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The dipeptidyl peptidase-4 inhibitor alogliptin in combination with pioglitazone improves glycemic control, lipid profiles, and increases pancreatic insulin content in *ob/ob* mice

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ABSTRACT

The combination of two agents with different but complementary mechanisms of action is a logical approach for treating patients with type 2 diabetes. Thus, we evaluated chronic combination therapy with alogliptin, a highly selective dipeptidyl peptidase-4 inhibitor that enhances the action of incretins, and pioglitazone, a thiazolidinedione that improves peripheral and hepatic insulin sensitivity. Studies were designed to investigate the chronic metabolic and pancreatic effects of alogliptin (0.03%) plus pioglitazone (0.003%) combination treatment in obese *ob/ob* mice. After 4–5 weeks of treatment, alogliptin significantly increased plasma active glucagon-like peptide-1 levels up to 4.1-fold and decreased plasma glucagon up to 25%, whereas pioglitazone significantly increased plasma adiponectin up to 1.3-fold. Combination treatment exhibited a complementary effect, increasing plasma insulin levels by 3.2-fold (alogliptin alone, 1.6-fold; pioglitazone alone, 1.5-fold) and decreasing glycosylated hemoglobin by 2.3% (alogliptin alone, 1.0%; pioglitazone alone, 1.5%), and non-fasting and fasting plasma glucose by 37% and 62% (alogliptin alone, 17% and 24%; pioglitazone alone, 30% and 45%), respectively. Combination treatment also decreased plasma triglycerides by 67% and non-esterified fatty acids by 25% (alogliptin alone, 24% and 11%; pioglitazone alone, 54% and 8%). Moreover, combination treatment increased pancreatic insulin content by 2.2-fold (alogliptin alone, 1.3-fold; pioglitazone alone, 1.6-fold), with no significant changes in body weight. These results indicate that combination treatment with alogliptin and pioglitazone improved glycemic control, lipid profiles and increased pancreatic insulin content in *ob/ob* mice by preventing incretin inactivation and improving insulin resistance. These results provide a strong argument for using alogliptin in combination with pioglitazone.

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1. Introduction

A major goal of diabetes management is to restore and maintain fasting and postprandial blood glucose levels to within normal ranges, thereby decreasing the risk of diabetic complications such as neuropathy, nephropathy, and retinopathy as well as macrovascular events (Meeuwisse-Pasterkamp et al., 2008), although recently published studies have shown that macrovascular events may not be decreased by the aggressive glucose control (Gerstein et al., 2008; Patel et al., 2008). Diabetes is a chronic disease in which secondary failure often occurs with single agent treatment, due to the progressive loss of β-cell function and increased insulin resistance over time (Giorgino et al., 2005; Wajchenberg, 2007). Therefore, combination therapy using antihyperglycemic drugs with different but complimentary mechanisms of action is often required to achieve and maintain adequate glycemic control (Charpentier, 2002; Turner et al., 1999).

Dipeptidyl-peptidase-4 (DPP-4) is a clinically validated target for the treatment of type 2 diabetes (Barnett, 2006). DPP-4 cleaves multiple peptide substrates, including the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Lambeir et al., 2003). GLP-1 and GIP are secreted from gut L- and K-cells in response to meal ingestion and serve as potent prandial stimulators of insulin secretion thereby playing a major role in glucose homeostasis (Holst, 2004). In addition, both peptides exhibit trophic affects on pancreatic islets, helping to maintain β-cell function and physiologic numbers (Farilla et al., 2002; Perfetti and Hui, 2004; Rolin et al., 2002). Despite the beneficial actions of GLP-1 and GIP, their use as antidiabetic agents was impractical due to their short half-lives as a result of their rapid inactivation by DPP-4 (Deacon, 2004; Drucker and Nauck, 2006). Therefore, DPP-4 inhibitors have emerged as a new class of antihyperglycemic agents. Clinical trials have demonstrated that DPP-4 inhibitors, such as alogliptin, sitagliptin, and vildagliptin, lower fasting and postprandial glucose levels and improve measures of β-cell function, resulting in clinically meaningful reductions in glycosylated

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hemoglobin levels in patients with type 2 diabetes (Covington et al., 2008; Madsbad et al., 2008). In addition, there appears to be a decreased risk of hypoglycemia with DPP-4 inhibitors compared with insulin secretagogues such as sulfonylureas (El-Ouaghlidi et al., 2007; Nauck et al., 2007).

Alogliptin is a orally-available, quinazolinone-based, non-covalent DPP-4 inhibitor under development as a once-daily treatment for type 2 diabetes (Christopher et al., 2008; Defronzo et al., 2008; Feng et al., 2007). Alogliptin has been shown to inhibit human DPP-4 with an IC_{50} of 6.9 nM *in vitro* and to exhibit >10,000-fold selectivity for DPP-4 over other, related serine proteases such as DPP-8 and DPP-9 (Drucker, 2007), indicating that it is a potent and highly selective inhibitor of DPP-4 (Lee et al., 2008). Studies have shown that a single dose of alogliptin improves oral glucose tolerance in both non-obese N-STZ-1.5 rats and obese Wistar fatty rats (Feng et al., 2007; Takeuchi et al., 2006). More recently, chronic treatment with alogliptin for 4 weeks has been shown to improve glycemic control and β-cell function in *ob/ob* mice (Moritoh et al., 2008).

Pioglitazone is a commercially available member of the thiazolidinedione (TZD) class of antihyperglycemic agents and is a specific, potent agonist for the peroxisome proliferator-activated receptor-γ (Wilding, 2006). Pioglitazone improves glycemic control in patients with type 2 diabetes primarily by improving peripheral and hepatic insulin resistance, thereby increasing insulin-dependent glucose disposal and reducing hepatic glucose production (Pfutzner et al., 2006).

The beneficial effects of chronic combination treatment with a DPP-4 inhibitor (vildagliptin) and a TZD (pioglitazone) were first reported by Burkey et al. (2002). In this study, the combination of vildagliptin and pioglitazone increased glucose clearance rate and normalized glucose concentrations in adult obese Zucker rats following glucose challenge (Burkey et al., 2002). More recently, clinical studies have revealed that glycemic control was significantly improved with the addition of alogliptin, sitagliptin, or vildagliptin to ongoing pioglitazone treatment in subjects whose type 2 diabetes was inadequately controlled with pioglitazone alone (Garber et al., 2007; Pratley et al., 2008; Rosenstock et al., 2006). Furthermore, initial treatment with vildagliptin plus pioglitazone in drug-naive patients with type 2 diabetes resulted in improved glycemic control when compared to monotherapy (Rosenstock et al., 2007). Although, combination treatment with DPP-4 inhibitors and pioglitazone appears to hold considerable promise for treatment of type 2 diabetes, the pharmacological effects of combination treatment have not been well documented.

The present study was thus designed to assess the chronic effects of alogliptin plus pioglitazone combination treatment on hormone profiles, glycemic control, lipid profiles, and pancreatic insulin content in *ob/ob* mice that exhibit abnormal glycemic control and lipid profile, hyperinsulinemia, and hyperglucagonemia (Herberg and Leiter, 2001).

2. Materials and methods

2.1. Compounds

Alogliptin benzoate (2-[[6-[(3*R*)-3-amino-1-piperidinyl]-3,4-dihydro-3-methyl-2,4-dioxo-1(2*H*)-pyrimidinyl]methyl]benzonitrile monobenzoate) was synthesized by Albany Molecular Research Institute (Albany, NY, USA). Pioglitazone hydrochloride was synthesized by Takeda Pharmaceutical Company, Ltd (Hikari, Japan). The doses of alogliptin and pioglitazone are expressed as the free base form. All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (Tokyo, Japan).

2.2. Mice

Male Lep^{ob}/Lep^{ob} (*ob/ob*) mice and their non-diabetic, untyped (*?/+*) male littermates were obtained from Clea Japan (B6.V-Lep^{ob}/Jic; colony in Central Institute for Experimental Animals, Japan). All mice were housed in individual metal cages in a room with controlled temperature (23 °C), humidity (55%), and lighting (lights on from 7:30 am to 7:30 pm) and were maintained on a laboratory chow diet (CE-2, Clea Japan). The care and use of the animals and the experimental protocols used in this research were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company, Ltd (Osaka, Japan).

2.3. Chronic administration protocol

After a 6-day acclimation period, 7-week old *ob/ob* mice were divided into 4 groups (8 mice per group) based on glycosylated hemoglobin, plasma glucose, plasma insulin, and body weight and fed a CE-2 diet containing 0.03% alogliptin (45.7 mg/kg/day) alone, 0.003% pioglitazone (4.0 mg/kg/day) alone, or 0.03% alogliptin plus 0.003% pioglitazone in combination (41.0 and 4.1 mg/kg/day, respectively) during the experimental period. Control *ob/ob* and *?/+* mice (8 and 5 mice, respectively) were fed a drug-free CE-2 diet (vehicle). After 28 days treatment, blood samples were collected from the orbital vein via capillary pipette for the measurement of plasma parameters and DPP-4 activity. After 29 days of treatment, the mice were fasted for

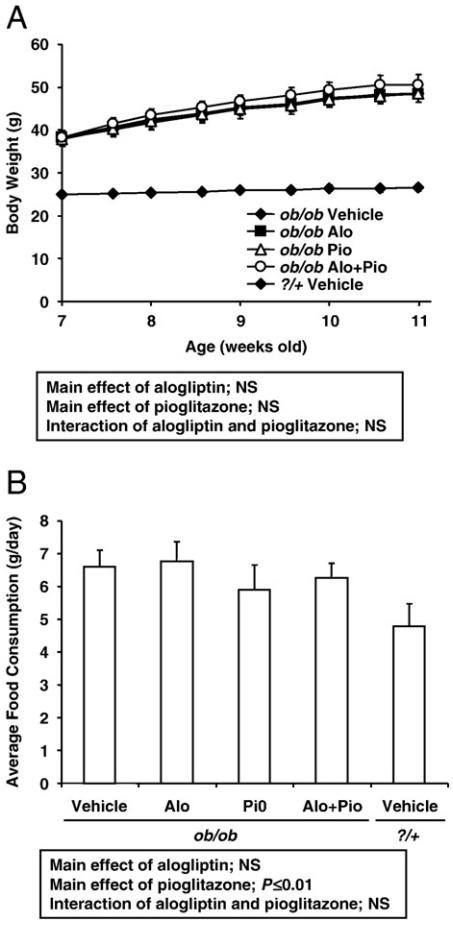
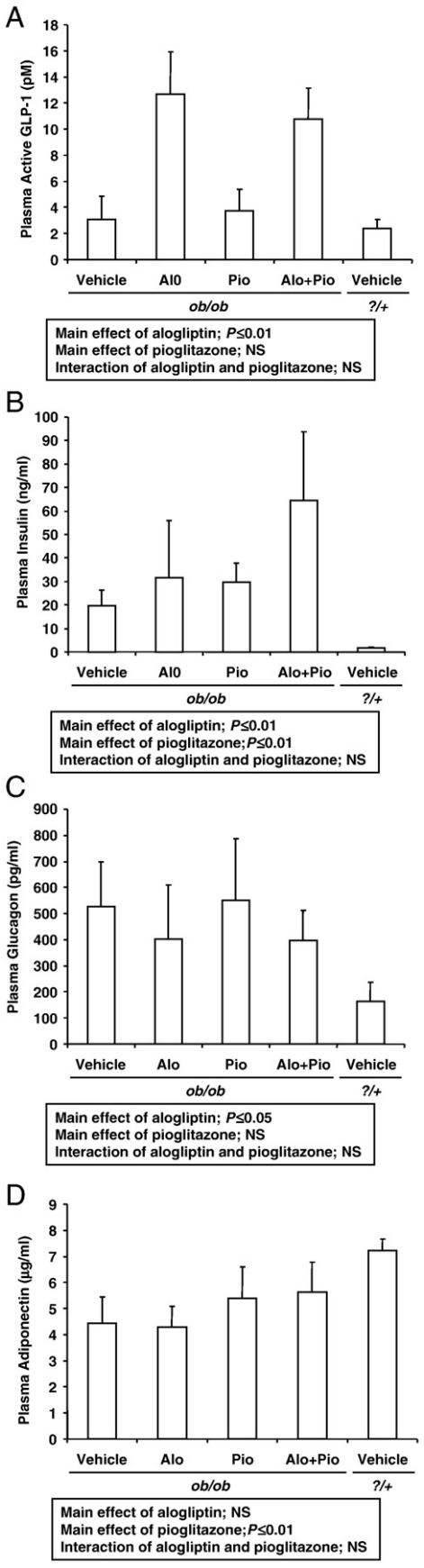


Fig. 1. Chronic effects of alogliptin, pioglitazone, and alogliptin plus pioglitazone combination treatment on body weight (A) and food consumption (B). Animals were administered CE-2 diet containing 0.03% alogliptin, 0.003% pioglitazone, a combination of alogliptin (0.03%) plus pioglitazone (0.003%), or vehicle for a study period of 33 days. Body weight and food consumption were measured periodically throughout the treatment period. Combination treatment with alogliptin and pioglitazone did not show any additive effects on body weight and food consumption in *ob/ob* mice. The results from a two-way ANOVA are presented in the figure inserts. Data are presented as means and S.D. (n=8 for *ob/ob* mice, n=5 for *?/+* mice).



17 h and fasting plasma glucose levels were determined. The mice were then re-fed with their corresponding diet and after 33 days of treatment, plasma glucagon and active (intact) GLP-1 levels were determined followed by pancreas isolation (fed state). Body weight and food consumption were measured at regular intervals. The animals were 11 weeks of age after 28 and 29 days of treatment, and approximately 12 weeks of age after the 33 day study period.

2.4. Assays of metabolic components

Glycosylated hemoglobin levels were analyzed by a high-performance liquid chromatography-based method using an automated analyzer HLC-723 G7 (Tosoh, Japan). Plasma glucose, triglyceride, and non-esterified fatty acid levels were measured using an autoanalyzer 7080 (Hitachi, Japan). Plasma insulin (Rat Insulin RIA Kit; Linco, USA) and adiponectin (Mouse Adiponectin RIA Kit; Linco, USA) levels were determined by radioimmunoassay (RIA). Plasma levels of active GLP-1 (Glucagon Like Peptide-1 [Active] ELISA Kit; Linco, USA) and glucagon (Glucagon ELISA Kit; Wako, Japan) were determined by enzyme-linked immunosorbent assay (ELISA).

2.5. Pancreas isolation and measurement of insulin content

At the end of the study, the mice were euthanized with carbon dioxide, and the pancreas was isolated, and homogenized in acid-ethanol containing 74% ethanol with 0.15 M HCl for the determination of insulin concentrations. The homogenized tissues were extracted overnight at 4 °C and centrifuged at 12,000 ×*g* for 10 min. The resultant supernatants were then diluted with phosphate-buffered saline containing 0.1% bovine serum albumin, and the insulin levels in the supernatants were measured by RIA kit (Linco, USA).

2.6. Statistical analysis

Statistical analysis was performed using the SAS Version 8.2 (SAS institute Inc.). To evaluate if combination treatment with alogliptin and pioglitazone had significant additive or synergistic effects, two-way ANOVA was performed, which generates main effects and interaction effect of alogliptin and pioglitazone. The evaluation of interaction effect aims to detect synergistic or attenuation effects statistically by combination of alogliptin and pioglitazone. The results of two-way ANOVA were interpreted as follows: 1) When significant interaction effect (alogliptin × pioglitazone, $P \leq 0.05$) was observed, the effect by combination treatment with alogliptin and pioglitazone was assessed to be synergistic or attenuation, which was determined from observed values. However no significant interaction was detected in the present study. 2) When no significant interaction was observed, the effect by combination treatment with alogliptin and pioglitazone was assessed to be neither synergistic nor attenuation. On the basis of no interaction observed, when both main effects of alogliptin treatment and pioglitazone treatment were significant ($P \leq 0.05$), the effect by combination treatment with alogliptin and pioglitazone was assessed to be additive. 3) When only one significant ($P \leq 0.05$) main effect was observed, the effect was assessed to be induced by only one drug, which was not affected by the other drug. Direct comparison

Fig. 2. Chronic effects of alogliptin-, pioglitazone-, and alogliptin plus pioglitazone combination treatment on plasma active GLP-1 (A), plasma insulin (B), plasma glucagon (C), and plasma adiponectin (D) levels. Animals were administered a CE-2 diet containing 0.03% alogliptin, 0.003% pioglitazone, a combination of alogliptin (0.03%) plus pioglitazone (0.003%), or vehicle for a study period of 33 days. Plasma insulin and adiponectin levels were determined after 28 days, and plasma active GLP-1 and glucagon levels were determined after 33 days. Alogliptin treatment increased plasma active GLP-1 levels and decreased plasma glucagon levels, while pioglitazone treatment increased plasma adiponectin levels. The combination with alogliptin and pioglitazone additively increased plasma insulin levels. The results from a two-way ANOVA are presented in the figure inserts. Data are presented as means and S.D. (n=8 for *ob/ob* mice, n=5 for *?/+* mice).

among study groups was not statistically tested in the combination study. All data were presented as the mean and S.D.

3. Results

3.1. Chronic effects of alogliptin plus pioglitazone combination treatment on body weight and food consumption

Alogliptin (45.7 mg/kg/day; 0.03%), pioglitazone (4.0 mg/kg/day; 0.003%), or alogliptin plus pioglitazone (41.0 and 4.1 mg/kg/day; 0.03% and 0.003%) were administered as a dietary admixture to *ob/ob* mice for 4 weeks. After the 4-week treatment period, regardless of treatment, no statistically significant changes were observed on body weight in *ob/ob* mice as determined by a two-way ANOVA (Fig. 1A). As shown in Fig. 1B, average food consumption was not changed in alogliptin-, decreased by 11% in pioglitazone-, and decreased by 5% in combination-treated *ob/ob* mice compared with the vehicle-treated *ob/ob* mice (alogliptin, NS; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS).

3.2. Chronic effects of alogliptin plus pioglitazone combination treatment on plasma hormone profiles

Alogliptin-treated *ob/ob* mice exhibited an 80% inhibition of plasma DPP-4 activity after 4 weeks of treatment, when compared with the vehicle-treated *ob/ob* mice (data not shown). After nearly 5 weeks of treatment, plasma active GLP-1 levels were increased by 3.5 to 4.1-fold by alogliptin alone and by the combination treatment; whereas pioglitazone alone resulted in only 1.2-fold increase on this parameter (Fig. 2A: alogliptin, $P \leq 0.01$; pioglitazone, NS; alogliptin × pioglitazone, NS). In the vehicle-treated *ob/ob* mice, baseline plasma insulin levels (31.8 ± 9.9 ng/ml) were decreased to 19.9 ± 6.4 ng/ml during the course of the 4-week study and corresponded with a rise in glucose levels (21.0 ± 1.3 mM to 28.8 ± 3.6 mM). In contrast, after 4 weeks of treatment, plasma insulin levels were increased by 1.6- and 1.5- fold in alogliptin- and pioglitazone-treated *ob/ob* mice, respectively, and additively increased by 3.2-fold in combination-treated *ob/ob* mice compared with vehicle-treated *ob/ob* mice (Fig. 2B: alogliptin, $P \leq 0.01$; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS). As shown in Fig. 2C and D, *ob/ob* mice exhibited hyperglucagonemia and reduced levels of plasma adiponectin when compared with the vehicle-treated *?/+* mice. As shown in Fig. 2C, plasma glucagon levels were decreased by 23% and 25% in alogliptin- and combination-treated *ob/ob* mice, respectively; however no decrease was observed in pioglitazone-treated *ob/ob* mice (alogliptin, $P \leq 0.05$; pioglitazone, NS; alogliptin × pioglitazone, NS) after nearly 5 weeks of treatment. Finally, plasma adiponectin levels were increased by 1.2- and 1.3-fold in pioglitazone- and combination-treated *ob/ob* mice, respectively, whereas alogliptin alone had no effect on this parameter (Fig. 2D: alogliptin, NS; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS) after 4 weeks of treatment. As shown, alogliptin plus pioglitazone combination treatment exhibited complementary and beneficial effects on metabolic hormone profiles in *ob/ob* mice.

3.3. Chronic effects of alogliptin plus pioglitazone combination treatment on glycemic parameters

After 4 weeks of treatment, the mean glycosylated hemoglobin levels (Fig. 3A), non-fasting plasma glucose concentrations (Fig. 3B), and fasting glucose concentrations (Fig. 3C) were higher in the vehicle-treated *ob/ob* mice when compared to the vehicle-treated *?/+* mice. As described above, hyperglycemia in the vehicle-treated *ob/ob* mice continued to worsen as the animals aged. In contrast, significant improvements in glycemic control were observed in *ob/ob* mice treated with alogliptin, pioglitazone, and the combination of alogliptin plus pioglitazone. After 4 weeks of treatment, glycosylated hemoglobin levels were decreased by 1.0% and 1.5% in alogliptin- and pioglitazone-treated *ob/ob* mice, respectively, and additively decreased by 2.3% in combination-treated *ob/ob* mice, compared with the vehicle-treated *ob/ob* mice (Fig. 3A: alogliptin, $P \leq 0.01$; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS). Non-fasting and fasting glucose levels were decreased by 17% and 24%, in alogliptin-

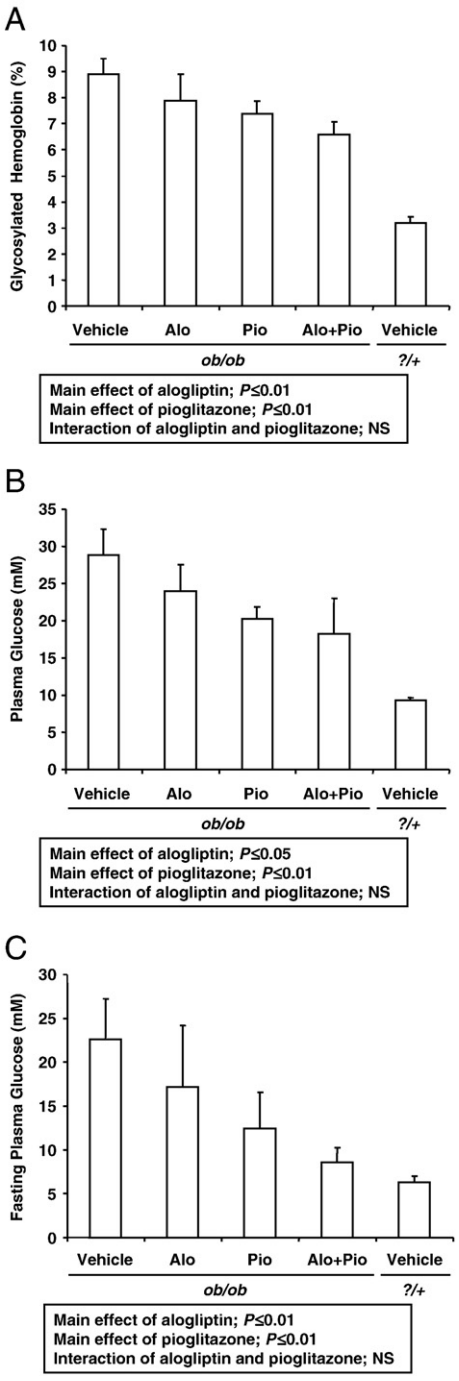


Fig. 3. Chronic effects of alogliptin-, pioglitazone-, and alogliptin plus pioglitazone combination treatment on glycosylated hemoglobin (A), non-fasting plasma glucose (B), and fasting plasma glucose (C) levels. Animals were administered a CE-2 diet containing 0.03% alogliptin, 0.003% pioglitazone, a combination of alogliptin (0.03%) plus pioglitazone (0.003%), or vehicle for a study period of 33 days. Glycosylated hemoglobin and non-fasting plasma glucose levels were determined after 28 days. After the 29 days, the animals were fasted for 17 h and fasting plasma glucose was determined. The combination with alogliptin and pioglitazone additively decreased glycosylated hemoglobin, non-fasting plasma glucose, and fasting plasma glucose levels. The results from a two-way ANOVA are presented in the figure inserts. Data are presented as means and S.D. ($n = 8$ for *ob/ob* mice, $n = 5$ for *?/+* mice).

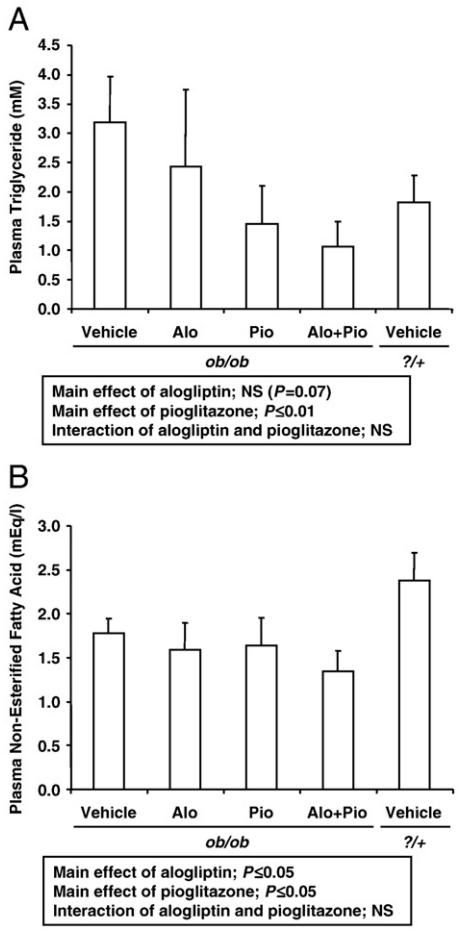


Fig. 4. Chronic effects of alogliptin-, pioglitazone-, and alogliptin plus pioglitazone combination treatment on plasma triglyceride (A) and plasma non-esterified fatty acid (B) levels. Animals were administered a CE-2 diet containing 0.03% alogliptin, 0.003% pioglitazone, a combination of alogliptin (0.03%) plus pioglitazone (0.003%), or vehicle for a study period of 33 days. Plasma triglyceride and plasma non-esterified fatty acid levels were determined after 28 days. Pioglitazone treatment significantly decreased plasma triglyceride levels, while combination with alogliptin and pioglitazone additively decreased plasma non-esterified fatty acid levels. The results from a two-way ANOVA are presented in the figure inserts. Data are presented as means and S.D. (n=8 for *ob/ob* mice, n=5 for *?/+* mice).

treated *ob/ob* mice, decreased by 30% and 45%, in pioglitazone-treated *ob/ob* mice, and additively decreased by 37% and 62%, respectively, in combination-treated *ob/ob* mice (Fig. 3B and C: alogliptin, $P \leq 0.05$ for non-fasting glucose and $P \leq 0.01$ for fasting glucose; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS).

3.4. Chronic effects of alogliptin plus pioglitazone combination treatment on lipid profiles

In addition to the beneficial effects observed for glycemic control, the combination treatment exhibited positive effects on lipid profiles. At the completion of the 4-week treatment period, plasma triglyceride concentrations in the vehicle-treated *ob/ob* mice were higher than those measured for the vehicle-treated *?/+* mice (Fig. 4A). After 4 weeks of treatment with alogliptin, pioglitazone, or a combination of alogliptin and pioglitazone, triglyceride levels were 24%, 54%, and 67%, respectively, lower than the vehicle-treated *ob/ob* mice (Fig. 4A: alogliptin, P=0.07; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS). Although the effect of combination treatment on plasma triglyceride levels was not statistically additive, combination treatment was more potent than treatment with alogliptin or pioglitazone alone. Moreover, plasma non-esterified fatty acid levels were decreased by 11% and 8% in alogliptin- and pioglitazone-treated *ob/ob* mice, respectively, and were additively decreased by 25% in combination-treated *ob/ob* mice, compared with the vehicle-treated *ob/ob* mice (Fig. 4B: alogliptin, $P \leq 0.05$; pioglitazone, $P \leq 0.05$; alogliptin × pioglitazone, NS).

3.5. Chronic effects of alogliptin plus pioglitazone combination treatment on pancreatic insulin content

At the end of the nearly 5-week treatment period, pancreatic insulin content in alogliptin- and pioglitazone-treated *ob/ob* mice was increased by 1.3- and 1.6-fold, respectively, and was additively increased by 2.2-fold in the combination treated *ob/ob* mice, compared with the vehicle-treated *ob/ob* mice (Fig. 5: alogliptin, $P \leq 0.01$; pioglitazone, $P \leq 0.01$; alogliptin × pioglitazone, NS). Interestingly, the pancreatic insulin content of combination-treated *ob/ob* mice was similar to that of the vehicle-treated *?/+* mice after the treatment period.

4. Discussion

In the present study, we characterized the pharmacological effects of combination treatment with the DPP-4 inhibitor alogliptin and the insulin sensitizer pioglitazone in *ob/ob* mice, an obese rodent model of type 2 diabetes that exhibits hyperglycemia, hyperinsulinemia, and hyperglucagonemia. Combination treatment with alogliptin plus pioglitazone, which have different but complementary mechanisms of action, resulted in increased plasma insulin levels, decreased plasma glucagon levels, and increased plasma adiponectin levels with no significant change in body weight. Combination treatment showed a more efficacious improvement of glycemic control and lipid profiles compared to monotherapy with either agent. In addition to improving metabolic profiles, combination treatment increased pancreatic insulin content compared to treatment with either alogliptin or pioglitazone alone. Hence, results from the present study provide preclinical support for the rationale use of alogliptin and pioglitazone combination therapy in patients with type 2 diabetes.

Contrary to previous studies reporting that pioglitazone decreases plasma insulin levels (Diani et al., 2004), this study observed an increase in plasma insulin levels as a result of treatment with pioglitazone alone. This apparent discrepancy needs to be considered when investigating the relationship between glucose levels, insulin demand, and β-cell function. As reported in pivotal studies conducted in *db/db* mice

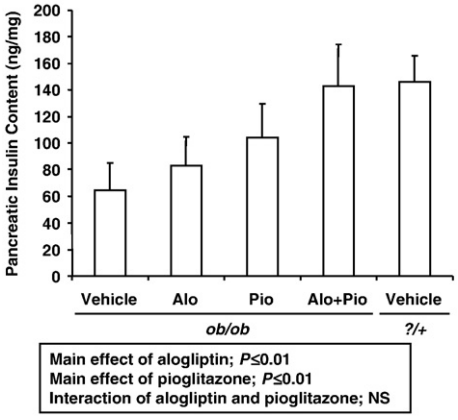


Fig. 5. Chronic effects of alogliptin-, pioglitazone-, and alogliptin plus pioglitazone combination treatment on pancreatic insulin content. Animals were administered a CE-2 diet containing 0.03% alogliptin, 0.003% pioglitazone, a combination of alogliptin (0.03%) plus pioglitazone (0.003%), or vehicle for a study period of 33 days. After the 33 day study period, the animals were euthanized with carbon dioxide in a fed state and the pancreata were isolated and pancreatic insulin content was determined. The combination with alogliptin and pioglitazone additively increased pancreatic insulin content. The results from a two-way ANOVA are presented in the figure insert. Data are presented as means and S.D. (n=8 for *ob/ob* mice, n=5 for *?/+* mice).

(Kjorholt et al., 2005), Zucker diabetic fatty rats (Harmon et al., 2001), and partially pancreatectomized rats (Jonas et al., 1999), glycemic control itself is a critical contributor for β-cell function. In fact, glycemic control by pioglitazone reportedly preserves β-cells in *ob/ob* and *db/db* mice (Diani et al., 2004). As shown in Fig. 3, glucose levels in *ob/ob* mice were decreased but still somewhat high when administered pioglitazone alone at a dose of 0.003%. This seems to suggest that the partially preserved β-cells that escaped glucose toxicity may have continued to actively secrete insulin into the blood in response to moderately high glucose levels. In fact, a high dose of pioglitazone has been shown to greatly decrease both plasma glucose and insulin levels in *ob/ob* mice (Diani et al., 2004).

Type 2 diabetes is often characterized by an inappropriate regulation of hepatic glucose production. (Jiang and Zhang, 2003; Sloop et al., 2005). Insulin resistance at the level of the liver is due to impaired insulin signaling, which is partly induced by elevated levels of plasma glucagon to insulin ratio in patients with type 2 diabetes (Baron et al., 1987). Thus, increasing the plasma insulin to glucagon ratio is a rational strategy for better glycemic control. In fact, the glucagon-lowering effect is considered an important and unique attribute of the pharmacological class of DPP-4 inhibitors (Ahren et al., 2004), indicating that DPP-4 inhibition not only targets insulin secretion but also suppresses inappropriate high glucagon levels possibly resulting from the effect of GLP-1 (Ahren, 2007). In the present study, alogliptin plus pioglitazone combination treatment was more effective at increasing plasma insulin levels compared with each drug alone. In addition, plasma glucagon levels were decreased, which was associated with increased plasma active GLP-1 levels, resulting in the highest plasma insulin to glucagon ratio in the combination-treated *ob/ob* mice. In addition to this, elevated circulating adiponectin levels were observed in the combination treatment group; this effect is most likely attributable to pioglitazone (Boden and Zhang, 2006). Pioglitazone-induced improvement of insulin resistance has been shown to occur in the liver via an adiponectin-dependent mechanism and in skeletal muscle via an adiponectin-independent mechanism in *ob/ob* mice (Kubota et al., 2006). Increased insulin to glucagon ratio in plasma, as described in a previous study (Moritoh et al., 2008), and increased adiponectin circulation taken together suggest that combination treatment with alogliptin and pioglitazone may have improved glycemic control as a result of their complementary mechanisms of action.

Combination treatment also resulted in additively increased pancreatic insulin content. As previously mentioned, improved glycemic control appears to be a critical factor in preserving β-cell function. Therefore, glucose control may be the primary factor contributing to the observed improvement in β-cell function found in this study. In addition, high levels of plasma active GLP-1, which has direct trophic effects on β-cells (Baggio and Drucker, 2007), may also be a plausible explanation for the beneficial effects on pancreatic insulin content observed with alogliptin and pioglitazone combination treatment.

Plasma glucose control is vital to maintaining the patient's health and reducing the risk of secondary diabetic complications, but is not necessarily enough to prevent cardiovascular complications. Patients with type 2 diabetes without known coronary artery disease have a similar 10-year risk for cardiac events as do patients with established coronary artery disease without diabetes (Brown, 2005). As complications resulting from atherosclerosis are responsible for approximately 80% of diabetic mortality, therapies that lowers plasma lipids are key in the primary and secondary prevention of atherosclerosis (Brown, 2005). In a recent clinical study, the use of pioglitazone in addition to an existing optimized macrovascular risk management plan significantly decreased the risk of secondary macrovascular events in a high risk patient population with type 2 diabetes and established macrovascular disease (Dormandy et al., 2005). The pioglitazone-induced improvement in adverse cardiovascular outcome appeared to be partly a result of decreased hyperlipidemia observed in these patients (Betteridge, 2007). In the present study, combination treatment with alogliptin and pioglitazone potently decreased plasma triglyceride levels in *ob/ob* mice, indicating that alogliptin plus pioglitazone combination treatment may be effective in patients with hypertriglyceridemia. Furthermore, clinical studies may be warranted to investigate the potential effects of combination therapy of alogliptin and pioglitazone on low-density lipoprotein and high-density lipoprotein cholesterol and the possible reduction in cardiovascular risk. In addition, plasma non-esterified fatty acid levels were also potently decreased by combination treatment. Because non-esterified fatty acids are thought to induce insulin resistance (Avramoglu et al., 2006), decreased plasma non-esterified fatty acid levels may have contributed to the overall efficacy of the combination treatment.

Activation of peroxisome proliferator-activated receptor-γ by pioglitazone triggers adipocyte differentiation, which is likely to contribute to body weight gain. Increase in body weight seems to be a class effect of TZDs, and dose-dependent in animals and humans (Wilding, 2006). In the present study, the relatively low dose of pioglitazone (0.003%) did not increase body weight, and alogliptin in combination with pioglitazone also showed no significant effect on body weight, indicating that alogliptin plus pioglitazone combination treatment may increase efficacy without concern about further body weight gain.

In conclusion, chronic treatment with a combination of alogliptin and pioglitazone potently increased active GLP-1 levels, increased insulin secretion, inhibited glucagon secretion, augmented adiponectin production, improved glycemic control and lipid profiles, and preserved pancreatic β-cells in *ob/ob* mice. These results suggest that alogliptin plus pioglitazone combination therapy may result in not only effective glycemic and lipid control but also β-cell preservation in patients with type 2 diabetes. β-cell preservation would represent an additional benefit of combination therapy that would promote better overall health and quality of life by delaying the common complications associated with this disease. In conjunction with the lipid lowering effects and, therefore, reduced cardiovascular risk factors, this combination therapy could be extremely beneficial to the overall risk outcome.

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