 5 weeks of treatment and at the end of the treatment period (13 weeks); five representative animals per group according to similar body weight and plasma parameters were placed individually into metabolic cages, without access to food but with free access to water, for 16–17 h urine collection using ice-refrigerated tubes.

An oral glucose tolerance test (OGTT) was carried out at 12 weeks in overnight-fasted animals, with measurements taken at 15, 30, 60 and 120 min after glucose challenge (1 g/kg). Homeostasis model assessment of insulin resistance (HOMA-IR) and β-cell function (HOMA-B) were estimated after overnight fasting and prior to performing OGTT using the following calculations: HOMA-IR = fasting insulin (ng/ml)/0.04 × fasting blood glucose (mM)/22.5; HOMA-B = 20 × fasting insulin (ng/ml)/0.04/fasting blood glucose (mM) – 3.5 [22].

Blood glucose levels were measured using a glucometer (GlucoTrend®, Roche Diagnostics, Burgdorf, Switzerland). Plasma concentrations of insulin (Rat Insulin, Mercodia, Uppsala, Sweden) and adiponectin (Mouse/Rat ELISA kit, K1002-1, B-Bridge, Cupertino, CA, USA) were quantified by ELISA. Metabolic plasma profiles were established by the Hitachi system using a fluorometric method: TC, TG, NEFA, HbA1c/Hb (turbimetric assay). HbA1c (%) was calculated as follows: 87.6 × (HbA1c/Hb) + 2.27 [23].

Histopathology and Immunohistochemistry

Tissue samples were fixed in 10% neutral-buffered formalin for at least 24 h. Eyes were fixed in modified Davidson’s fixative consisting of 13.3% ethyl alcohol, 6.3% glacial acetic acid and
13.9% formalin solution in distilled water, for at least 48 h. For histopathology examination, organs were embedded in Paraplast. Tissue sections (2–3 μm) were stained with haematoxylin eosin (pancreas, kidneys) or periodic acid-Schiff (PAS; kidneys). Glycogen within distal tubular epithelial cells was determined in adjacent kidney sections on two separate slides. One section was pretreated with diastase, and both were stained with PAS. Glycogen was determined in the non-diastase-treated section. The following immunofluorescent stainings were carried out to assess islet morphology: anti-insulin (DAKO, Glostrup, Denmark), anti-glucagon (R&D Systems, Minneapolis, MN, USA) and 4′, 6-diamidino-2-phenylindole (DAPI; DAKO); to assess β-cell apoptosis: anti-insulin (DAKO), transferease-mediated dUTP nick-end labelling (TUNEL; Roche Diagnostics) and DAPI (DAKO); and to assess islet fibrosis: a cocktail of anti-insulin (DAKO), anti-glucagon (R&D Systems), anti-pancreatic polypeptide (Millipore, Billerica, MA, USA), anti-somatostatin (Genetex, Irvine, CA, USA), anti-fibronectin (Sigma, St Louis, MO, USA) and DAPI (DAKO), followed by respective secondary fluorescent antibodies (Invitrogen, Carlsbad, CA, USA). Digital imaging fluorescence microscopy of the pancreas was performed using a scanning platform (MetaSystems, Altlußheim, Germany) with a Zeiss Imager Z.2 microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). Immunohistochemical staining of kidney sections was carried out using anti-desmin (Millipore), anti-synaptopodin (Acris Antibodies, Herford, Germany), anti-collagen IV (Millipore), anti-fibronectin (Genetex, San Antonio, TX, USA), anti-vimentin (Roche/Ventana) and anti-T-cell immunoglobulin mucin/kidney injury molecule-1 (TIM/KIM1) (R&D Systems) antibodies. Anti-desmin was used to detect glomerular podocyte damage; anti-synaptopodin, anti-collagen IV and anti-fibronectin to detect further glomerular alterations; and anti-vimentin, -TIM/KIM1, -collagen IV and -fibronectin to detect tubulo-interstitial damage. Digital brightfield imaging of the kidneys was performed using the Aperio Scanscope™ slidescanner (Aperio, Vista, CA, USA). Quantitative image analysis of islet and glomerular staining was performed using Definiens Architect XD (Definiens AG, Munich, Germany).

Statistical methods
Statistical analysis was performed by analysis of variance followed by Dunnett’s post hoc test. Any between-group differences versus vehicle were considered significant at p ≤ 0.05. Data are reported as mean ± standard error of the mean, unless otherwise stated.

Results
Glucose and Lipid Effects of Aleglitazar
ZDF rats at baseline had slightly elevated blood glucose levels compared with their non-diabetic ZL counterparts (Table 1). Blood glucose in untreated ZDF rats progressively increased from baseline to 4 and 12 weeks, while blood glucose in aleglitazar-treated ZDF rats remained normal. Aleglitazar significantly lowered HbA1c compared with vehicle-treated ZDF rats at both 4 and 12 weeks. At the end of the study period, blood glucose and HbA1c were similar in both non-diabetic ZL and aleglitazar-treated ZDF rats (Table 1). These data indicate that aleglitazar effectively prevents the development of hyperglycaemia.

At baseline, ZDF rats were already hyperinsulinaemic (Table 1). In untreated ZDF rats, plasma insulin levels progressively increased up to 4 weeks and declined thereafter. Aleglitazar reversed the development of early hyperinsulinaemia, maintaining near-normal plasma insulin levels at 4 weeks that were significantly lower than in control ZDF rats and only slightly elevated versus non-diabetic ZL rats. At 12 weeks, the difference between plasma insulin levels in aleglitazar-treated and vehicle-treated ZDF rats was not significant due to the advanced state of disease in the untreated animals. Aleglitazar strongly induced plasma adiponectin levels and showed significant effects on non-fasted plasma lipids (Table 1).

Table 1. Effect of aleglitazar on non-fasted metabolic parameters.

<table>
<thead>
<tr>
<th>Metabolic Parameter</th>
<th>ZDF (N = 10)</th>
<th>ZDF + aleglitazar (N = 10)</th>
<th>ZL (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mmol/l</td>
<td>7.9 ± 0.2</td>
<td>8.0 ± 0.3</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>4.6 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>4.8 ± 0.5</td>
<td>5.0 ± 0.7</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/l</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>Plasma non-esterified fatty acids, mmol/l</td>
<td>0.07 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Plasma total cholesterol, mmol/l</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.0</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>204 ± 3</td>
<td>205 ± 3</td>
<td>160 ± 4</td>
</tr>
<tr>
<td>At 4 weeks of treatment (age = 10 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>17.5 ± 1.9</td>
<td>7.6 ± 0.1†</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.5 ± 0.1</td>
<td>4.9 ± 0.0†</td>
<td>4.8 ± 0.0</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>8.3 ± 1.1</td>
<td>1.6 ± 0.3†</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/l</td>
<td>10.0 ± 0.5</td>
<td>0.6 ± 0.1†</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Plasma non-esterified fatty acids, mmol/l</td>
<td>0.32 ± 0.03</td>
<td>0.05 ± 0.02†</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Plasma total cholesterol, mmol/l</td>
<td>3.0 ± 0.0</td>
<td>3.6 ± 0.1†</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>354 ± 6</td>
<td>435 ± 7†</td>
<td>278 ± 4</td>
</tr>
<tr>
<td>At 12 weeks of treatment (age = 18 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>26.1 ± 1.0</td>
<td>8.3 ± 0.3†</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>9.2 ± 0.5</td>
<td>5.4 ± 0.1†</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>2.6 ± 0.5</td>
<td>1.9 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/l</td>
<td>8.5 ± 0.9</td>
<td>1.4 ± 0.1†</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Plasma non-esterified fatty acids, mmol/l</td>
<td>0.26 ± 0.04</td>
<td>0.09 ± 0.02†</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Plasma total cholesterol, mmol/l</td>
<td>5.0 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Plasma adiponectin, ng/ml</td>
<td>3.3</td>
<td>17.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Glucosuria, mmol/l†</td>
<td>81.3 ± 0.4</td>
<td>0.5 ± 0.1†</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Proteinurina, mg/ml</td>
<td>4.4 ± 1.2</td>
<td>0.3 ± 0.1†</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>428 ± 16</td>
<td>765 ± 16†</td>
<td>386 ± 5</td>
</tr>
</tbody>
</table>

ZDF, Zucker diabetic fatty; ZL, Zucker lean. Data are presented as means ± s.e.m.; ANOVA followed by Dunnett’s post hoc test. *N = 5 per group for glucosuria and proteinuria, evaluated at 13 weeks. †p < 0.01 ZDF + aleglitazar versus ZDF.
Glucose AUC0-120, mmol/l

Aleglitazar preserved islet integrity, maintaining normal endocrine cells dispersed within the exocrine tissue. In contrast, α-t-cells scattered throughout individual islets and vacuolated endocrine cells dispersed within the exocrine tissue. In contrast, aleglitazar preserved islet integrity, maintaining normal α-cell localization at the islet periphery while preserving β-cell area and number (figure 1 and Table 3). In aleglitazar-treated ZDF rats, β-cell area, number and insulin content were higher than in non-diabetic ZL rats (figure 1).

Aleglitazar significantly prevented islet fibrosis (indicated by percentage fibrotic islet area and fibronectin staining intensity) to a level comparable to that of ZL rats (figure 2). Aleglitazar treatment significantly protected β-cells from apoptosis (indicated by number of apoptotic β-cells and percentage apoptotic β-cell number per islet cross-section) compared with vehicle-treated ZDF rats (figure 2).

### Table 2. Effects on fasted parameters of glucose homeostasis and oral glucose tolerance test (OGTT) after 12 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>ZDF (N=10)</th>
<th>ZDF + aleglitazar (N=10)</th>
<th>ZL (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose, mmol/l</td>
<td>9.3 ± 1.1</td>
<td>5.8 ± 0.1±</td>
<td>4.4 ± 0.0</td>
</tr>
<tr>
<td>Fasting plasma insulin, ng/ml</td>
<td>2.84 ± 0.86</td>
<td>1.70 ± 0.19</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>Glucose AUC0-120, mmol/l 1min</td>
<td>2094 ± 265</td>
<td>971 ± 68±</td>
<td>883 ± 38</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>26.8 ± 5.9</td>
<td>11.0 ± 1.3±</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>322 ± 143</td>
<td>379 ± 36</td>
<td>564 ± 22</td>
</tr>
</tbody>
</table>

HOMA-B, homeostasis model assessment of β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance; ZDF, Zucker diabetic fatty; ZL, Zucker lean.

Data are presented as means ± s.e.m. ANOVA followed by Dunnett’s post hoc test.

\*p < 0.01 ZDF + aleglitazar versus ZDF.

At baseline, ZDF rats had elevated plasma TG and lower plasma NEFA levels compared with non-diabetic ZL controls. In ZDF rats, plasma TG progressively increased over time – an effect that was largely prevented by aleglitazar. At the end of the treatment period, aleglitazar-treated ZDF rats had plasma TG comparable to the ZL controls. Plasma cholesterol was elevated in controlled ZDF rats compared with ZL rats but was not significantly changed by aleglitazar.

**Fasting Parameters and OGTT.** Aleglitazar significantly reduced fasting blood glucose compared with vehicle-treated ZDF rats at 12 weeks (Table 2). Following an OGTT, aleglitazar significantly reduced glucose area under the curve compared with vehicle-treated ZDF rats at 12 weeks (Table 2) and normalized glucose excursions to a level comparable to vehicle-treated ZL rats. Fasting plasma insulin levels were slightly lower with aleglitazar compared with vehicle-treated ZDF rats after 12 weeks of treatment, but were higher than in non-diabetic ZL rats (Table 2; not significant vs. vehicle-treated ZDF rats). Reductions in HOMA-IR indicated a trend to improved insulin sensitivity with aleglitazar compared with vehicle-treated ZDF rats (Table 2; not significant vs. vehicle-treated ZDF rats).

**Body Weight.** At baseline, body weight was increased in ZDF rats compared with ZL controls (Table 1). Aleglitazar was associated with significantly increased body weight compared with vehicle-treated ZDF rats at both 4 weeks and 12 weeks.

**Effects of Aleglitazar on Islet Morphology, β-Cell Markers and β-Cell Function**

Severe β-cell destruction was notable in vehicle-treated ZDF rats. As shown in figure 1 and Table 3, loss of islet structural integrity and insulin content occurred in vehicle-treated ZDF rats after 13 weeks of treatment. Islets were enlarged, containing irregular projections into the exocrine pancreas, with α-cells scattered throughout individual islets and vacuolated endocrine cells dispersed within the exocrine tissue. In contrast, aleglitazar preserved islet integrity, maintaining normal α-cell localization at the islet periphery while preserving β-cell area and number (figure 1 and Table 3). In aleglitazar-treated ZDF rats, β-cell area, number and insulin content were higher than in non-diabetic ZL rats (figure 1).

Aleglitazar significantly prevented islet fibrosis (indicated by percentage fibrotic islet area and fibronectin staining intensity) to a level comparable to that of ZL rats (figure 2). Aleglitazar treatment significantly protected β-cells from apoptosis (indicated by number of apoptotic β-cells and percentage apoptotic β-cell number per islet cross-section) compared with vehicle-treated ZDF rats (figure 2).

**Effect of Aleglitazar on Cataract Formation**

Aleglitazar protected against cataract formation, which occurred in vehicle-treated ZDF rats (see figure 3 and Table 3). Clinical examination revealed vehicle-treated ZDF rats had lens opacity indicative of diabetic cataracts as compared with ZL rats. Histologically, cataracts were characterized by degeneration and swelling of lens fibres, with formation of Morgagnian globules, abnormally retained nuclei (bladder cells) and hyperplasia of the lens epithelium underlying the anterior capsule. Lens opacity or histopathologic changes indicative of cataract formation were absent in aleglitazar-treated rats.

**Effects of Aleglitazar on Renal Function, and Glomerular and Tubulo-Interstitial Markers**

ZDF rats developed renal dysfunction, as indicated by high levels of urinary protein. At 13 weeks, aleglitazar significantly reduced urinary protein and glucose compared with vehicle-treated ZDF rats to levels characteristic of non-diabetic ZL rats (Table 1).

Vehicle-treated ZDF rats exhibited evidence of glomerular hypertrophy, PAS + glomerular absorption droplets within degenerating podocytes, mesangial expansion and glomerulosclerosis (Table 3). PAS staining revealed minimal focal segmental glomerulosclerosis in vehicle-treated ZDF rats, which was prevented by treatment with aleglitazar (figure 4). Glomerular desmin staining was increased in vehicle-treated ZDF rats, suggesting podocyte damage, and was largely prevented by aleglitazar treatment (figure 4). Quantitative image analysis of desmin demonstrated that both desmin marker area and percentage desmin marker area per glomerulum was reduced in aleglitazar-treated ZDF rats to levels comparable to those in non-diabetic ZL rats (figure 5). Other markers, most notably of fibrosis and glomerular dysfunction [collagen IV and fibronectin (figure 4) and synaptopodin (data not shown)] were not significantly different in any of the groups.

Aleglitazar prevented tubular basophilia, dilation and luminal proteinaceous casts accompanied by interstitial fibrosis, and vacuolation associated with PAS + droplets indicative of glycogen storage within renal distal tubular epithelial cells, all of which were present in vehicle-treated ZDF rats (Table 3). Immunohistochemistry for vimentin, TIM/KIM1, collagen IV and fibronectin revealed increased tubular expression of these markers in vehicle-treated ZDF rats (figure 6), correlating with tubular damage, also observed with haematoxylin eosin and PAS staining. These
Figure 1. Preservation of islet structure in aleglitazar-treated rats. Assessment of islet morphology, β-cell area, β-cell number and insulin staining intensity by digital imaging quantitative fluorescence microscopy using anti-insulin or anti-glucagon antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) staining. Scale bar, 100 μm. N = 179–291 islets/group, ***p < 0.001 versus vehicle; data are shown as mean ± s.e.m.; ANOVA followed by Dunnett’s test.
Discussion

Several landmark studies have demonstrated a relationship between improvements in metabolic abnormalities and prevention of diabetic complications in patients with T2DM. The direct relationship between glycaemia and diabetes-related complications was shown in an epidemiologic analysis from the UKPDS [24], while, in Steno-2, patients with T2DM and microalbuminuria who were receiving intensive therapy for control of glucose, lipids and blood pressure had a significantly lower risk of CV disease, nephropathy, retinopathy and autonomic neuropathy [25]. Results from these studies suggest that a significant portion of the beneficial effect of the multifactorial intervention on CV disease was attributable to lipid lowering on top of effective blood pressure and glycaemic control. The strengths of this study are its evaluation of multiple immunohistochemical markers of pancreatic β-cell health, and renal glomerular and interstitial changes, in a well-established rodent disease model of T2DM. A limitation of this study is inability to dissect the relative contributions of improved glycaemic control versus improved lipids, or of the contributions of direct mechanisms on β-cell or renal protection conferred by aleglitazar.

Aleglitazar prevented β-cell apoptosis in this study by up to 87%. Histologic and immunofluorescent examination of the pancreas showed that aleglitazar was able to maintain β-cell area and number to levels comparable to prediabetic ZDF rats. After 13 weeks of treatment, aleglitazar preserved islet integrity, maintained insulin content and significantly reduced islet fibrosis. These effects are likely the result of multiple factors leading to reduced gluco- and lipotoxicity, related to improved insulin sensitivity, as well as significantly reduced glucose, TG and NEFA. These beneficial effects on β-cell morphology and function are in agreement with previous studies of PPAR-γ agonists, which showed that rosiglitazone preserves β-cell mass by preventing the rise in net β-cell death [14,15]. In one study, rosiglitazone, but not metformin, preserved β-cell mass while both agents reduced glucose levels, suggesting a protective effect of PPAR-γ that is independent of the effects on glucose [14].

Podocyte injury plays an early and important role in progressive renal dysfunction in the ZDF model [10,29].

Table 3. Summary of histopathologic findings after 13 weeks of treatment.

<table>
<thead>
<tr>
<th>Pancreas</th>
<th>ZDF (N = 10)</th>
<th>ZDF + aleglitazar (N = 10)</th>
<th>ZL (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islets enlarged with irregular projections into exocrine pancreas, endocrine cells scattered within exocrine tissue (HE)</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vacuolated endocrine cells (HE) corresponding to reduced insulin content (insulin IHC)</td>
<td>++++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α-cells at periphery of islet (glucagon IHC)</td>
<td>−</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Glomerular mesangial expansion</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Glomerulosclerosis, focal, segmental</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Vacuolation and PAS+ droplets within distal tubular epithelial cells (corresponding to glycogen storage)</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tubular dilation and lumenal proteinaceous casts</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tubular basophilia</td>
<td>++++/++/++</td>
<td>(+)/+</td>
<td>(+)</td>
</tr>
</tbody>
</table>

HE, haematoxylin eosin; IHC, immunohistochemistry; PAS, periodic acid-Schiff; ZDF, Zucker diabetic fatty; ZL, Zucker lean.
− = absent; (+) = minimal, individual animals only; + = minimal; ++ = slight; +++ = moderate; ++++ = marked.

markers were decreased in aleglitazar-treated animals to levels comparable to non-diabetic ZL rats, indicating prevention of tubulo-interstitial fibrosis and pathologic changes.
Figure 2. Aleglitazar inhibits islet fibrosis and β-cell apoptosis in the pancreas. Assessment of islet fibrosis and β-cell apoptosis by digital imaging quantitative fluorescence microscopy using anti-insulin, anti-glucagon, anti-pancreatic polypeptide, anti-somatostatin antibodies and anti-fibronectin antibody (for fibrosis) and anti-insulin antibody, transferase-mediated dUTP nick-end labelling (TUNEL) and 4′, 6-diamidino-2-phenylindole (DAPI) staining (for apoptosis). Scale bar, 100 μm. Fibrosis: N = 227–562 islets/group, Apoptosis: N = 190–344 islets/group, * p < 0.05, ** p < 0.01 versus vehicle; data are shown as mean ± s.e.m.; ANOVA followed by Dunnett’s test.
Figure 3. Aleglitazar prevents development of cataracts. Histologic characterization of the lens following haematoxylin eosin (HE) staining, showing development of cortical cataracts in untreated Zucker diabetic fatty (ZDF) rats but prevention of cataracts in aleglitazar treated ZDF rats. Scale bar, 50 μm.

Figure 4. Aleglitazar prevents glomerulosclerosis. Assessment of glomerular pathology by periodic acid-Schiff (PAS) staining and immunohistochemical staining. Evidence of glomerulosclerosis indicated by black arrow. Evidence of podocyte damage indicated by PAS+ absorption droplets (open arrow) and by immunohistochemical staining with anti-desmin antibody. Extent of fibrosis was assessed by immunohistochemical staining with anti-collagen IV or anti-fibronectin antibodies. Scale bar, 50 μm.

Although early podocyte damage may be influenced by high glucose and dyslipidaemia [10], agonism of PPAR-α [20,21] and PPAR-γ [16–18] may also have direct renoprotective actions. PPAR-α and PPAR-γ are both expressed in the kidney, with PPAR-α predominantly expressed in proximal tubules and medullary thick ascending limbs and PPAR-γ in medullary collecting ducts, pelvic urothelium and glomerular mesangial cells [19]. In diabetic PPAR-α-knockout mice, diabetic nephropathy is more severe than in wild-type mice [30]. There is substantial evidence that PPAR-α and PPAR-γ agonists have direct effects on several critical pathways contributing to the development and progression of diabetic kidney disease, as reviewed by Thomas et al. [30]. For example, both gemfibrozil and rosiglitazone have been shown to reduce albuminuria, glomerulosclerosis, tubulointerstitial expansion and collagen IV deposition in streptozotocin-induced diabetes, where the effects on glycaemic control and lipids are minimal, suggesting direct protective actions [30]. Other glucose-independent renoprotective mechanisms that have been attributed to PPAR-γ agonism include reductions in blood pressure, oxidative stress, inflammation, fibrosis and positive effects on podocyte function, including increased expression of nephrin [30].

In this study, aleglitazar prevented renal glomerular hypertrophy, podocyte degeneration and glomerulosclerosis, as well as tubular basophilia, dilation and lumenal proteinaceous casts accompanied by interstitial fibrosis. Aleglitazar also prevented vacuolation associated with PAS+ droplets, indicative of hyperglycaemia-related glycogen storage within renal distal tubular epithelial cells that may, in part, be consistent with the ‘Armanni–Ebstein phenomenon’ in diabetic states [31]. Quantitative immunohistochemistry using an extensive panel of markers provided detailed insights regarding both glomerular and tubular protection. In the glomerulus, aleglitazar normalized desmin levels, suggesting prevention of podocyte dysfunction. Our data also indicated a tubulo-interstitial-protective effect of aleglitazar, evidenced by decreases in a range of markers...
for tubular damage, including vimentin, TIM/KIM1, collagen IV and fibronectin. These results concur with previous findings in studies with both PPAR-α and -γ agonists [16–18,21,32], although, to our knowledge, previous investigations have not explored the effect of PPAR agonists on such a comprehensive panel of markers. In the case of fenofibrate, one mechanism that has been hypothesized to explain its effects is the inhibition of transforming growth factor β1/Smad3 and nuclear factor κB signalling pathways, resulting in suppression of inflammation and fibrosis [21].

The reduction in renal fibrosis may, in part, be mediated by decreased fibrinogen, which has been observed in preclinical and clinical studies of aleglitazar [7,33]. This concurs with recent data suggesting that fibrinogen promotes renal fibrosis by triggering resident fibroblast proliferation [34]. These effects are similar to observations from studies [35] of fenofibrate such as the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial, in which fenofibrate reduced albuminuria and slowed estimated glomerular filtration rate loss over 5 years, indicating a potential to retard loss of kidney function despite initial reversible increases in serum creatinine [36]. Serum creatinine levels were not monitored in the present study; however, aleglitazar has been shown to increase serum creatinine in clinical studies [7,37]. Notwithstanding, these effects have been shown to be fully reversible, even at the dose of 600μg (four times the dose currently being

Figure 5. Aleglitazar decreases glomerular desmin expression. Quantitative image analysis of glomerular desmin staining measured as average desmin marker area (μm²/glomerulum) and percentage marker area per glomerulum. N = 240 glomeruli/group; *** p < 0.001 versus Zucker diabetic fatty (ZDF); data are shown as mean ± s.e.m.; ANOVA followed by Dunnett’s test.

Figure 6. Prevention of kidney fibrosis with aleglitazar. Assessment of tubular pathology by immunohistochemical staining of kidney sections using anti-vimentin, anti-KIM-1, anti-collagen IV and anti-fibronectin antibodies. Scale bar, 200μm.
evaluated in phase III trials), with creatinine levels returning to pretreatment levels following cessation of treatment within a 4- to 8-week follow-up period [37].

In conclusion, aleglitazar strongly improves glycaemic and lipid parameters in ZDF rats while protecting key tissues, including the pancreas, eyes and kidneys, against diabetes-associated structural and functional changes. Aleglitazar protected β-cell morphology, as evidenced by effects on islet integrity, β-cell apoptosis and islet fibrosis. Aleglitazar prevented the development of cataracts and prevented renal glomerular hypertrophy, podocyte degeneration, glomerulosclerosis and tubulo-interstitial lesions. Together, these data suggest that aleglitazar may be effective in reducing the progression of T2DM, and macrovascular and microvascular complications of the disease.

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Conflict of Interest


References
