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ASP8497 is a novel selective and competitive dipeptidyl peptidase-IV inhibitor with antihyperglycemic activity

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ABSTRACT

Dipeptidyl peptidase (DPP)-IV inhibitors are expected to become a useful new class of antidiabetic agent. The aim of the present study is to characterize the *in vitro* and *in vivo* profile of ASP8497, (2S,4S)-4-fluoro-1-({[4-methyl-1-(methylsulfonyl)piperidin-4-yl]amino}acetyl)pyrrolidine-2-carbonitrile monofumarate, which is a novel DPP-IV inhibitor. ASP8497 inhibited DPP-IV in plasma from mice, rats, dogs and humans with IC_{50} values of 3.86, 2.36, 5.53 and 5.30 nM, respectively. In contrast, ASP8497 did not potently inhibit DPP8 or DPP9 activity ($IC_{50} > 200$ nM). Kinetic analysis indicated that ASP8497 inhibits DPP-IV activity in a competitive manner. In streptozotocin–nicotinamide-induced diabetic mice, ASP8497 (3 mg/kg) significantly reduced glucose excursion during the oral glucose tolerance test conducted 0.5 and 8.5 h after administration, with increases in plasma insulin and active glucagon-like peptide-1 (GLP-1) levels. In contrast, ASP8497 (3 and 30 mg/kg) did not cause hypoglycemia in fasted normal mice. Furthermore, administration of exogenous GLP-1 induced significant inhibition of gastric emptying and small intestinal transit rates, but ASP8497 (30 mg/kg) had no significant effects in normal mice. These present preclinical studies indicate that ASP8497 is a novel selective DPP-IV inhibitor with long-acting antidiabetic effect that might be a potential agent for type 2 diabetes.

1. Introduction

Impaired insulin secretion in type 2 diabetes is often characterized by a decreased first-phase insulin response which leads to postprandial hyperglycemia [1]. Sulfonylureas, which are widely used as potent hypoglycemic agents for type 2 diabetes, stimulate insulin secretion irrespective of blood glucose levels, and thus, cause hypoglycemia [2]. To complement the diabetes treatments currently available, approaches that operate through incretins, which potentiate physiological glucose-dependent insulin release, are beginning to show promise. Although several gastrointestinal regulatory peptides have been proposed as incretins, only glucagon-like peptide 1 [GLP-1 or GLP-1(7–36)amide] and glucose-dependent insulinotropic polypeptide-1 [GIP or GIP(1–42)] appear to meet the requirements for consideration as physiological stimulants of postprandial insulin release [3]. Among these, GLP-1, which is the most potent endogenous insulinotropic peptide, is secreted from L cells in the intestine in response to oral ingestion of nutrients [4]. GLP-1 exerts multiple actions, including glucose-dependent stimulation of insulin biosynthesis and secretion, regulation of GLUT-2 and glucokinase gene expression, trophic effect of pancreatic β-cells, slowing of gastric emptying and inhibition of appetite [5–8]. However, the active form of GLP-1 is rapidly degraded ($t_{1/2} < 1$ min) to an inactive form [GLP-1(9–36)amide] by dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5), which catalyzes the cleavage of two residues from the NH_2-terminal end of the peptide, NH_2-His-

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Ala, and it has been demonstrated that this cleavage is the primary physiological route of GLP-1 degradation in both humans and animals [9,10]. Therefore, the inhibition of DPP-IV prevents degradation of biologically active GLP-1 and enhances glucose-dependent insulin secretion from pancreatic β-cells, which leads to a reduction in postprandial glucose levels without affecting fasting blood glucose levels. For these reasons, orally active DPP-IV inhibitors are currently being actively explored as a novel approach to the treatment of type 2 diabetes. Indeed, it has been reported that several DPP-IV inhibitors attenuate glucose excursion with the elevation of plasma GLP-1 and insulin levels in animal models of diabetes [11–13]. Furthermore, clinical efficacy has been reported for inhibitors such as sitagliptin (MK-0431) and vildagliptin (LFA237) [14,15].

DPP-IV is a member of the family of DPP-IV activity and/or structure homologue (DASH) proteins, which are enzymes unified by their common post-proline cleaving, serine dipeptidyl peptidase mechanism [16]. Family members include DPP8 [17] and DPP9 [18]. Recently, Lankas et al. suggested that inhibition of DPP8 and DPP9 may be associated with multiorgan toxicity and the production of profound immunotoxicity in preclinical studies [19]. Therefore, it is important to characterize enzyme selectivity in the preclinical stage to avoid any potential risk due to DPP8 and/or DPP9 inhibition. In addition, recent studies have suggested that exogenous GLP-1 or GLP-1 derivatives cause a delay in gastric emptying both in healthy volunteers and type 2 diabetes patients [8,20,21]. On the other hand, no delay in gastric emptying occurred when the endogenous GLP-1 level increased following administration of DPP-IV inhibitors [22]. One possible explanation for this discrepancy is that there may be a difference in the plasma GLP-1 levels that induce the incretin and gastrointestinal effects. Therefore, it is important to investigate the effect of DPP-IV inhibitors on gastrointestinal functions and plasma GLP-1 levels. The purpose of the present study is to characterize the pharmacological profile of ASP8497, (2*S*,4*S*)-4-fluoro-1-({[4-methyl-1-(methylsulfonyl)piperidin-4-yl]amino}acetyl)pyrrolidine-2-carbonitrile monofumarate (Fig. 1), which is a novel DPP-IV inhibitor that was discovered in our laboratories. This characterization was conducted with regard to the following points: (1) assessment of inhibitory potency against DPP-IV, DPP8 and DPP9, and analysis of the DPP-IV inhibition kinetics, (2) investigation of the effects on blood glucose, plasma insulin and GLP-1 levels after oral glucose loading in streptozotocin–nicotinamide-induced diabetic mice, (3) investigation of effects on gastric emptying and small intestinal transit in normal mice.

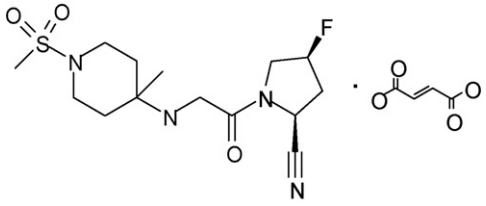

Fig. 1 – Chemical structure of ASP8497, (2*S*,4*S*)-4-fluoro-1-({[4-methyl-1-(methylsulfonyl)piperidin-4-yl]amino}acetyl)pyrrolidine-2-carbonitrile monofumarate.

2. Materials and methods

2.1. Materials

ASP8497, vildagliptin (LAF237; 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(*S*)-pyrrolidine), sitagliptin (MK-0431; (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro [1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine phosphate), saxagliptin (BMS-477178; (1*S*,3*S*,5*S*)-2-[(2*S*)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo[3.1.0]-hexane-3-carbonitrile hydrochloride) and DPP8/9-selective inhibitor ((2*S*,3*R*)-1-(1,3-dihydro-2*H*-isoindol-2-yl)-3-methyl-1-oxo-2-pentanamine (2*E*)-2-butenedioate) were synthesized by Astellas Pharma Inc. (Ibaraki, Japan). Gliclazide was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). These compounds were dissolved or suspended in purified water, and then orally administered. Active GLP-1(7–36 amide) was purchased from the Peptide Institute, Inc. (Osaka, Japan), dissolved in physiological saline, and administered intraperitoneally. *H*-glycyl-prolyl-7-amino-4-methylcoumarine (Gly-Pro-AMC) was purchased from Bachem Bioscience, Inc. (King of Prussia, PA, USA).

2.2. Animals

Male ICR mice (6 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used at the age of 7 weeks. Streptozotocin–nicotinamide-induced diabetic mice were created by treating the mice as follows: fasting overnight and intraperitoneally administering a nicotinamide solution (1000 mg/kg), followed by a streptozotocin solution (150 mg/kg) 90 min later. Normal control mice were intraperitoneally administered physiological saline. Blood glucose levels were measured in the diabetic mice 1 week later, after which they were grouped so that the blood glucose levels were uniform among the groups. All mice were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet (CE-2; Oriental Yeast, Tokyo, Japan) and water (ad libitum). All experimental procedures were conducted according to the Animal Ethical Committee of Astellas Pharma Inc.

2.3. DPP-IV inhibition assay

Mouse, rat, dog and human plasma were used to measure DPP-IV activity. Each plasma was mixed with a compound or vehicle in an assay buffer (25 mM HEPES, 140 mM NaCl, 80 mM $MgCl_2$, 0.5% BSA, pH 7.3). The enzyme reaction was started by adding 0.05 mM Gly-Pro-AMC as a substrate, and then incubating for 20 min at room temperature. The fluorescence intensities (excitation: 355 nm, emission: 460 nm) were measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Plasma DPP-IV activity was measured in the same manner and catalytic DPP-IV activity in plasma was calculated as the cleavage rate of AMC from the substrate Gly-Pro-AMC (nmol/(min mL)) and expressed as the inhibitory rate from the value of the vehicle group. Basal plasma DPP-IV activities in normal and diabetic mice were 11.7 ± 0.2 and 11.9 ± 0.3 nmol/(min mL), respectively.

2.4. DPP8 and DPP9 inhibition assay

The recombinant human DPP8 and DPP9 were expressed by HEK-293 (EBNA) cells, and then purified [23]. Mouse, rat and dog DPP8 and DPP9 were purified from liver and testis tissues from male ICR mice, SD rats and beagle dogs. Briefly, all purification steps were conducted at 4 °C, and at each step, the activity of DPP was measured as described above. Liver and testis tissues were homogenized with six volumes of 0.25 M sucrose solution and centrifuged (1000 × *g*, 10 min), after which the supernatant was centrifuged further (100,000 × *g*, 60 min). The supernatant was brought to 70% saturation with sodium ammonium sulfate, and after stirring for an additional 60 min, recentrifuged (10,000 × *g*, 45 min). The precipitate obtained was dissolved in the minimum volume of 25 mM Tris–HCl (pH 8.4) buffer and applied at a flow rate of 0.2 mL/min to a Resource Q column attached to an AKTA purifier system (Amersham Pharmacia Biotech, NJ, USA) pre-equilibrated with Tris–HCl buffer. A linear gradient was then formed with Tris–HCl buffer containing 0.5 M NaCl. Fractions with DPP activity were collected and dialyzed overnight against assay buffer (25 mM HEPES, 140 mM NaCl, 80 mM $MgCl_2$, 0.5% BSA, pH 7.3). A DPP8 and DPP9 inhibition assay was performed as described in Section 2.3.

2.5. Inhibitory kinetics study

Human plasma DPP-IV activity was measured at various concentrations of ASP8497 (1–30 nM). For each concentration, measurements were conducted in the presence of various concentrations of Gly-Pro-AMC (0–3200 μM) as a substrate. The inhibition pattern was evaluated using a curve-fitting program (GraphPad Software, Inc., San Diego, CA, USA).

2.6. Specificity assay

Binding and enzyme assays for several receptors, channels and enzymes were performed according to the procedures of MDS Pharma Services (Bothell, WA, USA).

2.7. Effects of ASP8497 on blood glucose levels during the oral glucose tolerance test (OGTT) in diabetic mice

Blood samples were collected from normal and diabetic mice fasted overnight for the measurement of blood glucose levels and to which either the vehicle or the test compound (ASP8497: 3 mg/kg; gliclazide: 10 mg/kg) had been orally administered. After 30 min, blood glucose levels were measured again, after which glucose solution (2 g/kg) was orally administered (1st OGTT). At 0.5, 1, 2 and 3 h after glucose loading, blood glucose levels were measured. At 8 h after the first glucose loading (8.5 h after drug administration), blood glucose levels were measured, and then glucose solution was orally administered (2nd OGTT). The blood glucose levels were again measured at 0.5, 1, 2 and 3 h after the second glucose loading.

2.8. Effects of ASP8497 on plasma insulin and GLP-1 levels during the OGTT in diabetic mice

Normal and diabetic mice were fasted overnight, and blood samples (basal value) were collected to measure plasma insulin and GLP-1 levels. Either the vehicle or the test compound (ASP8497: 3 mg/kg; gliclazide: 10 mg/kg) was administered orally, and blood samples were collected 30 min later (1st OGTT-pre value). Glucose solution (2 g/kg) was then orally administered, and blood samples were collected 10 min later (1st OGTT-10 min value). Eight hours after the first glucose loading, blood samples were collected (2nd OGTT-pre value). Glucose solution was then orally administered, and blood samples were collected 10 min later (2nd OGTT-10 min value).

2.9. Effects of ASP8497 on fasting blood glucose levels in normal mice

Either the vehicle or the test compound (ASP8497: 3 and 30 mg/kg; gliclazide: 10 mg/kg) was orally administered to mice fasted overnight and the blood glucose levels were measured before (0 h) and 0.5, 1, 2, 4 and 6 h after administration. In addition, effects of ASP8497 and gliclazide on blood glucose levels during the OGTT were investigated by a similar method described in Section 2.7. In brief, blood samples were collected from mice fasted overnight and to which either the vehicle or the test compound has been orally administered. After 5 min, glucose solution was orally administered, and blood glucose levels were measured at 0.5, 1, 2, 4 and 6 h after glucose loading.

2.10. Effects of GLP-1 and ASP8497 on gastrointestinal functions in normal mice

Either the vehicle or the test compound (GLP-1: 25, 50, 100 and 200 μg/kg; ASP8497: 30 mg/kg) was administered to normal mice that had been fasted overnight. Five minutes later (GLP-1 treatment examination) or 30 min later (ASP8497 treatment examination), glucose solution (0.2 g/mL glucose, 0.25% methylcellulose, 1 mg/mL phenol red and 10 mg/mL charcoal) was orally administered at a volume of 15 mL/kg. After 15 min, blood samples for the measurement of blood glucose, plasma insulin and active GLP-1 levels were collected. Under ether anesthesia, the stomach was ligated and removed, after which it was transferred to a tube and cryopreserved. The entire length of the small intestine (between the pylorus of the stomach and the end of the ileum) and the distance to the charcoal front were measured. The mice in the control group were given vehicle solution in order to measure the total amount of glucose solution injected into the stomach. At 5 or 30 min after administration, blood samples were collected, and the pylorus of the stomach was ligated under ether anesthesia. Glucose solution was then orally administered, after which the stomach was immediately removed, and small intestinal transit was checked. To measure the gastric emptying rate, 0.1 mol/L NaOH solution (5 mL) was added to the stomach sample, and they were homogenized. After centrifugation (3000 rpm, 10 min), 20% TCA solution (50 μL) was added to a 500-μL aliquot of the supernatant. The mixture was then stirred and centrifuged (15,000 rpm, 10 min). A 100-μL aliquot of the supernatant was then dispensed into a 96-well assay plate, and 0.5 mol/L NaOH solution (50 μL) was added. After stirring, phenol red concentration in the sample was determined from a phenol red (0–1000 μg/mL) calibration

Table 1 – Inhibitory concentrations of ASP8497 against DPP-IV

	IC_{50} (nM)			
	Mouse	Rat	Dog	Human
ASP8497	3.86 ± 0.48	2.36 ± 0.08	5.53 ± 0.22	5.30 ± 0.54
Vildagliptin	3.24 ± 0.33	1.67 ± 0.06	3.47 ± 0.07	5.28 ± 1.04
Sitagliptin	21.3 ± 1.0	11.5 ± 0.2	10.8 ± 0.3	9.96 ± 1.03
Saxagliptin	2.57 ± 0.13	1.71 ± 0.06	4.22 ± 0.20	3.37 ± 0.90
DPP8/9 selective inhibitor	22900 ± 1200	18300 ± 200	20100 ± 1400	35800 ± 3300

Values are expressed as the means ± S.E. obtained from three independent experiments.

Table 2 – Inhibitory concentrations of ASP8497 against DPP8

	IC_{50} (nM)			
	Mouse	Rat	Dog	Human
ASP8497	5370 ± 180 (1400)	4860 ± 150 (2100)	4420 ± 110 (800)	2830 ± 271 (530)
Vildagliptin	1910 ± 40 (590)	1260 ± 140 (750)	1240 ± 70 (360)	1112 ± 50 (210)
Sitagliptin	28400 ± 800 (1300)	32800 ± 8300 (2900)	31900 ± 3500 (3000)	26800 ± 3000 (2700)
Saxagliptin	358 ± 19 (140)	226 ± 16 (130)	191 ± 16 (45)	244 ± 8 (72)
DPP8/9 selective inhibitor	22.1 ± 0.8 (0.001)	19.3 ± 1.9 (0.001)	15.9 ± 1.0 (0.0008)	11.9 ± 0.5 (0.0003)

Values are expressed as the means ± S.E. obtained from three independent experiments. Values in parentheses are the IC_{50} ratio of DPP8/DPP-IV.

Table 3 – Inhibitory concentrations of ASP8497 against DPP9

	IC_{50} (nM)			
	Mouse	Rat	Dog	Human
ASP8497	224 ± 10 (58)	349 ± 14 (150)	655 ± 14 (120)	436 ± 91 (82)
Vildagliptin	271 ± 36 (84)	120 ± 6 (72)	117 ± 7 (34)	66.2 ± 7.3 (13)
Sitagliptin	25900 ± 400 (1200)	36600 ± 2900 (3200)	57900 ± 4800 (5400)	48500 ± 5700 (4900)
Saxagliptin	93.5 ± 2.2 (36)	58.8 ± 2.5 (34)	130 ± 4 (31)	104 ± 7 (31)
DPP8/9 selective inhibitor	14.7 ± 1.5 (0.0006)	8.16 ± 0.57 (0.0005)	14.8 ± 0.6 (0.0007)	12.2 ± 1.4 (0.0003)

Values are expressed as the means ± S.E. obtained from three independent experiments. Values in parentheses are the IC_{50} ratio of DPP9/DPP-IV.

curve. The gastric emptying rate (%) was then calculated using the following equation: [(mean value of the control group) − (the sample value)]/(mean value of the control group). The small intestinal transit rate (%) was calculated using the following equation: (the distance traveled by the charcoal front)/(the entire length of the small intestine).

2.11. *Biochemical determination*

Blood glucose levels were measured using Glucose CII-Test reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma insulin and active GLP-1 levels were determined using a Biotrak rat insulin RIA kit (Amersham Biosciences Corp., NJ, USA) and an active GLP-1 ELISA kit (Linco Research, Inc., MO, USA).

2.12. *Statistical analysis*

The experimental results are expressed as the means ± S.E. The IC_{50} values were calculated using regression analysis. The significance of differences between the two groups was determined using the Student's *t*-test. The significance of differences between multiple groups was assessed using Dunnett's multiple range test. A value of $P < 0.05$ was taken to be significant. Statistical and data analysis were conducted using the SAS 8.2 software package (SAS Institute Japan, Ltd., Tokyo, Japan).

3. Results

3.1. *DPP-IV inhibition assay*

ASP8497 potently inhibited DPP-IV in mouse, rat, dog and human plasma, with IC_{50} values of 3.86, 2.36, 5.53 and 5.30 nM, respectively (Table 1). Vildagliptin, sitagliptin and saxagliptin also potently inhibited DPP-IV activity in all species. In contrast, the DPP8/9-selective inhibitor had no potent inhibitory effect ($IC_{50} > 10{,}000$ nM).

3.2. *DPP8 and DPP9 inhibition assay*

ASP8497 did not potently inhibit DPP8 or DPP9 activity in any species (Tables 2 and 3). IC_{50} values of ASP8497 for human

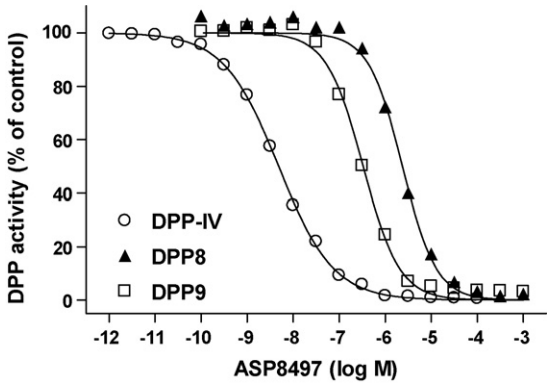

Fig. 2 – The inhibitory effects of ASP8497 on human DPP-IV, DPP8 and DPP9. Results are representative data from three independent experiments.

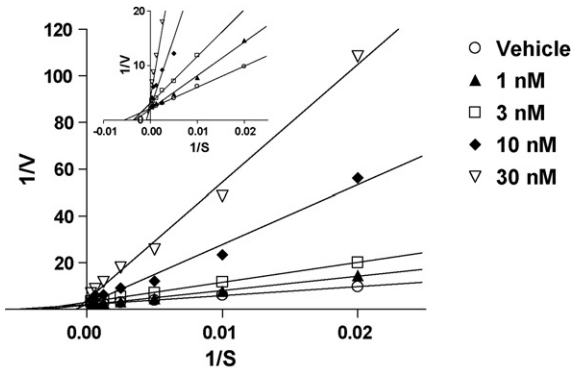

Fig. 3 – Inhibition kinetics of human plasma DPP-IV by ASP8497. Different concentrations of ASP8497 (1, 3, 10 and 30 nM) were incubated in the presence of various concentrations of Gly-Pro-AMC (0–3200 μM) as the substrate. Initial rates of the reaction were measured, and the results are expressed as a Lineweaver–Burk plot. The insert shows an enlargement of the plot. Data represent the mean of four independent experiments.

DPP8 and DPP9 activity were 2830 and 436 nM, respectively; this proved to be 530 and 82 times more selective for human DPP-IV (Fig. 2). In addition, vildagliptin, sitagliptin and saxagliptin did not also potently inhibit DPP8 or DPP9 activity and exhibited selectivity for DPP-IV. Among these, sitagliptin exhibited the highest selectivity for DPP-IV. In contrast, the DPP8/9-selective inhibitor potently inhibited DPP8 and DPP9 activity in all species.

3.3. *Inhibitory kinetics study*

To identify the DPP-IV inhibition kinetic patterns of ASP8497, we carried out inhibition kinetic analyses using human plasma as the enzyme source. ASP8497 showed a competitive inhibition pattern well-fitted to a Lineweaver–Burk plot, and its K_i value was estimated to be 1.32 nM (Fig. 3).

3.4. *Specificity assay*

To confirm the specificity, effects of ASP8497 on several representative receptors, channels and enzymes were examined using radioligand binding and enzyme assays. At 10 μM, ASP8497 showed no significant activity for the following receptors, channels or enzymes: adrenergic (α1, α2 and β), angiotensin (AT_1 and AT_2), atrial natriuretic factor, bombesin, bradykinin (B_1 and B_2), calcitonin, calcitonin gene-related peptide, calcium channel (L-type and N-type), dopamine (D_1–D_5), endothelin (ET_A and ET_B), galanin (GAL_1 and GAL_2), glucagon-like peptide 1, motilin, muscarinic (M_1–M_5), neuropeptide (Y_1 and Y_2), neurotensin, nicotinic acetylcholine, serotonin (5-HT_1 to 5HT_7), vasoactive intestinal peptide, vasopressin (V_{1A}, V_{1B} and V_2), acetylcholinesterase, choline acetyltransferase and angiotensin-converting enzyme.

3.5. *Effects of ASP8497 on blood glucose levels during the OGTT in diabetic mice*

In streptozotocin–nicotinamide-induced diabetic mice, ASP8497 (3 mg/kg) significantly inhibited increases in the blood glucose level during the OGTT conducted both 0.5 h (1st)

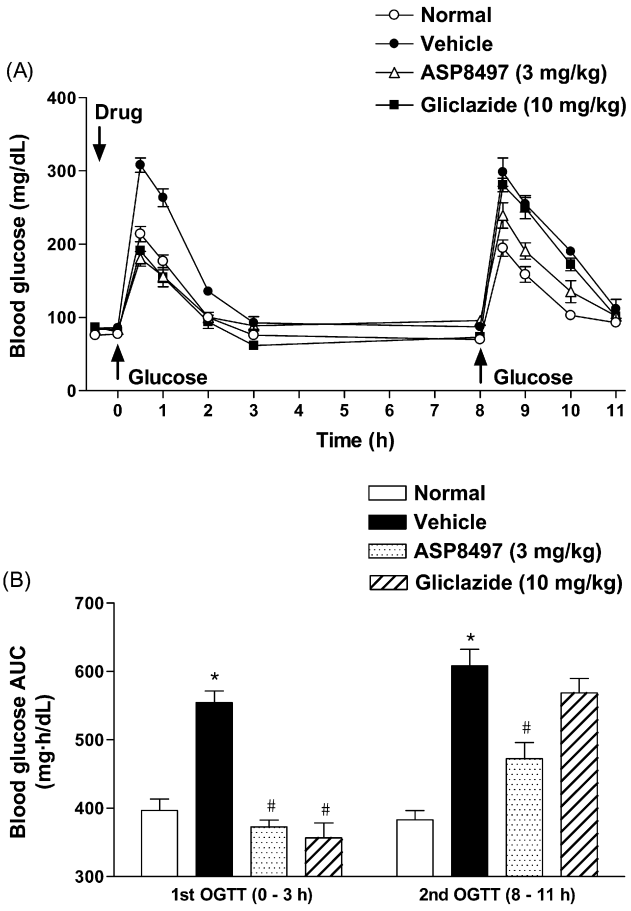

Fig. 4 – Effects of ASP8497 on blood glucose levels during the oral glucose tolerance test in streptozotocin–nicotinamide-induced diabetic mice: (A) time course of changes in blood glucose levels during the oral glucose tolerance test (OGTT) and (B) the area under the blood glucose concentration–time curve (AUC) during the OGTT. The values are the means ± S.E. from five animals in each group. *$P < 0.05$ vs. normal group, #$P < 0.05$ vs. vehicle group.

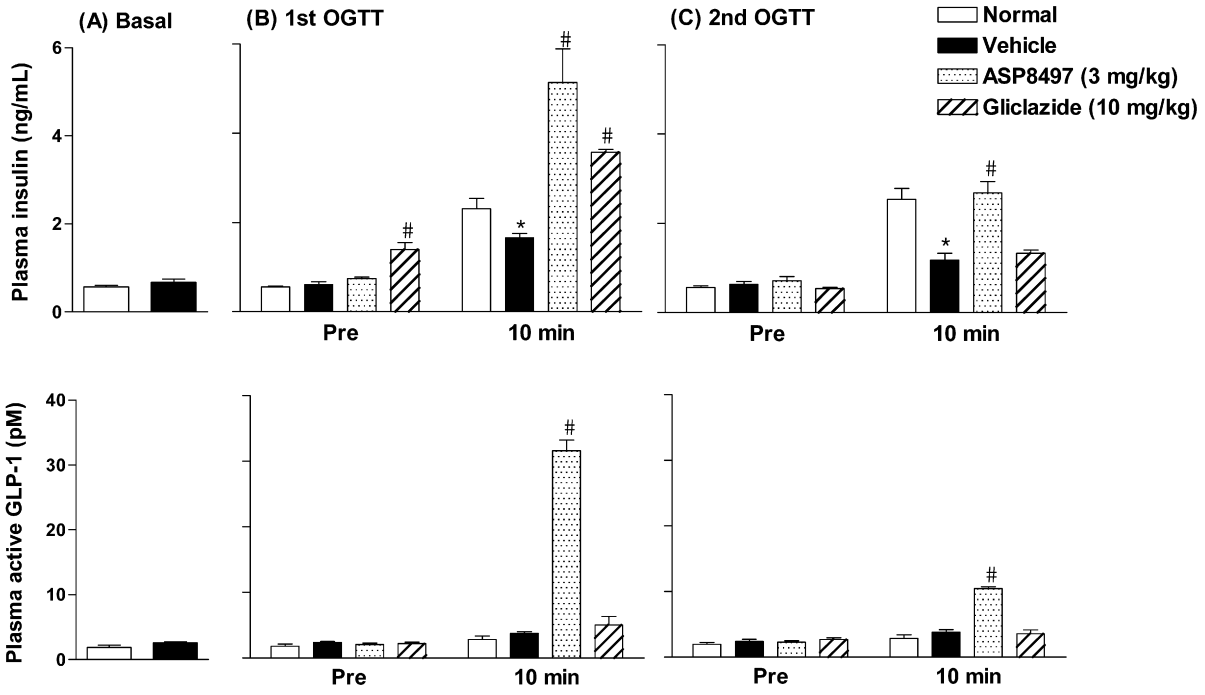

Fig. 5 – Effects of ASP8497 on plasma insulin and active GLP-1 levels during the oral glucose tolerance test in streptozotocin–nicotinamide-induced diabetic mice: (A) basal (fasted) plasma insulin and active GLP-1 levels. Plasma insulin and active GLP-1 levels at 0 min (pre) and 10 min during the oral glucose tolerance test (OGTT) conducted (B) 0.5 h (1st OGTT) and (C) 8 h (2nd OGTT) after drug administration. The values are the means ± S.E. from five animals in each group. *P < 0.05 vs. normal group, #P < 0.05 vs. vehicle group.

and 8.5 h (2nd) after drug administration (Fig. 4). In contrast, gliclazide (10 mg/kg) significantly improved glucose tolerance during the 1st OGTT, but had no significant effect during the 2nd OGTT.

3.6. *Effects of ASP8497 on plasma insulin and GLP-1 levels during the OGTT in diabetic mice*

ASP8497 did not change plasma insulin and GLP-1 levels at pre values, but significantly increased at 10 min values during the OGTT conducted both 0.5 h (1st) and 8 h (2nd) after drug administration (Fig. 5). In addition, ASP8497 significantly inhibited plasma DPP-IV activity, even after the 2nd OGTT (1st OGTT: 90.7 ± 0.5% inhibition; 2nd OGTT: 65.2 ± 3.1% inhibition). In contrast, gliclazide significantly increased plasma insulin levels at pre and 10 min values during the 1st OGTT, but had no significant effect during the 2nd OGTT. In addition, gliclazide did not influence plasma GLP-1 levels during both the 1st and 2nd OGTT.

3.7. *Effects of ASP8497 on fasting blood glucose levels in normal mice*

In normal mice, ASP8497 (3 and 30 mg/kg) and gliclazide (10 mg/kg) significantly inhibited increases in the blood glucose level during the OGTT. In contrast, ASP8497 had no significant effect on fasting blood glucose levels even at a dose of 30 mg/kg, but gliclazide significantly reduced (Fig. 6).

3.8. *Effects of GLP-1 and ASP8497 on gastrointestinal functions in normal mice*

Intraperitoneal administration of active GLP-1 (25–200 μg/kg) dose-dependently increased the plasma insulin level and decreased the blood glucose level with a concomitant increase in the plasma active GLP-1 level. These effects were significant even at the lowest dose of 25 μg/kg (Fig. 7). In addition, GLP-1 dose-dependently inhibited gastric emptying and small intestinal transit rates, with significance at doses of 100 μg/kg or higher. Oral administration of ASP8497 (30 mg/kg) also significantly increased the plasma insulin level and decreased the blood glucose level with concomitant increases in the plasma active GLP-1 level (Fig. 8). However, ASP8497 did not significantly influence gastric emptying or small intestinal transit rates.

4. Discussion

DPP-IV inhibition *in vivo* is expected to result in the elevation of plasma insulin levels by inhibiting the degradation of active GLP-1 after oral glucose loading. This, in turn, leads to the suppression of blood glucose elevation. Indeed, several previous studies have indicated that orally active DPP-IV inhibitors such as vildagliptin [13], K579 [24] and saxagliptin [25] cause a significant reduction in glucose excursion, concomitant with elevations in plasma insulin and active GLP-1 levels during the OGTT in Zucker fatty rats, which is the

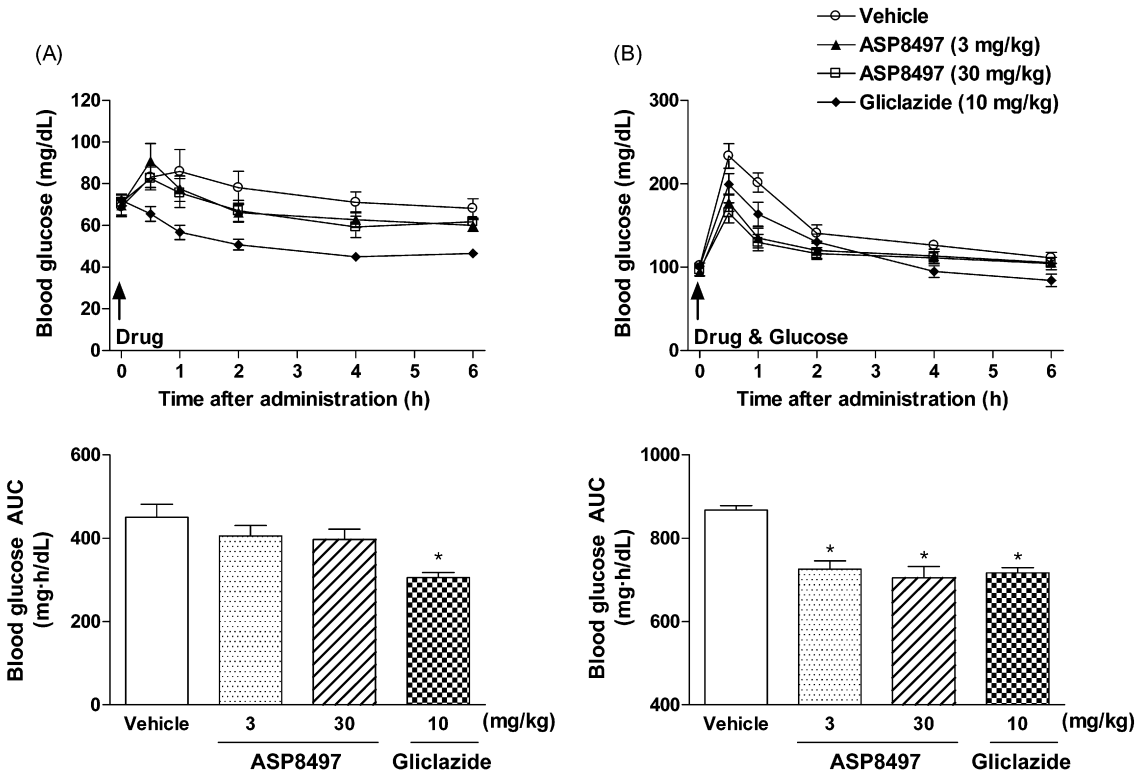

Fig. 6 – Effects of ASP8497 on fasting blood glucose levels in normal mice. Time course of changes in blood glucose levels and the area under the blood glucose concentration-time curve (AUC) (A) under fasting conditions and (B) during the oral glucose tolerance test. The values are the means ± S.E. from five animals in each group. *P < 0.05 vs. vehicle group.

characteristic animal model of obesity and insulin resistance. Clinical proof of concept for the efficacy of DPP-IV inhibitors has also been obtained for vildagliptin [26,27] and sitagliptin [28]. Therefore, DPP-IV inhibitors are expected to become a new class of antihyperglycemic drugs. In this study, we report the *in vitro* and *in vivo* characteristics of a novel DPP-IV inhibitor, ASP8497. ASP8497 potently inhibited DPP-IV activity in mouse, rat, dog and human plasma with IC_{50} values in the

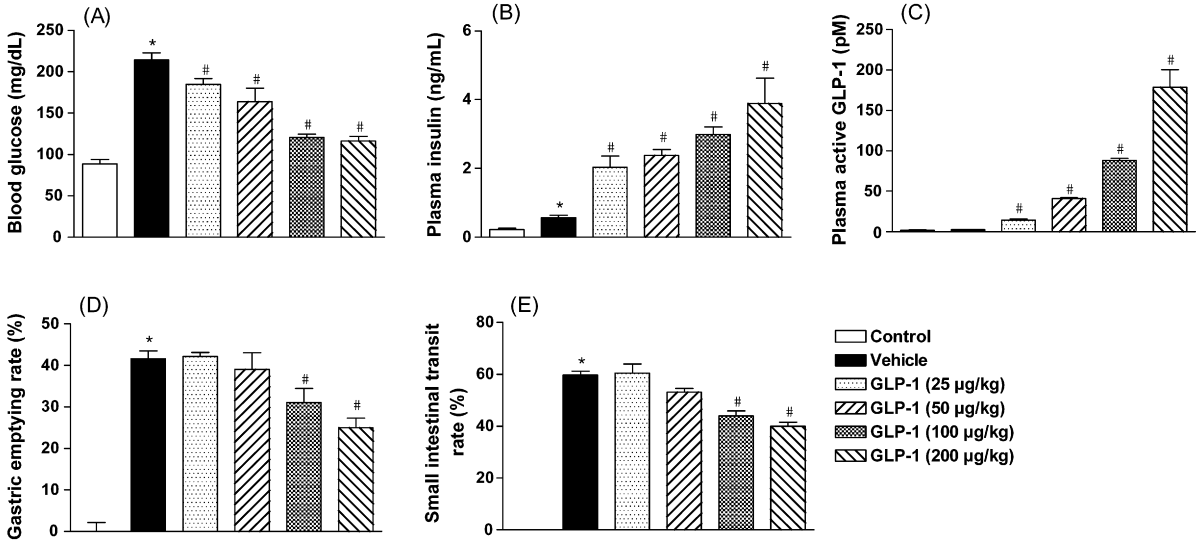

Fig. 7 – Effects of exogenous GLP-1 on glucose metabolism and gastrointestinal functions in normal mice. Vehicle or GLP-1 was intraperitoneally administered, and glucose solution was orally administered 5 min later. Fifteen minutes after glucose loading (A) blood glucose, (B) plasma insulin and (C) active GLP-1 levels, (D) gastric emptying and (E) small intestinal transit rates were measured. The values are the means ± S.E. from five animals in each group. *P < 0.05 vs. control group, #P < 0.05 vs. vehicle group.

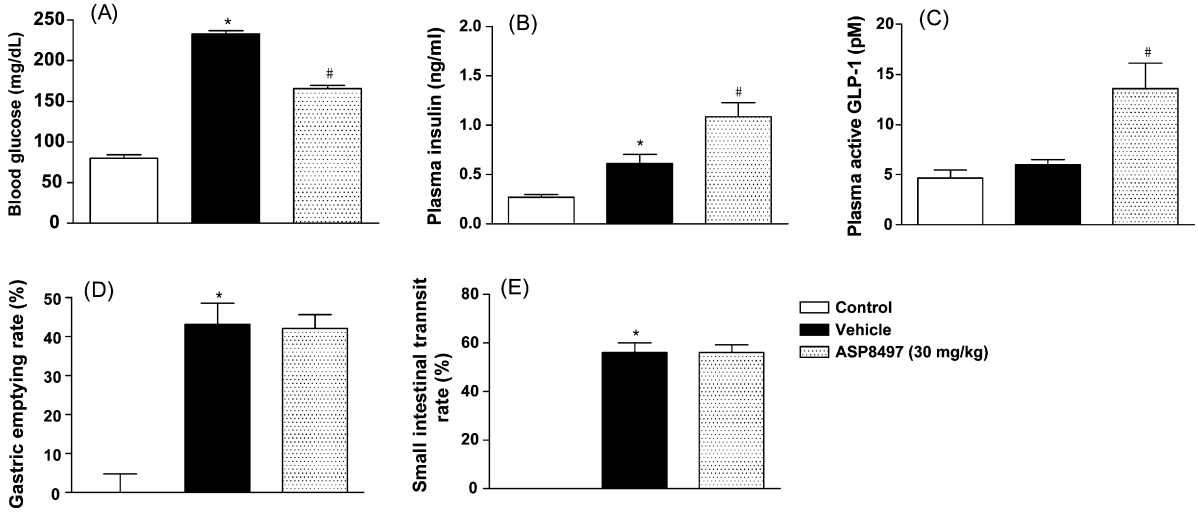

Fig. 8 – Effects of ASP8497 on glucose metabolism and gastrointestinal functions in normal mice. Vehicle or ASP8497 was orally administered, and glucose solution was orally administered 30 min later. Fifteen minutes after glucose loading (A) blood glucose, (B) plasma insulin and (C) active GLP-1 levels, (D) gastric emptying and (E) small intestinal transit rates were measured. The values are the means ± S.E. for five animals in each group. *P < 0.05 vs. control group, #P < 0.05 vs. vehicle group.

nanomolar range; however, it did not potently inhibit DPP8 or DPP9 activity in any species, nor did it affect any of the numerous representative receptors, channels or enzymes. In addition, the kinetics study indicated that ASP8497 inhibited DPP-IV competitively. These *in vitro* studies showed that ASP8497 is a potent, selective and competitive DPP-IV inhibitor.

Next, we investigated the antihyperglycemic effects of ASP8497 in streptozotocin–nicotinamide-induced diabetic mice, which exhibited a mild decline in glucose tolerance due to loss of early-phase insulin secretion [29]. These diabetic mice experienced an approximately 50% decrease in pancreatic insulin content. In addition, because body weight, non-fasting plasma insulin levels and insulin resistance index values (HOMA-R), calculated from blood glucose and plasma insulin levels under fasting conditions, were similar to those in normal mice, insulin resistance was barely detected. Furthermore, fasting plasma GLP-1 levels or levels after glucose loading did not differ between normal and diabetic mice. ASP8497 caused significant decreases in the blood glucose levels during both the 1st and 2nd OGTT in diabetic mice. Inhibition of plasma DPP-IV activity and increases in both the plasma insulin and active GLP-1 levels were also noted. In contrast, gliclazide also caused significant decreases in the blood glucose levels during the 1st OGTT, which increased plasma insulin levels, but did not cause significant effects during the 2nd OGTT. As previously reported, gliclazide potently stimulated insulin secretion and reduced the postprandial blood glucose level; however, the duration of this effect was not long-lasting [30]. These results suggest that ASP8497 improves glucose tolerance potently and sustainably through the elevation of plasma insulin and active GLP-1 levels via the inhibition of DPP-IV activity. In normal mice, ASP8497 and gliclazide significantly inhibited increases in the blood glucose level during the OGTT. In contrast, ASP8497 had no significant effect on fasting blood glucose levels, but gliclazide decreased significantly. Sulfonylureas, including gliclazide, strongly inhibit ATP-sensitive K^+ channel activity by binding to the high-affinity sulfonylurea receptors in pancreatic β-cells, which stimulates insulin secretion glucose-independently. Hypoglycemia has been reported as a side effect with the use of sulfonylureas in diabetic patients [2]. It is presumed that the reduction in the fasting blood glucose level that occurs at the effective dose for gliclazide is related to this hypoglycemia. Since ASP8497 has no effect on fasting blood glucose levels, there should be no risk of hypoglycemia, which, unlike the existing insulin secretagogue sulfonylurea, makes it safe for use as an antihyperglycemic.

Another incretin, GIP, is secreted from K cells in the duodenum and jejunum in response to oral ingestion of nutrients [31]. Like GLP-1, GIP potentiates glucose-stimulated insulin release, and is degraded by DPP-IV to a biologically inactive form [GIP(3–42)] [9]. It has been reported that DPP-IV inhibitors increased the plasma insulin level and decreased the postprandial blood glucose level in both GLP-1 receptor-deficient mice and GIP receptor-deficient mice [32]. However, in double GLP-1 and GIP receptor-deficient mice, the DPP-IV inhibitors had no postprandial blood glucose-lowering effect. These results suggest that both GLP-1 and GIP contribute to the improvement in glucose tolerance elicited by DPP-IV inhibitors. Although the effects of ASP8497 on plasma GIP levels were not investigated in this study, GIP may contribute to the antihyperglycemic efficacy of ASP8497 in diabetic mice.

In addition to potentiating the effects of GLP-1 and GIP, DPP-IV inhibitors may also prolong the actions of other peptide hormones, such as neuropeptide Y, substance P and growth hormone-releasing hormone, as well as chemokines [33]. Therefore, potential side effects associated with the reduced degradation of other peptide hormones and chemokines need to be considered. However, animals lacking DPP-IV

consistently display healthy phenotypes [34,35], and to date, no serious side effects due to DPP-IV inhibition have been reported in clinical studies [28,36]. Hence, DPP-IV inhibition may not produce undesirable changes in downstream biological pathways, despite altering the relative levels of intact-to-cleaved peptide substrates. In addition, DPP-IV is a member of the family of serine peptidase, which includes DPP8 and DPP9. Recently, Lankas et al. used two structurally distinct DPP8/9 inhibitors, L-allo-isoleucyl thiazolidide and (2S,3R)-2-(2-amino-3-methyl-l-oxopentan-l-yl)-1,3-dihydro-2*H*-isoindole hydrochloride to shown that DPP8/9 inhibition results in similar toxicities, such as alopecia, thrombocytopenia, anemia and enlarged spleens in rats, as well as bloody diarrhea in dogs [19]. In contrast, DPP-IV-selective inhibitors were comparatively safe and did not produce any changes. These results suggest that characterizing the enzyme selectivity of DPP-IV inhibitors for DPP8 and DPP9 in the preclinical stage is important for avoiding any potential risk of clinical side effects based on DPP8/9 inhibition. In this study, ASP8497 did not potently inhibit DPP8 or DPP9 activity ($IC_{50} > 200$ nM). In addition, ASP8497 showed no significant activity for representative receptors, channels or enzymes. These results suggest that ASP8497 had potent inhibitory activity and high selectivity for DPP-IV, with a low risk of DPP8/9-related toxicity.

GLP-1 not only stimulates insulin secretion glucose-dependently, but also acts as a physiological mediator for various gastrointestinal functions. Recent studies revealed that, in addition to the incretin effect, exogenous GLP-1 or GLP-1 derivatives also caused a delay in gastric emptying, which was considered to be partially responsible for the inhibition of postprandial hyperglycemia [8,21]. However, there was no delay in gastric emptying when the incretin effect was induced through increased endogenous GLP-1 levels after administration of a DPP-IV inhibitor [22]. In this study, intraperitoneal administration of active GLP-1 dose-dependently exerted an incretin effect, which was significant, even at the lowest dose of 25 μg/kg, and corresponds to the plasma active GLP-1 level (approximately 14 pM). In addition, GLP-1 dose-dependently inhibited gastric emptying and small intestinal transit rates, with significance at doses of 100 μg/kg or higher, which corresponds to the plasma active GLP-1 level (starting around 90 pM). This result suggests that there is an approximately sixfold separation between the plasma active GLP-1 levels associated with the occurrences of the incretin and gastrointestinal effects. In contrast, oral administration of ASP8497 exerted an incretin effect, but did not significantly influence gastric emptying or small intestinal transit rates. At this time, the plasma active GLP-1 level was approximately 14 pM. This correlated with the level required to induce the incretin effect after exogenous administration of GLP-1, and was far lower than that required to induce gastrointestinal effects. Because of this difference in the plasma active GLP-1 levels, none of the DPP-IV inhibitors induced gastrointestinal effects. In clinical studies, nausea was the most common adverse event resulting from a high dose of GLP-1 derivatives administered to induce a long-lasting incretin effect [37–39]. This may be due to a delay in gastric emptying and reduced small intestinal transit, which leads to a feeling of fullness. Therefore, unlike GLP-1 derivatives, DPP-IV inhibitors including ASP8497 would be an antidiabetic drug with few adverse events such as nausea.

In conclusion, the present study shows that ASP8497 is a competitive and selective DPP-IV inhibitor with potent antihyperglycemic activity and no effect on fasting blood glucose levels or gastrointestinal function. The results suggest the usefulness of ASP8497 for further development as a therapeutic agent for impaired glucose tolerance and type 2 diabetes.

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