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ORIGINAL ARTICLE

Combination treatment with alogliptin and voglibose increases active GLP-1 circulation, prevents the development of diabetes and preserves pancreatic beta-cells in prediabetic *db/db* mice

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Aim: Alogliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, and voglibose, an alpha-glucosidase inhibitor, have different but complementary mechanisms of action on glucagon-like peptide-1 (GLP-1) regulation and glucose-lowering effects. The present study evaluated the chronic effects of combination treatment with alogliptin and voglibose in prediabetic *db/db* mice.
Methods: Alogliptin (0.03%) and voglibose (0.001%) alone or in combination were administered in the diet to prediabetic *db/db* mice.
Results: After 3 weeks, voglibose treatment increased GLP-1 secretion (voglibose alone, 1.6-fold; alogliptin plus voglibose, 1.5-fold), while it decreased plasma glucose-dependent insulinotropic polypeptide (GIP) (voglibose alone, −30%; alogliptin plus voglibose, −29%). Alogliptin, voglibose and combination treatment decreased plasma DPP-4 activity by 72, 15 and additively by 80%, respectively, and increased plasma active GLP-1 levels by 4.5-, 1.8- and synergistically by 9.1-fold respectively. Combination treatment increased plasma insulin by 3.6-fold (alogliptin alone, 1.3-fold; voglibose alone, 1.8-fold), decreased plasma glucagon by 30% (alogliptin alone, 11%; voglibose alone, 8%), and prevented the development of diabetes, much more effectively than either agent alone. After 4 weeks, alogliptin, voglibose and combination treatment increased pancreatic insulin content by 1.6-, 3.4- and synergistically by 8.5-fold respectively. Furthermore, combination treatment resulted in an increased expression of insulin, pancreatic and duodenal homeobox 1 (PDX1) and glucose transporter 2 (GLUT2), and maintenance of normal beta/alpha-cell distribution in the pancreatic islet.
Conclusions: Chronic treatment with alogliptin in combination with voglibose concurrently increased active GLP-1 circulation, increased insulin secretion, decreased glucagon secretion, prevented the onset of the disease, and preserved pancreatic beta-cells and islet structure in prediabetic *db/db* mice.
Keywords: alogliptin, dipeptidyl peptidase-4, glucagon-like peptide-1, prediabetes, voglibose

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Introduction

Type 2 diabetes mellitus is a major health problem and is associated with high morbidity and excess mortality. Recent epidemiological studies have shown the critical contribution of hyperglycaemia in the development and progression of micro and macrovascular complications in type 2 diabetes [1]. As the prevalence of type 2 diabetes is rapidly increasing and current treatment fails to stabilize the disease in the majority of patients, prevention of diabetes should be considered one of the primary objectives [2]. Disease progression is generally associated with impaired pancreatic beta-cell function; this impairment results in a failure to compensate for insulin resistance thereby resulting in abnormally high glucose levels and progression to overt diabetes. Preventing beta-cell dysfunction, therefore, may prevent elevated blood glucose levels associated with co-morbidities, and remains an unmet need in the diabetes therapy. Recent drug treatment strategy for type 2 diabetes includes targeting the incretin system. The incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are released from the enteroendocrine cells and enhance insulin secretion; a phenomenon known as the incretin effect [3,4]. As the insulinotropic action of GLP-1 remains largely preserved compared to that of GIP, most pharmaceutical efforts directed at potentiating the incretin action have focused on agonism of the GLP-1 receptor.

In addition to its insulinotropic action, GLP-1 has been reported to stimulate proliferation, enhance differentiation and inhibit apoptosis of beta-cells [5,6]. Thus unlike other classes of drugs, GLP-1-based treatment is expected to induce direct trophic or protective effects on beta-cells in addition to its glucose-lowering effects in type 2 diabetes. Therefore,

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determining whether or not activation of GLP-1 signalling delays or prevents diabetes is of special interest. Thus, this study focused on two different mechanisms by which endogenous GLP-1 is regulated, inhibition of dipeptidyl peptidase-4 (DPP-4) and inhibition of alpha-glucosidase.

DPP-4 inhibitors have emerged as a new class of agents and have demonstrated the ability to improve glycaemic control, principally by potentiating the action of endogenously secreted incretin [7]. Alogliptin is an orally available, highly selective, quinazolinone-based, non-covalent DPP-4 inhibitor under development as a once-daily treatment for type 2 diabetes [8,9]. Alogliptin has been shown to improve glycaemic control in type 2 diabetic rodents and patients [10,11].

Alpha-glucosidase is a membrane-bound enzyme located in the epithelium of the small intestine, and is involved in the digestion of carbohydrates. By competitively inhibiting the break down of carbohydrates, alpha-glucosidase inhibitors delay the absorption of digested carbohydrates from the small intestine and thus lower both postprandial glucose and insulin levels [12]. Clinical trials have shown that treatment with alpha-glucosidase inhibitors dose-dependently increased GLP-1 circulation [13–17]. Voglibose is a clinically available alpha-glucosidase inhibitor, which has been shown to improve glycaemic control in both animal and human studies [18–20].

It is likely that combination of alogliptin and voglibose, which would prevent the inactivation of intact GLP-1 and enhance its release, would result in an increase in active GLP-1 levels in circulation. Recently, Yamazaki et al. have demonstrated that combination with a DPP-4 inhibitor and voglibose resulted in higher plasma active GLP-1 levels compared with each agent alone in the meal tolerance test in high-fat diet-fed mice [21]. Considering the different but complementary mechanisms of action by which alogliptin and voglibose lower glucose and increase GLP-1 action, combination therapy with these agents may provide a valuable means of treating diabetes. However, the effects of combination with DPP-4 inhibitors and alpha-glucosidase inhibitors on diabetic indices, diabetes prevention and pancreatic beta-cell preservation remain poorly understood. The present study was conducted to evaluate the effects of combination therapy with alogliptin and voglibose on diabetic indices, diabetes prevention and beta-cell preservation in prediabetic *db/db* mice.

Material and Methods

Test Materials

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 (Albany, NY, USA). Voglibose was manufactured at Takeda Pharmaceutical Company (Hikari, Japan). The quality of alogliptin benzoate and voglibose was analysed and found to be acceptable by Albany Molecular Research and Takeda Pharmaceutical Company respectively. All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated.

Mice

Male $Lepr^{db}/Lepr^{db}$ (*db/db*; BKS.Cg-*m* +/+ $Lepr^{db}$/Jcl) mice and their non-diabetic heterozygous (*db*/+; BKS.Cg-*m* +/+ $Lepr^{db}$/Jcl) littermates were obtained from CLEA Japan (Tokyo, Japan). All mice were housed in individual metal cages in a room with controlled temperature (23 °C), humidity (55%) and lighting (lights on from 07:30 am to 07:30 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 (Osaka, Japan).

Combination Study in *db/db* Mice

After an acclimation period of 6 days, 6-week old *db/db* mice with glucose levels comparable to those of the *db*/+ mice, which were defined as prediabetic, were divided into four groups (six mice in each group) based on the levels of glycosylated haemoglobin, plasma glucose, plasma insulin and body weight. Once-daily administration of 25 mg alogliptin induced sustained DPP-4 inhibition throughout the day in normal subjects (74.3% DPP-4 inhibition after 24 h of dosing) [22] and in patients with type 2 diabetes (78.3–81.8% DPP-4 inhibition after 24 h of dosing) [23]. Thus, 0.03% alogliptin in the diet, which induced around 80% DPP-4 inhibition in *db/db* mice [24], was selected as the study dose. A previous report showed that over 0.0025% of voglibose-induced diarrhoea and soft faeces in rodent diabetic model [20]. Thus, 0.001% voglibose in the diet, which did not induce soft faeces in *db/db* mice, was selected as the study dose. The mice were fed a powder CE-2 diet containing 0.03% alogliptin alone (equivalent to 72.8 mg/kg/day), 0.001% voglibose alone (equivalent to 1.8 mg/kg/day), or in combination (equivalent to alogliptin: 53.8 mg/kg/day + voglibose: 1.8 mg/kg/day) for 27 days. Control *db/db* and non-diabetic *db*/+ mice (six and five mice, respectively) were fed a drug-free powder CE-2 diet (vehicle). Blood samples were collected after 7, 14 and 21 days of treatment (08:00 am, non-fasting condition), and metabolic parameters were analysed. After 23 days of treatment, blood samples were collected (08:00 am, non-fasting condition), and the plasma levels of total GIP, DPP-4 activity and active GLP-1 were analysed. After 26 days of treatment, blood samples were collected for the measurement of total GLP-1 levels (08:00 am, non-fasting condition). After 27 days of treatment, all mice were fasted for 17 h and fasting plasma glucose levels were determined (08:00 am; after the 28-day study period); the pancreas was then isolated for measurement of insulin and glucagon content and immunohistochemical analysis. Body weight and food consumption were measured at regular intervals. Average food consumption was calculated using the following formula [(total weight of added food) − (total weight of remaining food)]/experimental day.

Assays for Plasma Metabolic Parameters

Glycosylated haemoglobin levels were analysed using an autoanalyzer HLC-723 GHb G7 (Tosoh, Tokyo, Japan). Plasma

glucose, triglyceride, total cholesterol and non-esterified fatty acid (NEFA) levels were determined using an autoanalyzer 7080 (Hitachi, Tokyo, Japan). Plasma insulin and total GLP-1 levels were analysed with a radioimmunoassay (RIA) kit (Millipore, Billerica, MA, USA). Plasma glucagon, total GIP and active GLP-1 levels were determined by enzyme-linked immunosorbent assays (Wako, Osaka, Japan for glucagon; Millipore for total GIP and active GLP-1).

Plasma DPP-4 Enzymatic Assay

Plasma DPP-4 activity was measured as described previously [24]. Plasma DPP-4 activity of the compound-treated *db/db* mice was compared with that of the vehicle-treated *db/db* mice, which was defined as 100%.

Pancreas Isolation and Measurement of Insulin and Glucagon Content

At the end of the chronic study, the mice were euthanized with carbon dioxide, and the pancreas was isolated and cut into two sections. One section was homogenized in acid–ethanol containing 74% ethanol with 0.15 mol/l HCl for the determination of insulin and glucagon concentrations. The other section was placed in Bouin's fixative solution (Polysciences, Warrington, PA, USA) for immunohistochemical analysis. 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% (wt/vol) bovine serum albumin, and the insulin and glucagon levels in the supernatants were determined by RIA kits (Millipore for insulin, and TFB, Tokyo, Japan for glucagon).

Immunohistochemical Analysis

After the overnight fixation, tissue samples were placed in 70% ethanol for 2 days, and subsequently embedded in paraffin. The tissue sections were dried on slides overnight at 37 °C. Paraffin sections [6 μm for insulin, glucagon, pancreatic and duodenal homeobox 1 (PDX1), and 3 μm for glucose transporter 2 (GLUT2)] were cut, deparaffinized and rehydrated at room temperature. The sections were then heated for 15 min at 90 °C in a microwave oven for glucagon and PDX1 staining, and autoclaved for 15 min at 121 °C for GLUT2 staining. Then the endogenous peroxidase was blocked with 80% methanol containing 0.6% hydrogen peroxide for 15 min. The sections were rinsed with distilled water and placed in 3% hydrogen peroxide for 15 min. Next, the sections were rinsed with distilled water and washed in Tris-buffered saline with Tween (TBST; 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) for 5 min. The sections were then reacted with the primary antibodies, ready-to-use guinea pig antiinsulin antibody (Dako, Tokyo, Japan), ready-to-use rabbit antiglucagon antibody (Dako), rabbit anti-PDX1 antibody (5 μg/ml; Transgenic, Kumamoto, Japan), or rabbit anti-GLUT2 antibody (1 μg/ml; Santa Cruz, CA, USA) for overnight at 4 °C. The sections were washed with TBST, and bound antibody was detected using a ready-to-use polymer-labelled Envision+ system (Dako) for 30 min. The sections were rinsed with TBST and developed for 1 min using 3,3′-diaminobenzidine tetrahydrochloride substrate. Finally, slides were washed with distilled water, counterstained with haematoxylin and mounted.

Statistical Analysis

Statistical analysis was performed using the SAS Version 8.2 (SAS Institute Inc., Cary, NC, USA). To evaluate if combination treatment with alogliptin and voglibose had significant additive or synergistic effects, a two-way ANOVA was performed, which generates main effects and interaction effect of alogliptin and voglibose. The evaluation of interaction effect by two-way ANOVA aims to detect synergistic or attenuation effects statistically by combination of alogliptin and voglibose. The results of two-way ANOVA were interpreted as follows: (i) When significant interaction effect (alogliptin × voglibose, $p < 0.05$) was observed, the effect by combination treatment with alogliptin and voglibose was assessed to be synergistic (it is when the effect of the combination therapy exceeds the sum of the effect of the monotherapy), or attenuation (it is when the effect of the combination therapy falls below the sum of the effect of the monotherapy), which can be determined from observed values; (ii) When no significant interaction was observed, the effect by combination treatment with alogliptin and voglibose was assessed to be neither synergistic nor attenuation. On the basis of no interaction observed, when both main effects of alogliptin treatment and voglibose treatment were significant ($p < 0.05$), the effect by combination treatment with alogliptin and voglibose was assessed to be additive (it is when the effect of the combination therapy equals the sum of the effect of the monotherapy); (iii) When only one significant ($p < 0.05$) 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 among study groups was not statistically tested in the combination study. All data are presented as the mean ± s.d.

Results

Average Food Consumption and Body Weight

Chemical structures of alogliptin and voglibose are shown in figure 1. As shown in figure 2A, average food consumption was unchanged in alogliptin-treated *db/db* mice, and decreased by 37 and 35%, respectively, in voglibose- and combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (alogliptin, N.S.; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). Body weight was unchanged in alogliptin-, decreased by 12% in voglibose-, and decreased by 7% in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (figure 2B: alogliptin, N.S.; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). Results of a two-way ANOVA indicated that voglibose treatment decreased food consumption and body weight.

Plasma Incretin and DPP-4 Activity

As shown in figure 3A, plasma total GIP levels were decreased by 30 and 29%, respectively, in voglibose- and combination-treated *db/db* mice, whereas no change was observed in

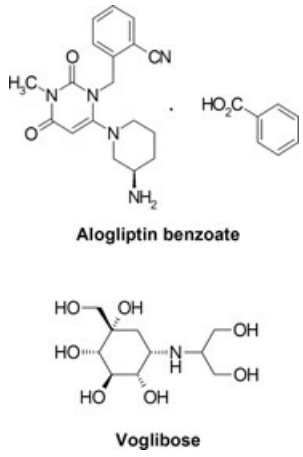


Figure 1. Chemical structures of alogliptin benzoate and voglibose.

alogliptin-treated *db/db* mice compared with vehicle-treated *db/db* mice (alogliptin, N.S.; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). In contrast to GIP levels, plasma total GLP-1 levels were increased by 1.6- and 1.5-fold in voglibose- and combination-treated *db/db* mice, respectively, whereas no change was observed in alogliptin-treated *db/db* mice, compared with vehicle-treated *db/db* mice (figure 3B: alogliptin, N.S.; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). Results of a two-way ANOVA indicated that voglibose treatment decreased plasma total GIP and increased plasma total GLP-1 levels. Interestingly, plasma DPP-4 activities were decreased by 72 and 15% in alogliptin- and voglibose-treated *db/db* mice, respectively, and additively decreased by 80% in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (figure 3C: alogliptin, $p < 0.01$; voglibose, $p < 0.05$; alogliptin × voglibose, N.S.). Plasma active GLP-1 levels were increased by 4.5- and 1.8-fold, respectively, in alogliptin- and voglibose-treated *db/db* mice, and synergistically increased by 9.1-fold in combination-treated *db/db* mice compared with vehicle-treated *db/db* mice (figure 3D: alogliptin, $p < 0.01$; voglibose, $p < 0.01$; alogliptin × voglibose, $p < 0.05$).

Plasma Insulin and Glucagon

As in some type 2 diabetic patients, *db/db* mice exhibit abnormal secretion of plasma insulin and glucagon; therefore, we traced the insulin profiles through the study. After 3 weeks of treatment (9 weeks of age), plasma insulin levels were increased by 1.3- and 1.8-fold, respectively, in alogliptin- and voglibose-treated *db/db* mice, and additively increased by 3.6-fold in combination-treated *db/db* mice compared with vehicle-treated *db/db* mice (figure 4: alogliptin, $p < 0.05$; voglibose, $p < 0.01$; alogliptin × voglibose, $p = 0.08$). Treatment with alogliptin alone, voglibose alone or vehicle alone resulted in a decrease in plasma insulin levels compared with pretreatment, however, insulin levels in combination-treated *db/db* mice were increased (figure 4). After 3 weeks of treatment with alogliptin, voglibose or a combination of alogliptin and voglibose, plasma glucagon levels were lower by 11% (276 ± 79 pg/ml), 8% (285 ± 96 pg/ml) and 30% (217 ± 42 pg/ml), respectively, than the vehicle-treated *db/db* mice (309 ± 28 pg/ml: alogliptin, $p = 0.08$; voglibose, N.S.; alogliptin × voglibose, N.S.). Although this effect of combination treatment on plasma glucagon levels was not statistically additive, combination treatment was more potent than treatment with alogliptin or voglibose alone on lowering plasma glucagon.

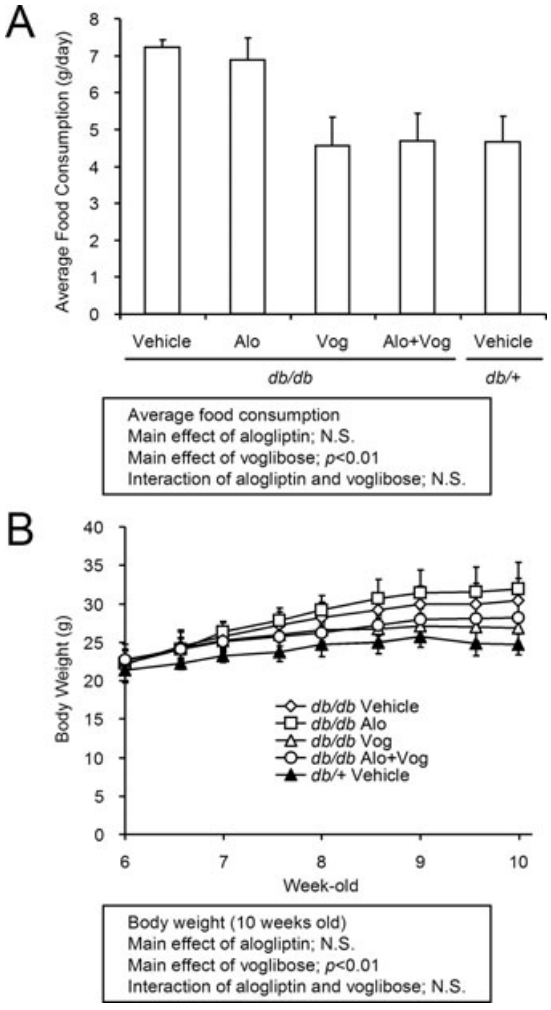
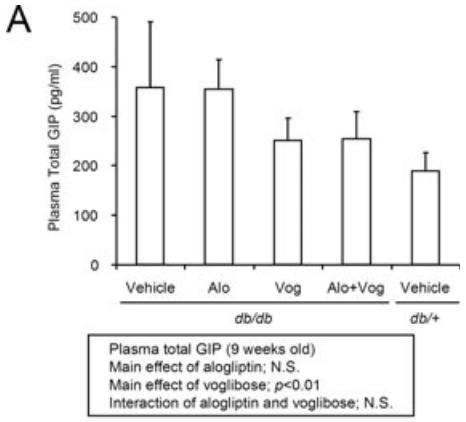
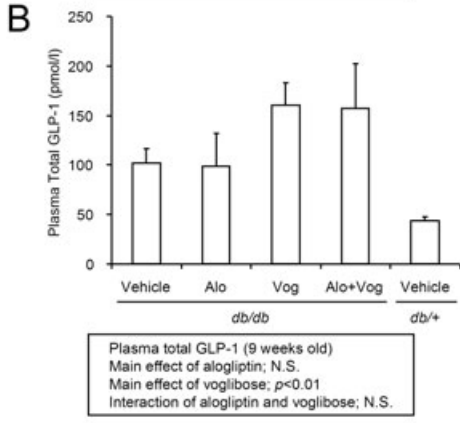


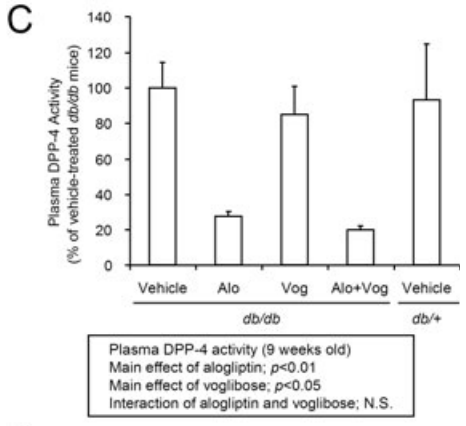
Figure 2. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on food consumption (A) and body weight (B). Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. Average food consumption and body weight were measured at designated intervals throughout the treatment period. The results of a two-way ANOVA, which are presented in the figure inserts, indicated that voglibose treatment decreased average food consumption and body weight. Alogliptin showed neutral effect on body weight and food consumption. Data are presented as means ± s.d. (n = 6 for *db/db* mice, n = 5 for *db/+* mice)

Glycaemic Parameters

Glycaemic profiles were investigated throughout the study. At 6 weeks of age (before treatment), the average levels of glycosylated haemoglobin in *db/db* and *db/+* mice were 2.6 and 2.8%, respectively, and plasma glucose in *db/db* and







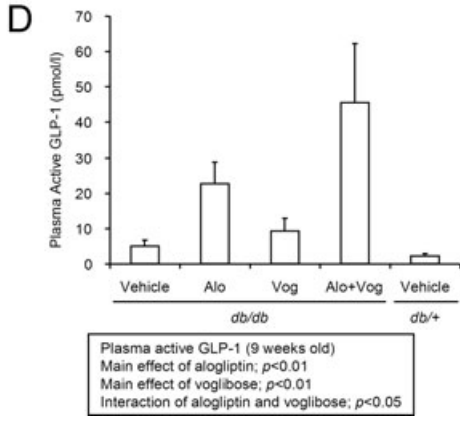


Figure 3. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on plasma levels of total GIP (A), total GLP-1 (B), DPP-4 activity (C), and active GLP-1 (D). Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. Blood samples collected after 23 days of treatment were used for the measurement of total GIP, DPP-4 activity and active GLP-1 levels. Plasma total GLP-1 levels were determined after 26 days of treatment. The results of a two-way ANOVA, which are presented in the figure insert, indicated that voglibose treatment decreased plasma total GIP levels and increased plasma total GLP-1 levels. In addition, the results of a two-way ANOVA indicated that alogliptin in combination with voglibose additively decreased plasma DPP-4 activity and synergistically increased plasma active GLP-1 levels. Data are presented as means ± s.d. (n = 6 for *db/db* mice, n = 5 for *db/+* mice).

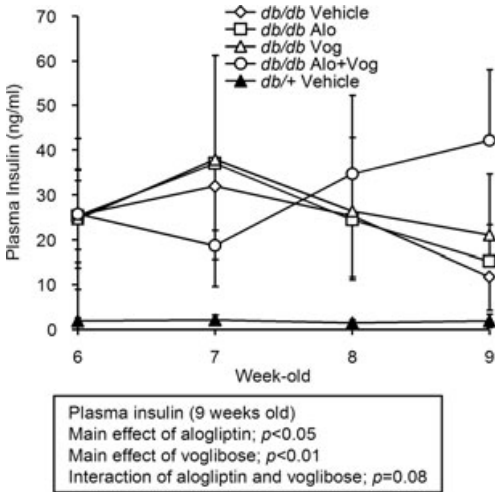


Figure 4. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on plasma insulin levels. Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. Blood samples collected after 7, 14 and 21 days of treatment were used for plasma insulin. The results of a two-way ANOVA, which are presented in the figure insert, indicated that combination with alogliptin and voglibose additively increased plasma insulin levels. Data are presented as means ± s.d. (n = 6 for *db/db* mice, n = 5 for *db/+* mice).

db/+ mice were 7.6 and 9.7 mmol/l, respectively, indicating that *db/db* mice were in the prediabetic stage. Glycosylated haemoglobin and non-fasting plasma glucose levels were analysed after 3 weeks of treatment (9 weeks of age), and fasting plasma glucose levels were analysed after 4 weeks of treatment (10 weeks of age). As shown in figure 5A, the levels of glycosylated haemoglobin were lower by 0.5 and 1.6% in alogliptin- and voglibose-treated *db/db* mice, respectively, and additively lower by 2.1% in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (alogliptin, $p < 0.05$; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). Non-fasting plasma glucose levels were lower by 2 and 32% in alogliptin- and voglibose-treated *db/db* mice, respectively, and additively lower by 61% in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (figure 5B: alogliptin, $p < 0.05$; voglibose, $p < 0.01$; alogliptin × voglibose, $p = 0.07$). As shown in figure 5C, fasting plasma

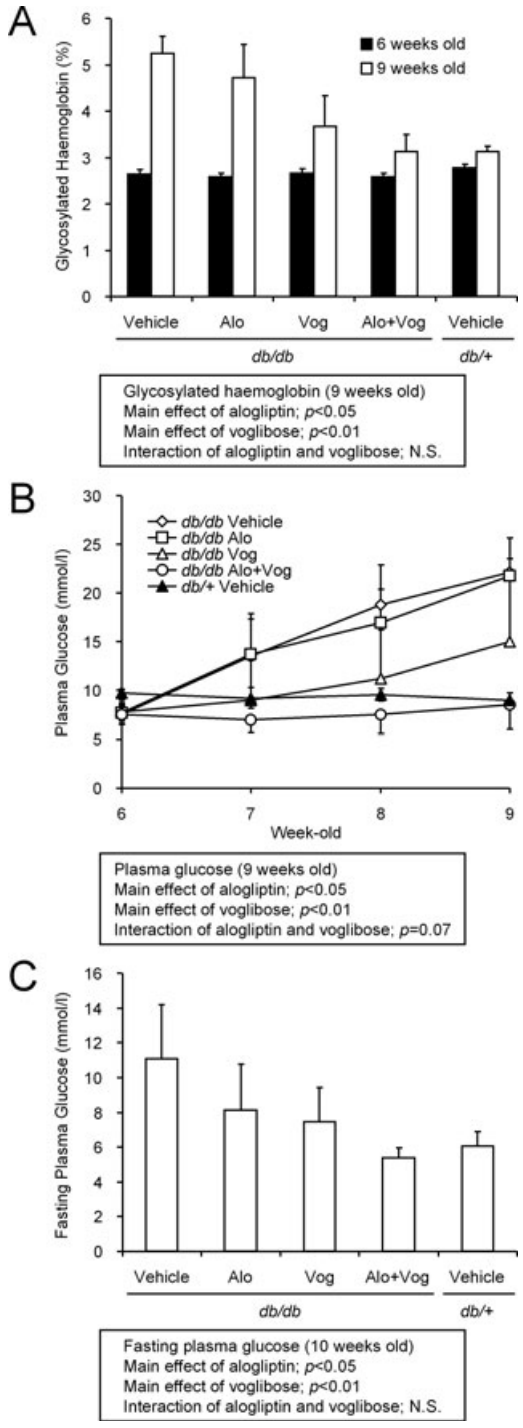


Figure 5. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on glycosylated haemoglobin (A), plasma glucose (B) and fasting plasma glucose (C). Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. Blood samples collected after 21 days of treatment were used for glycosylated haemoglobin, after 7, 14 and 21 days of treatment were used for plasma glucose, and blood samples collected after 27 days of treatment followed by 17 h fast were used for fasting plasma glucose. The results of a two-way ANOVA, which are presented in the figure inserts, indicated that combination with alogliptin and voglibose additively improved glycaemic control. Data are presented as means ± s.d. (n = 6 for *db/db* mice, n = 5 for *db/+* mice).

glucose levels were lower by 26 and 32% in alogliptin- and voglibose-treated *db/db* mice, respectively, and additively lower by 51% in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (alogliptin, $p < 0.05$; voglibose, $p < 0.01$; alogliptin × voglibose, N.S.). In the combination-treated *db/db* mice, the levels of glycosylated haemoglobin, non-fasting and fasting plasma glucose levels were equivalent to those of *db/+* mice.

Plasma Lipid Profiles

Although the combination treatment prevented hyperglycaemia as shown above, no significant combination effects were observed in the levels of plasma triglyceride (vehicle, 1.82 ± 0.87; alogliptin, 2.19 ± 0.58; voglibose, 1.45 ± 0.86; alogliptin + voglibose, 1.37 ± 0.48 mmol/l), NEFA (vehicle, 1.08 ± 0.38; alogliptin, 1.13 ± 0.20; voglibose, 1.47 ± 0.30; alogliptin + voglibose, 1.17 ± 0.27 mEq/l), or total cholesterol (vehicle, 3.00 ± 0.63; alogliptin, 3.11 ± 0.21; voglibose, 2.82 ± 0.60; alogliptin + voglibose, 2.97 ± 0.15 mmol/l) by two-way ANOVA.

Pancreatic Insulin and Glucagon Content

After 4 weeks of treatment (10 weeks of age), the levels of pancreatic insulin and glucagon content were analysed in overnight-fasted mice. Pancreatic insulin content was increased by 1.6- and 3.4-fold in alogliptin- and voglibose-treated *db/db* mice, respectively, and synergistically increased by 8.5-fold in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice (figure 6: alogliptin, $p < 0.01$; voglibose, $p < 0.01$; alogliptin × voglibose, $p < 0.05$). Pancreatic

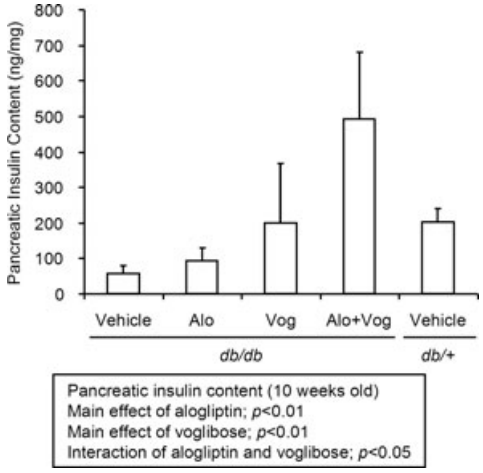


Figure 6. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on pancreatic insulin content. Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. After the treatment period, the animals were fasted for 17 h and the pancreas was isolated and homogenized to measure pancreatic insulin content. The results of a two-way ANOVA, which are presented in the figure insert, indicated that alogliptin in combination with voglibose synergistically increased pancreatic insulin content. Data are presented as means ± s.d. (n = 6 for *db/db* mice, n = 5 for *db/+* mice).

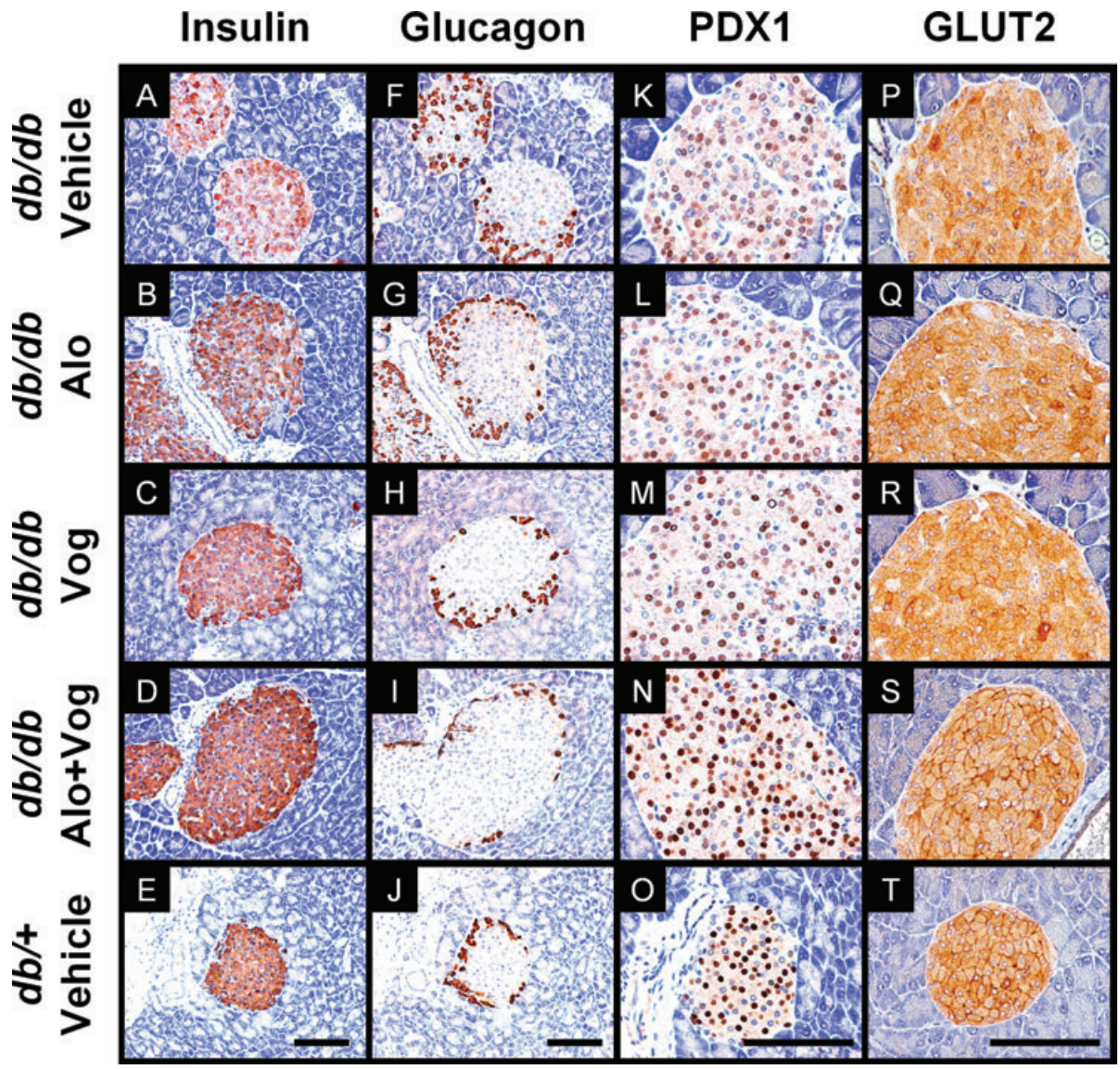


Figure 7. Chronic effects of alogliptin-, voglibose- and alogliptin plus voglibose combination treatment on insulin expression, alpha-cell localization, PDX1 expression and GLUT2 membrane localization in pancreatic islets. Animals were fed a diet containing alogliptin (0.03%), voglibose (0.001%) or alogliptin (0.03%) plus voglibose (0.001%) for 27 days. After the treatment period, the animals were fasted for 17 h and the pancreas was isolated and sectioned. The sections were stained with anti-insulin antibody (A–E), anti-glucagon antibody (F–J), anti-PDX1 antibody (K–O) or anti-GLUT2 antibody (P–T). Representative images for each group of mice are shown. Combination-treated *db/db* mice exhibited increased expression of insulin and PDX1, membrane localization of GLUT2, and normal beta/alpha-cell distribution, which were comparable to those observed in the vehicle-treated non-diabetic *db/+* mice. Scale bars = 100 µm.

glucagon content was not changed in alogliptin- or voglibose-treated *db/db* mice, whereas a slight increase (1.3-fold) was observed in combination-treated *db/db* mice, compared with vehicle-treated *db/db* mice. However, two-way ANOVA showed no significant main effect and no interaction of these compounds on pancreatic glucagon content.

Insulin, Glucagon, PDX1, and GLUT2 Staining in Islets

After the final blood sampling, the pancreas was isolated from each fasted animal and analysed by immunohistochemistry using antiinsulin, antiglucagon, anti-PDX1 and anti-GLUT2 antibodies (10 weeks of age). This analysis revealed reduced and unhomogeneous insulin staining, decreased expression of both PDX1 and GLUT2 in the beta-cells in islets of the vehicle-treated *db/db* mice when compared with the vehicle-treated non-diabetic *db/+* mice (figure 7A, E, K, O, P, T). In contrast, greatly increased insulin staining, PDX1 staining and GLUT2 membrane localization in the beta-cells were observed in the combination-treated *db/db* mice, compared with the vehicle-treated *db/db* mice (figure 7D, N, S). Glucagon staining revealed an abnormal distribution of alpha-cells in islets of the

vehicle-treated diabetic *db/db* mice compared with the vehicle-treated non-diabetic *db*/+ mice (figure 7F, J). In contrast, the distribution of glucagon-positive cells in islets was similar in the combination-treated *db/db* mice and vehicle-treated *db*/+ mice after 4 weeks of treatment (figure 7I).

Discussion

In the present study, combination treatment with alogliptin and voglibose, agents with different but complementary mechanisms of action on glucose lowering and GLP-1 regulation resulted in elevated plasma active GLP-1 levels, increased plasma insulin levels, decreased plasma total GIP and glucagon levels, and prevention of the development of diabetes. In addition to the improved metabolic profiles, combination treatment potently increased pancreatic insulin content, augmented expression of insulin, PDX1 and GLUT2, and maintained normal distribution of beta/alpha-cells in islets, some of which showed statistically significant additive or synergistic changes, demonstrating that alogliptin plus voglibose is the intensely favourable combination for treating prediabetes.

A number of studies have suggested that hyperglycaemia plays a critical role in beta-cell deterioration in diabetes [25], and improving glycaemic control has been shown to indirectly improve beta-cell function in *db/db* mice [26], whereas GLP-1 has been reported to have direct trophic or protective effects on beta-cells [5,6]. In alogliptin alone-treated *db/db* mice, over fourfold increase of active GLP-1 circulation resulted in neither robust prevention of hyperglycaemia nor sufficient beta-cell preservation. In contrast, voglibose alone moderately increased active GLP-1 levels, but still partially improved glycaemic control most likely by inhibiting the absorption of carbohydrates from the gut; this resulted in increased pancreatic insulin content that was comparable to normal mice. These results suggest that glycaemic control rather than sustained activity of endogenous incretins may be the primary mechanism by which beta-cells are preserved in this model. Consistent with alogliptin, administration of the DPP-4 inhibitors sitagliptin and vildagliptin in the diet also failed to improve glycaemic parameters in *db/db* mice (Moritoh Y et al., unpublished observations), suggesting that the increased circulating active GLP-1 levels, which were induced by DPP-4 inhibition, may not provide significant trophic or protective effects against glucose toxicity in beta-cells of this model. Conversely, alogliptin clearly improved glycaemic control and beta-cell function in *ob/ob* mice [10], which have a different disease background than *db/db* mice, indicating that an increase in active GLP-1 may induce a varying degrees of efficacy in different disease backgrounds. Thus, comparison in drug efficacy among different human races would be useful in clinical practice.

The highest levels of plasma insulin circulation, pancreatic insulin content, islets expression of insulin, PDX1 and GLUT2 [27] indicate that alogliptin plus voglibose preserved pancreatic beta-cells in *db/db* mice. The combination of highly controlled glycaemia with synergistically increased active GLP-1 may be a potential mechanism by which alogliptin plus voglibose preserved pancreatic beta-cells. The present study did not investigate the effects of alogliptin plus voglibose combination on beta-cell secretary capacity or glucose metabolism in extrapancreatic organs, both of which seem to be affected by incretin hormones [28] and glycaemic control. Thus measuring glucose tolerance, insulin secretion in response to glucose, and insulin sensitivity may elucidate the effects of alogliptin plus voglibose combination on beta-cell function and extrapancreatic site. Furthermore, clinical evaluation in prediabetic human individuals is essential, because the effect of DPP-4 inhibitors and alpha-glucosidase inhibitors on beta-cell mass remains to be directly demonstrated in patients with type 2 diabetes [29].

Oral ingestion of carbohydrates promotes the secretion of both GLP-1 and GIP from the gut L- and K-cells respectively [30]. The sugars that cross the intestinal epithelium stimulate GLP-1 release, whereas non-transportable sugars do not [31]. Furthermore, the intestinal glucose absorption rate was correlated with GIP secretion in healthy men [32]. These observations indicate that gut absorption of digested carbohydrates has a pivotal role on incretin secretion. Considering that GIP- and GLP-1-secreting cells are abundant in the upper- and lower-gut, respectively, and the majority of glucose is absorbed before reaching the ileum under normal feeding condition [33], voglibose-induced delay of carbohydrate degradation may have led to decreased GIP secretion in the upper-intestine and increased GLP-1 secretion from the lower part of the gut in *db/db* mice. In the present study, the levels of peptide tyrosine–tyrosine (PYY), which has effects on appetite regulation and energy homeostasis [34], were not measured because of the limited sample volume. Considering that PYY is secreted from the gut L-cells together with GLP-1 and cleaved by DPP-4, measurement of PYY levels may explain some of the observations that are a result of alogliptin plus voglibose combination.

The combination of GIP and GLP-1 accounts for the full incretin effect in normal subjects [35], whereas patients with type 2 diabetes have one or more defects in the incretin axis [4]. Although the insulinotropic effect of GLP-1 is preserved to a much greater extent than that of GIP, which is reduced or even lost, GLP-1 secretion is still slightly but significantly decreased in some but not all patients with type 2 diabetes [4,36]. In addition, increasing evidence has shown that GIP has a significant effect on adipocytes, which have functional GIP receptors, by increasing deposition rather than mobilization of fat stores [37]. Interestingly, genetic knockout or chemical antagonism of GIP receptor resulted in improved metabolic profiles in obesity-related diabetic models [38,39], indicating that GIP antagonism may be effective for obesity-related diabetes. In the present study, the combination with alogliptin and voglibose co-ordinately increased plasma active GLP-1 levels, and decreased plasma total GIP levels as well as body weight. Thus, the incretin changes observed as a result of the combination of alogliptin plus voglibose suggest that combination treatment may provide a favourable and useful option for treatment of obesity-related type 2 diabetes.

In the present study, alogliptin had no effect on plasma total GLP-1 levels but potently inhibited plasma DPP-4 activity as

was expected, resulting in an increase in the active forms of GLP-1 by up to 4.5-fold. Unlike alogliptin, voglibose alone increased plasma total GLP-1 levels, suggesting it stimulated secretion of GLP-1 from gut L-cells. Voglibose also slightly decreased (−15%) plasma DPP-4 activity. Thus, the 1.8-fold increase in plasma active GLP-1 levels observed in voglibose-treated *db/db* mice can be interpreted as a result of both stimulated GLP-1 secretion and decreased plasma DPP-4 activity. Interestingly, voglibose was unable to directly inhibit DPP-4 enzymatic activity *in vitro*, but decreased plasma DPP-4 activity by reducing circulating DPP-4 protein in *ob/ob* mice [40]. Therefore, the voglibose-induced decrease in DPP-4 activity is likely because of a reduction in the circulation of the DPP-4 protein in *db/db* mice. In fact, DPP-4 activity was additively decreased (−80%) by combination of alogliptin and voglibose, resulting in synergistically increased active GLP-1 circulation (9.1-fold).

GLP-1 has the ability to inhibit gastric empting and food intake [3], thus reducing body weight. In the present study, alogliptin alone had neutral effects on body weight; combination treatment with alogliptin and voglibose had no additive effect on body weight when compared to voglibose alone. This suggests that the decrease in food intake, which seemed to be a result of abdominal symptoms caused by voglibose administration, may play a major role for decreasing body weight in *db/db* mice.

Plasma GIP levels were increased in prediabetic and diabetic humans [41–43] similarly to those observed in *db/db* mice in this study. Although physiological explanation for the increased GIP levels in prediabetic and diabetic humans still awaits further investigation, *db/db* mice may be the good model for studying abnormal GIP secretion.

In conclusion, these studies have shown that combination treatment with alogliptin and voglibose was able to improve diabetic indices, prevent the development of diabetes, and preserved pancreatic beta-cells and islet structure in prediabetic *db/db* mice. Both increased active GLP-1 and highly controlled glycaemia may have been the mechanisms by which the beneficial effects were achieved in this model. Although an appropriate clinical trial is essential to further elucidate the relevance of the present preclinical study, clinical combination using these drugs may provide a useful therapeutic option for the treatment and/or prevention of type 2 diabetes.

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Conflicts of Interest

Y. M., K. T. and M. H. are employees of Takeda Pharmaceutical Company. K. T. and M. H. own Takeda stock and/or stock options at the time of manuscript preparation.

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