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Inhibition of dipeptidyl peptidase IV by novel inhibitors with pyrazolidine scaffold

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Abstract

Inhibition of dipeptidyl peptidase IV (DPP-IV) activity has been reported to improve nutrient-stimulated insulin secretion through the stabilization of glucagon-like peptide (GLP-1). In the present study, we identified novel DPP-IV inhibitors of pyrazolidine derivatives (Compounds **1** and **2**) and characterized their biological effects in vitro and in vivo. Compound **1**, an isoleucine pyrazolidide with a phenyl urea group, inhibited rat plasma DPP-IV, porcine kidney DPP-IV, as well as human Caco-2 DPP-IV with IC_{50} values of 1.70, 2.26, and 2.02 μM, respectively. Because of the poor pharmacokinetic properties of Compound **1**, further optimization was carried out, leading to the discovery of Compound **2**, which had similar in vitro activities. Compound **2** acted as a selective and competitive inhibitor of DPP-IV. MALDI-TOF mass spectrometric analysis proved that the compound (20 μM) effectively blocked the degradation of active GLP-1 peptide by 61%. Although similar in in vitro potency, marked improvement of in vivo efficacy and pharmacokinetic properties was seen with Compound **2**. Oral administration of Compound **2** resulted in potent and rapid inhibition of circulating DPP-IV in C57BL/6J mice, with ED_{50} values of 26 mg/kg (s.c.) and 42 mg/kg (p.o.). In addition, this compound improved glucose tolerance in ob/ob mice, as determined by an oral glucose tolerance test (OGTT). These results indicate that Compound **2** is a potent and selective DPP-IV inhibitor with oral anti-hyperglycemic activity in vivo.
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Keywords: Dipeptidyl peptidase IV; GLP-1; Oral glucose tolerance test; ob/ob mice; Insulin secretion; Pharmacokinetics

1. Introduction

Insulin secretion from pancreatic beta cells is tightly regulated by several hormones, which are released from various organs. Among these hormones, the most prominent are the peptide hormones released by the digestive tract in response to nutrient ingestion, namely gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP is released by K-cells in the duodenum and jejunum, whereas GLP-1 is mainly released from the L-cells of the small intestine. Both GLP-1 and GIP sensitize beta cells to initiate glucose-stimulated insulin secretion [1]. GLP-1 also inhibits glucagon secretion, slows gastric emptying and reduces appetite [2]. These actions act in concert, resulting in general improvement of glucose tolerance.

The serine peptidase dipeptidyl peptidase IV (DPP-IV, CD26, and EC 3.4.14.5), is expressed ubiquitously, and alters biological levels of several peptide hormones, chemokines and neuropeptides, by specifically cleaving penultimate amino acids of alanine or proline residues, and thereby modulating their activities [3]. The involvement of DPP-IV in the degradation of GLP-1 is well supported by several studies. In 1993, Mentlein et al. [4] found that DPP-IV removed the N-terminal dipeptide from GLP-1.

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In another report, they clarified that DPP-IV degraded GLP-1 and played a role in inactivating the peptide in human plasma in vitro [5]. Furthermore, it was shown that glucose tolerance was improved in animals lacking DPP-IV following glucose treatment, and increased GLP-1 levels and insulin secretions were achieved [6,7]. Taken together, it appears that DPP-IV directly modulates bioactive GLP-1 levels in vivo, thus significantly influencing glucose tolerance and insulin secretion.

In patients with type 2 diabetes, intravenous infusion of GLP-1 resulted in the plasma glucose lowering effect. Because it is rapidly eliminated from the circulation [8], active GLP-1 itself is not suitable for curing type 2 diabetes. Therefore, DPP-IV inhibition may be a more feasible alternative in the treatment of type 2 diabetes, acting instead via the preservation of active GLP-1 levels. Demonstration of the potential use of oral DPP-IV inhibitors for the treatment of diabetes has been shown by the results of clinical trials of several inhibitors [9].

In this paper, we report new and potent pyrazolidine-derived DPP-IV inhibitors, which show high selectivity against several related peptidases. 2-(2-(*S*)-Amino-3-methylpentanoyl)pyrazolidine-1-carboxylic acid (3-nitrophenyl)amide·HCl (Compound **1**; Fig. 1A) is one of the potent inhibitors of this class, but has low oral bioavailability, likely resulting from the breakage of its urea bond. In an attempt to slow down the breakage of this urea bond in Compound **1**, we replaced it with various heterocyclic rings instead of the phenyl ring, resulting in the representative compound, 2-(2-(*S*)-amino-3-methylpentanoyl)pyrazolidine-1-carboxylic acid (5-methylisoxazol-3-yl)amide·HCl (Compound **2**; Fig. 1B). Compound **2** significantly protected active GLP-1 peptide in vitro and in vivo, and oral administration resulted in improvement of glucose tolerance in ob/ob mice, a well-known type 2 diabetes animal model. Consistent with this, an improvement in oral bioavailability in the rat was also achieved. Preliminary results of these series of derivatives on their structure–activity relationship were published previously in part [10].

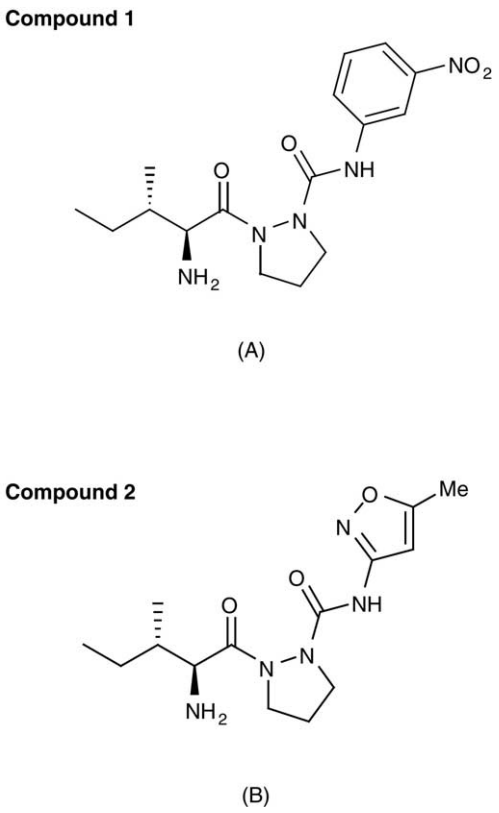

Fig. 1. Chemical structures of Compound **1** (A) and Compound **2** (B).

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial suppliers: Ala-Pro-7-amino-4-trifluoromethylcoumarin (AFC) (Enzyme system products); *Z*-Gly-Pro-methylcoumaryl-7-amide (MCA) and Cbz-Glu-Tyr (Fluka Co.), DPP-IV (porcine kidney), elastase, trypsin, N^{α}-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), aminopeptidase N (APN), leucine-*p*-nitroanilide, carboxypeptidase P, and ninhydrin (Sigma-Aldrich Co.). Prolyl oligopeptidase (POP) was prepared from Sprague–Dawley rat brain cortex, and the supernatant obtained from the centrifugation of the homogenates used as the enzyme source. DPP-II was prepared from the crude lysates of COS-7 cells transfected with pcDNA3.1-DPP-II cDNA. To determine DPP-IV activity from Caco-2 cells (human colonic adenocarcinoma cells), the cells were cultured to induce differentiation, as previously described [11], lysed with lysis buffer (0.15 M NaCl, 1% Triton X-100, 10 mM Tris–HCl, pH 7.4, 10% glycerol) and centrifuged at 12,000 rpm for 10 min. The supernatant was then used as the enzyme source. Experimental protocols concerning the use of laboratory animals were followed in accordance with National Institute of Health guidelines.

2.2. DPP-IV enzyme assay

DPP-IV enzyme preparations (10 μl) of rat plasma, porcine kidney DPP-IV, or Caco-2 cell lysate, were suspended in Tris–HCl buffer (pH 7.5) and 40 μM Ala-Pro-AFC (ICN Biomedicals, Inc) added. After the addition of compound (i.e. Compounds **1** or **2**), the mixture was incubated for 60 min at 24 °C. AFC, as an indicator of DPP-IV activity, was detected fluorometrically at 405/510 nm (Ex/Em) (Synergy HT fluorometer, Biotek). IC_{50} values were calculated using Prism 4.0 software (GraphPad Software, Inc).

2.3. Selectivity over DPP-IV related enzymes

The selectivity of Compound **2** was determined against several enzymes including elastase, trypsin, DPP-II, and

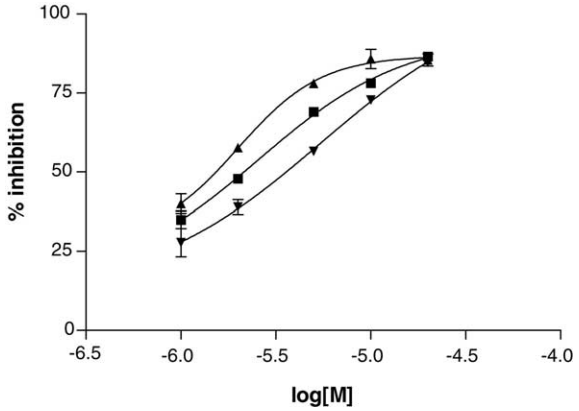

Fig. 2. Concentration-dependent inhibition of DPP-IV by Compound **2**. Different enzyme preparations (rat plasma; ▼; porcine kidney; ▲; Caco-2 cells; ■) were incubated with different concentrations of Compound **2**. The enzyme activity was measured by the fluorescence of AFC released by the enzyme reaction, and results expressed as percentage inhibition (mean ± S.E.M.) ($N = 3$ separate experiments).

post-proline-cleaving enzymes. For DPP-II enzyme assays, the enzyme (10 μg/well) was incubated in a buffer containing 50 mM sodium acetate buffer (pH 5.0) and 40 μM Ala-Pro-AFC, with or without compound, for 1 h at room temperature. The fluorescence of AFC was determined (excitation 405 nm; emission 510 nm). For the trypsin enzyme assay, the enzyme (2 μg/well) was incubated in a buffer containing 50 mM Tris buffer (pH 8.0) and 2 mM N^{α}-benzoyl-L-arginine-*p*-nitroanilide, with or without Compound **2**, for 1 h at room temperature. The absorbance at 405 nm was measured for the detection of *p*-nitroanilide released from the enzyme reaction. For the elastase assay, the enzyme (0.8 units/ml) was incubated in a buffer containing 90 mM Tris buffer (pH 8.0), 10 mM $CaCl_2$ and succinyl-ala-ala-ala-*p*-nitroanilide (1 mM), with or without Compound **2**, for 1 h at room temperature. The absorbance at 405 nm was measured. For the APN enzyme assay, the enzyme (0.2 μg/well) was incubated in a buffer containing 50 mM Tris buffer (pH 8.0) and L-leucine-*p*-nitroanilide (0.5 mM), with or without Compound **2**, for 30 min at room temperature. The absorbance at 405 nm was measured. For the POP enzyme assay, the enzyme (10 μg/well) was incubated in a buffer containing 25 mM potassium phosphate buffer (pH 7.0) and *Z*-Gly-Pro-MCA (200 μM) for 1 h at room temperature, with or without Compound **2**. The fluorescence of MCA produced by the enzyme reaction was measured (excitation 360 nm; emission 485 nm). For carboxypeptidase P enzyme assay, the enzyme (0.02 units/200 μl) was incubated in a buffer containing 50 mM sodium acetate buffer (pH 3.7) and 0.5 mM Cbz-glu-Tyr (0.5 mM), with or without Compound **2**, for 20 min at room temperature. The amount of tyrosine released from the enzyme reaction was measured by ninhydrin reaction. The absorbance at 570 nm was measured.

Table 1
Selectivity of Compound **2** over DPP-IV related enzymes

Isozyme	K_i (μM)
DPP-II	67
Trypsin	>100
Elastase	>100
APN	>100
POP	>100
Prolidase	>100
Carboxypeptidase P	>100
DPP-IV (porcine kidney)	1.2

2.4. Enzyme kinetics

Initial rates at five different fixed inhibitor concentrations (1, 2, 4, 8, and 16 μM) were measured at seven different concentrations of Ala-Pro AFC (12.5, 25, 50, 100, 200, 400, and 800 μM) in a buffer containing 50 mM Tris (pH 7.5). The inhibition pattern was evaluated by Lineweaver–Burk plot, and K_i determined using the curve fitting program, GraFit (Erithacus Software, Inc.).

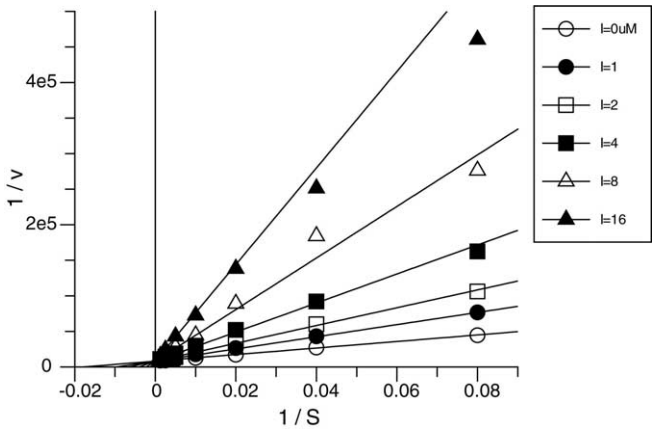

Fig. 3. Inhibition kinetics of DPP-IV by Compound **2**. Different concentrations of Compound **2** (0, 0.25, 0.5, 1, 2, and 4 μM) were incubated in the presence of various concentrations of Ala-Pro-AFC. Initial rates of the reaction were measured, and the results expressed as a Lineweaver–Burk plot. Data represent means of three separate experiments performed in triplicate.

2.5. In vivo DPP-IV assay

Male C57BL/6J mice (9 weeks old), bred at in-house animal facilities, were fasted for 18 h and then treated either orally or subcutaneously with or without Compound **2**. Control animals received vehicle only (0.5% carboxy methyl cellulose; CMC). Each group was allocated seven animals. Blood was taken from the eye vein either at 60 min (p.o.) or at 30 min (s.c.) after administration of the compound, and subjected to the measurement of DPP-IV activity after centrifugation. To determine the effect of Compound **2** on intact GLP-1 and insulin levels in plasma, the compound was administered orally 30 min before oral glucose challenge (2 g/kg), and plasma withdrawn at 5 min (GLP-1) or 10 min (insulin) after oral glucose challenge.

2.6. MALDI-TOF MS analysis

One microunit (μU) of porcine DPP-IV (Sigma) was incubated with GLP-1 (15 μM) in 0.1 mM Tricine buffer, pH 7.5, in the presence or absence of Compound **2** (20 μM). After 24 h incubation, an equal volume of assay sample and matrix (CHCA; cyano-4-hydroxycinnamic acid) was combined and the mixture analyzed by MALDI-TOF mass spectrometry (PE Biosystems Voyager System 4095). Signals were defined and quantified by software in Voyager System.

2.7. Oral glucose tolerance test (OGTT)

C57BL/6J mice (lean) and ob/ob mice were bred at in-house animal facilities. Mice of 9–10 weeks were used to investigate the effect of Compound **2** on plasma glucose levels after their body weights and plasma glucose levels were matched. After overnight fasting, lean or ob/ob mice were orally administered with various doses of Compound **2**, 30 min before being given an oral glucose challenge, at a dose of 2 g/kg. Compound **2** was suspended in 0.5% CMC. Blood samples from the eye veins of mice were collected into heparinized capillary tubes at 0, 15, 30, 60, and 120 min after oral glucose administration. Blood samples were centrifuged at 4 °C, and plasma glucose levels analyzed immediately.

2.8. In vivo pharmacokinetic profile of Compound 2

Sprague–Dawley rats (adult males, 250–300 g) were fasted overnight, and the femoral vein (for compound administration for intravenous study) and jugular vein (for blood sampling) of each rat cannulated with polyethylene tubing. Animals were administered Compound **2** (20 mg/kg) dissolved in 30% polyethyleneglycol (PEG) in saline either intravenously or orally. At various time points after administration, blood samples were collected from the jugular vein, transferred to heparin-coated tubes and centrifuged to separate off the plasma. The plasma was stored at −80 °C until assayed. Compound concentrations in plasma were determined by LC/MS/MS analysis (Q TRAP mass spectrometer, Applied Biosystem, USA).

2.9. Analysis

Plasma glucose concentrations were measured by colorimetric assay using the Selectra 2 (Vital Scientific N.V., Spankeren, The Netherlands). Plasma insulin concentrations were determined with an insulin ELISA kit (Shibayagi Co, Japan). Plasma GLP-1 levels were measured with a glucagon-like peptide (active) ELISA kit (Linco Research, St Charles, MO).

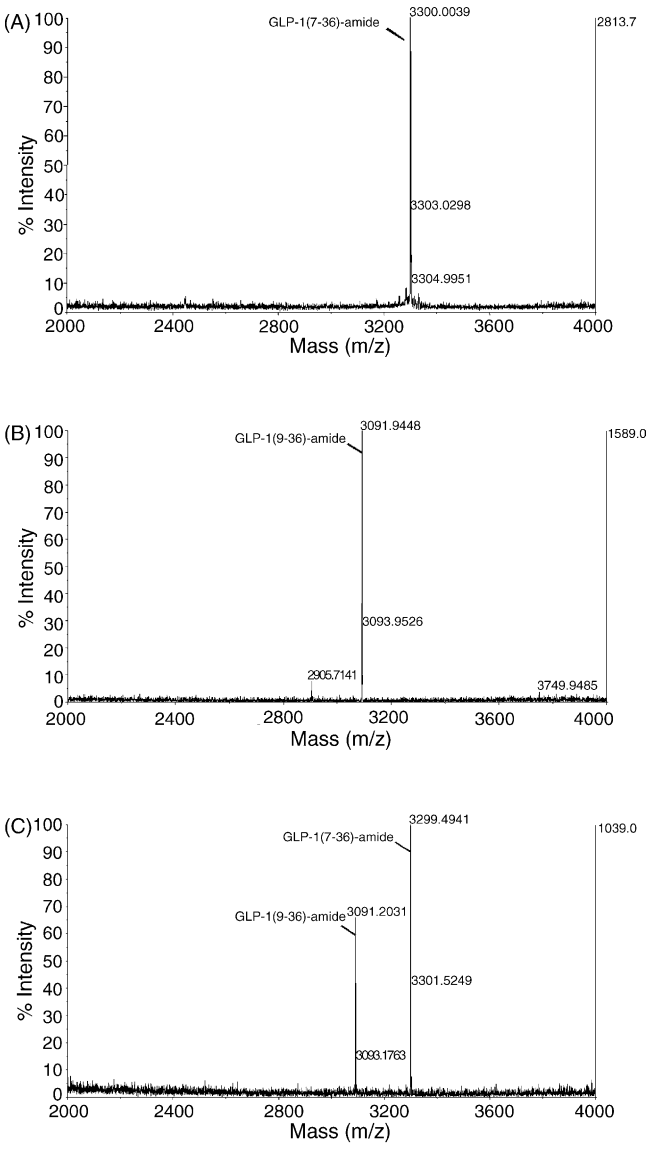

Fig. 4. Effect of Compound **2** on GLP-1 degradation by DPP-IV in vitro. GLP-1 (15 μM) was incubated with porcine kidney DPP-IV (1 μU) in the presence or absence of 20 μM Compound **2**. The amount of intact GLP-1 was quantified after MALDI-TOF mass spectrometry. (A) GLP-1 alone; (B) GLP-1 plus DPP-IV; and (C) GLP-1 plus DPP-IV in the presence of 20 μM Compound **2**.

2.10. Statistical analysis

Data are expressed as mean ± S.E.M., and statistical significance was assessed by one-way analysis of variance (ANOVA), followed by Dunnett's test.

3. Results

3.1. Inhibitory activity of pyrazolidine derivatives on DPP-IV

To find novel DPP-IV inhibitors, we carried out high throughput screening (HTS) using a compound collection from the Korea Chemical Bank and selected several hit compounds. Among them, a compound with a pyrazolidine scaffold was selected for further chemical modification. At the earliest stage of modification, Compound **1** was discovered (Fig. 1A), and its IC_{50} values were 1.70 μM in rat plasma, 2.26 μM in porcine kidney, and 2.02 μM in human Caco-2 cells. Compound **1**, however, exhibited in vivo oral activity only at high doses (results not shown), thus further optimization was carried out to enhance its in vivo oral efficacy. As a result, Compound **2** with improved oral bioavailability was discovered (Fig. 1B), and its biochemical and pharmacological activity profiles characterized. Compound **2** inhibited rat plasma DPP-IV, porcine kidney DPP-IV, and human Caco-2 DPP-IV, with IC_{50} values of 3.4, 1.5, and 2.2 μM, respectively (Fig. 2).

3.2. Selectivity profile of the compounds over related peptidases

We examined the effects of Compound **2** on several DPP-IV related peptidases including DPP-II, APN, POP, prolidase, carboxypeptidase P, trypsin, and elastase. Compound **2** had considerable selectivity for DPP-IV (at least 50-fold) except DPP-II (30-fold selectivity) (Table 1).

3.3. Inhibition kinetics of Compound 2

To identify enzyme inhibition kinetic patterns of Compound **2**, we carried out inhibition kinetic analyses with porcine kidney DPP-IV. As shown in Fig. 3, Compound **2** showed a competitive inhibition pattern well fitted to a Lineweaver–Burk plot and had a K_i value of 1.2 μM.

3.4. Compound 2 protected GLP-1 peptide from DPP-IV in vitro

To investigate the effect of Compound **2** on DPP-IV-mediated degradation of GLP-1, we used purified recombinant GLP-1 peptide (NH_2-7-36) and porcine kidney

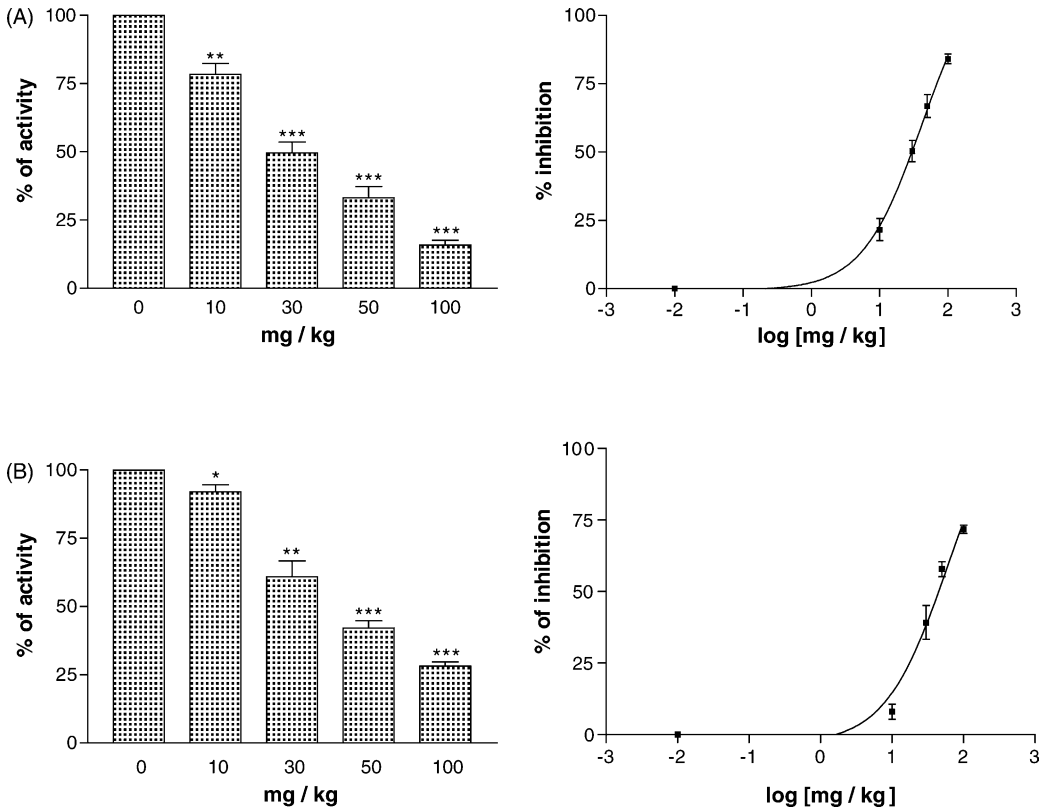

Fig. 5. In vivo inhibitory effect of Compound **2** on DPP-IV activity. Compound **2** was given to C57BL/6J mice either subcutaneously (A) or orally (B) at the doses indicated. Plasma DPP-IV activity was measured at 30 min (A) or 1 h (B) after Compound **2** administration. Data are presented as mean ± S.E.M. for three separate experiments ($N = 7$ for each group). ★P value <0.05 vs. control; ★★P value <0.01 vs. control; and ★★★P value <0.005 vs. control.

DPP-IV. The extent of degradation of GLP-1 was analyzed by the change in the molecular weight of active GLP-1 peptide, using MALDI-TOF mass spectrometry. The results show that approximately 61% of active GLP-1 was recovered in the presence of 20 μM of Compound **2** (Fig. 4). These results indicate that Compound **2** can protect active GLP-1 from degradation mediated by DPP-IV in vitro.

3.5. Compound 2 inhibited plasma DPP-IV activity in vivo

To investigate the DPP-IV inhibitory effects of Compound **2** in vivo, we administered various doses of Compound **2** to C57BL/6J mice, either subcutaneously or orally. Oral administration of this compound at 100 mg/kg resulted in 75% inhibition of plasma DPP-IV activity ($P < 0.0001$). The ED_{50} values of Compound **2** for DPP-IV were 26 mg/kg (s.c.) and 42 mg/kg (p.o.) (Fig. 5). Next, we examined whether DPP-IV inhibition increased active

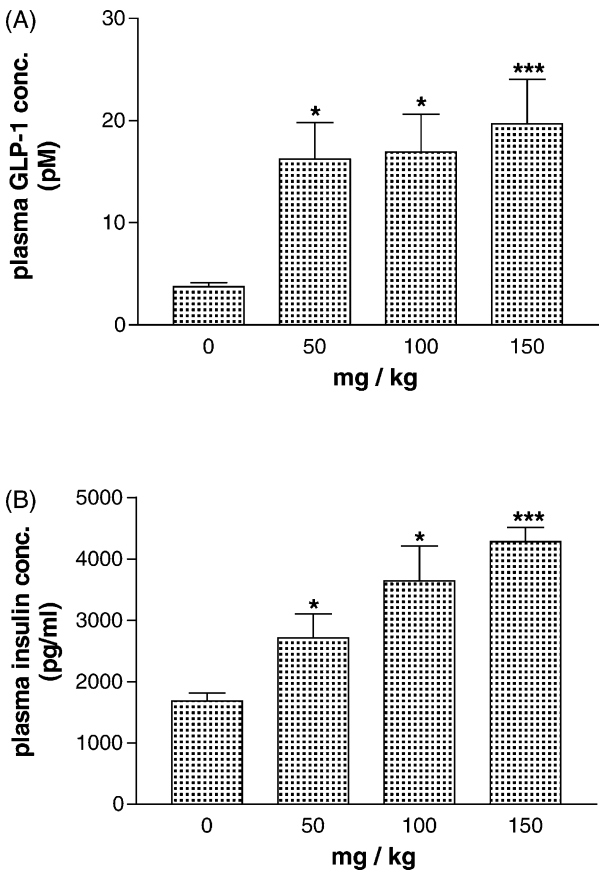

Fig. 6. Effect of Compound **2** on plasma GLP-1 and insulin levels. Compound **2** was orally given to C57BL/6J mice 30 min before oral glucose challenge (2 g/kg) at the doses indicated. Plasma was withdrawn at 5 min and 10 min for the measurement of GLP-1 and insulin levels. Intact GLP-1 and insulin levels were determined, as described in Section 2. (A) Plasma active GLP-1 concentration and (B) Plasma insulin concentration. Data are presented as mean ± S.E.M. for three separate experiments ($N = 7$ for each group). $^{\star}P$ value <0.05 vs. control; and $^{\star\star\star}P$ value <0.005 vs. control.

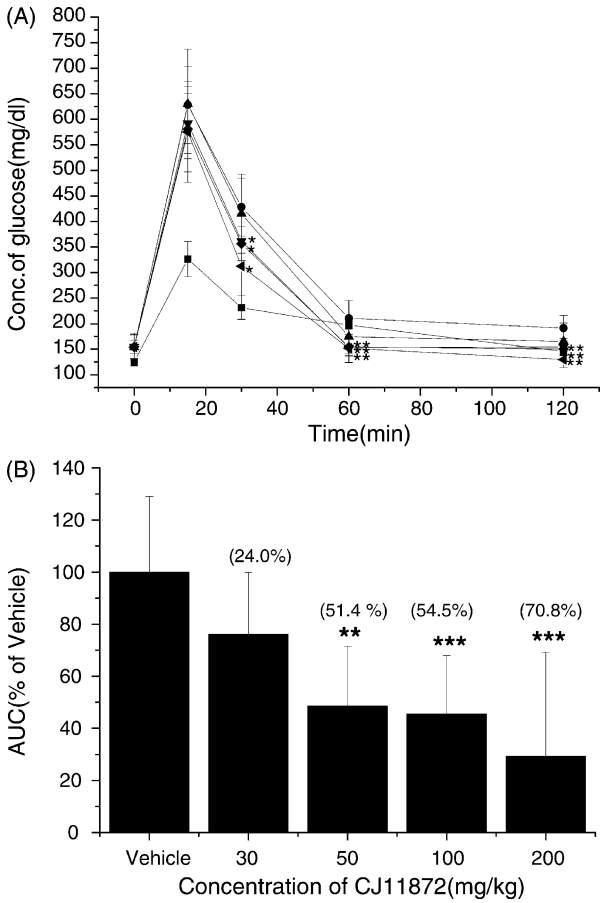

Fig. 7. OGTT of Compound **2** using ob/ob mice. Various doses of Compound **2** were administered orally to ob/ob mice, and glucose (2 g/kg, p.o.) given 30 min later (0 min). Plasma glucose concentration was then measured at the times indicated. (A) (■) C57BL/6J (lean) mice vehicle, (●) ob/ob mice vehicle, (▲) ob/ob mice Compound **2** 30 mg/kg, (▼) ob/ob mice Compound **2** 50 mg/kg, (◆) ob/ob mice Compound **2** 100 mg/kg, (◀) ob/ob mice Compound **2** 200 mg/kg. Data are presented as mean ± S.E.M. for three separate experiments ($N = 8$ for each group). $^{\star}P$ value <0.05 vs. control and $^{\star\star}P$ value <0.01 vs. control. (B) The AUC of the glucose concentration curve (A) was calculated. In (B), the percent inhibition values for each treatment (in parentheses) were generated from the AUC data normalized to vehicle-treated lean C57BL/6J mice. $^{\star\star}P$ value <0.01 vs. control and $^{\star\star\star}P$ value <0.005 vs. control.

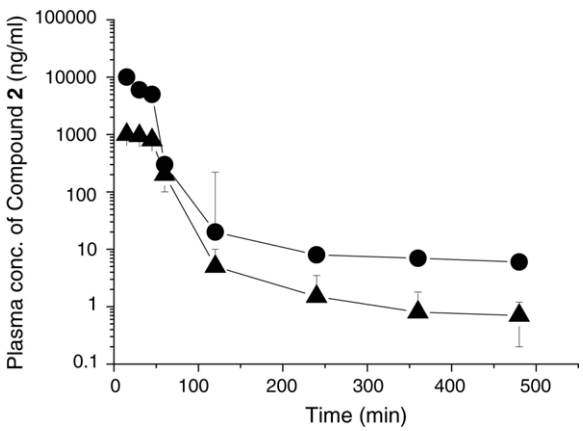

Fig. 8. Plasma concentration–time profiles of Compound **2** after i.v. (20 mg/kg, ●) and p.o. (20 mg/kg, ▲) administration to male Sprague–Dawley rats (mean ± S.D.; $N = 3$ animals/route of administration).

Table 2
Pharmacokinetic parameters of Compound **2** and Compound **2** salt form (Compound **2a**)

Parameters	Compound **2**		Compound **2a**	
	i.v. (20 mg/kg)	p.o. (20 mg/kg)	i.v. (20 mg/kg)	p.o. (20 mg/kg)
AUC_{0-8h} (μg min/ml)	184.6 ± 13.5	54.1 ± 7.3	273.6 ± 44.6	105.6 ± 32.8
C_{max} (μg/ml)	16.6 ± 12.6	1.1 ± 0.13	11.5 ± 1.2	3.0 ± 1.2
T_{max} (min)	0	20 ± 8.7	0	15.0 ± 0
$T_{1/2}$ (min)	84.6 ± 85.7	120 ± 32.2	113.2 ± 1.0	191.3 ± 24.6
CL (ml/kg min)	108.5 ± 8.0	–	73.1 ± 11.4	–
V_{ss} (ml/kg)	2331 ± 351	–	1647 ± 141	–
F (%)		29.3 ± 3.9		36.5 ± 12.0

GLP-1 levels and insulin secretion, via an OGTT using C57BL/6J mice, with or without Compound **2**. The increase in plasma GLP-1 after glucose loading was potentiated by Compound **2** in a dose-dependent manner, along with the enhancement of plasma insulin response to gastric glucose (Fig. 6). These results support the correlation between DPP-IV inhibition, increased GLP-1 and insulin levels and an improvement of glucose tolerance.

3.6. Compound 2 improved glucose tolerance in ob/ob mice

To examine whether the inhibitory activity of Compound **2** on DPP-IV could ameliorate glucose intolerance, the effect of Compound **2** on glucose tolerance was tested using ob/ob mice via an OGTT. Oral treatment with Compound **2** resulted in increased glucose clearance in a dose-dependent manner. Plasma glucose levels, determined by the AUC of the glucose concentration curve, were reduced 71% compared to controls by the administration of Compound **2** at 200 mg/kg. The ED_{50} value for Compound **2** was 40 mg/kg (Fig. 7).

3.7. In vivo pharmacokinetic profiles of Compound 2

When Sprague–Dawley rats received an oral dose of 20 mg/kg of Compound **2**, the C_{max} was 1.1 μg/ml with a T_{max} of 20 min, indicating rapid absorption. Absolute bioavailability was 29% with a half-life of 2 h, showing an overall fair pharmacokinetic profile (Fig. 8). To improve the pharmacokinetic profile of Compound **2** further, a maleic acid salt form (Compound **2a**) was prepared, and its pharmacokinetic studies revealed that Compound **2a** exhibited even better pharmacokinetic profiles, with an oral bioavailability of 37%. A summary of pharmacokinetic parameters is shown in Table 2.

4. Discussion

DPP-IV inhibition increases the amount of active GLP-1 that then stimulates insulin secretion from beta cells [6]. Moreover, DPP-IV-deficient rats or mice show improved glucose tolerance, via enhanced insulin release mediated by higher levels of active GLP-1, compared with wild type animals [6,7]. Since the discovery of the functional significance of DPP-IV in GLP-1 degradation, several small molecule inhibitors of DPP-IV have been discovered and are under development as oral anti-diabetic drugs [2].

However, the major concerns for DPP-IV inhibitors raised are due to the role of DPP-IV in the degradation of many other biologically active peptides other than GLP-1 and GIP. Such peptides, for example, are various cytokines, substance P and bradykinin [12,13]. Fortunately, recent reports suggest that the side effects of DPP-IV inhibitors, observed in clinical trials, may not be related to their mechanism-of-action, but rather being compound specific [14]. Nonetheless, selectivity against other peptidases is also an important issue for the risk of side effects from DPP-IV inhibitors. Compound **2**, a novel inhibitor reported in the present study demonstrated fair selectivity against these peptidases.

To confirm the functional consequences of Compound **2**, several experiments were carried out. MALDI-TOF mass spectrometric analysis of the in vitro incubation mixture in the presence or absence of Compound **2**, showed that the compound protected GLP-1 from degradation by DPP-IV. Based on the kinetic assays, Compound **2** acts as a competitive inhibitor, with a K_i value of 1.2 μM. Furthermore, the inhibitor improved glucose tolerance, as determined by the OGTT in ob/ob mice, in accordance with the in vivo inhibition of DPP-IV activity, increased active GLP-1 levels, and enhanced insulin secretion. Taken together, these results suggest that Compound **2**, with a novel scaffold structure, is functionally active in vivo, thus ameliorating glucose clearance rates. Acute toxicity studies with either 1000 mg/kg (p.o.) or 500 mg/kg (i.p.) did not show any adverse effects.

So far, the reported low molecular weight inhibitors of DPP-IV can be divided into two main groups based on their structural characteristics. The first group is comprised of compounds with nitrile groups, and the second includes compounds without nitrile groups. The inhibitors reported in the present study have a novel scaffold (pyrazolidine) structure without nitrile functional groups, which may be chemically more stable. Upon the initial optimization of our hit compound, we discovered Compound **1**. Although Compound **1** had moderate activity as a DPP-IV inhibitor,

its oral bioavailability was about 10%, possibly due to the cleavage of its urea bond. Thus, we carried out further optimization to improve oral bioavailability by the introduction of an isoxazole ring in place of the phenyl ring in Compound **1**. As a result, we improved oral bioavailability of the compound (i.e. Compound **2**) to 29%. Furthermore, the maleic acid salt form of Compound **2** (i.e. Compound **2a**) further enhanced its oral activity, as determined by OGTT using ob/ob mice (results not shown), with an oral bioavailability of 37%.

In conclusion, we report here novel pyrazolidine-derived DPP-IV inhibitors, and show that Compound **2** is a potent inhibitor of DPP-IV resulting in improved glucose tolerance, accompanied by markedly increased GLP-1 and insulin levels. Further optimization of Compound **2** to improve its in vivo efficacy will lead to the development of novel anti-diabetic agents by enhancing incretion activities, namely with respect to GLP-1.

Acknowledgments

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