Bioorganic & Medicinal Chemistry 20 (2012) 5033–5041

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Fused bicyclic heteroarylpiperazine-substituted L-prolylthiazolidines as highly potent DPP-4 inhibitors lacking the electrophilic nitrile group

Tomohiro Yoshida [a,†], Fumihiko Akahoshi [a,*], Hiroshi Sakashita [a,‡], Shuji Sonda [a,§], Masahiro Takeuchi [a], Yoshihito Tanaka [a], Mika Nabeno [a], Hiroyuki Kishida [b], Ikuko Miyaguchi [b], Yoshiharu Hayashi [a,†]

[a] Research Division, Mitsubishi Tanabe Pharma Corporation, 2-2-50, Kawagishi, Toda-shi, Saitama 335-8505, Japan
[b] R&D Strategy Dept., Corporate Strategy Division, Mitsubishi Chemical Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan

ARTICLE INFO

*Article history:*
Received 28 April 2012
Revised 7 June 2012
Accepted 8 June 2012
Available online 5 July 2012

*Keywords:*
DPP-4 inhibitor
Thiazolidine
Bicyclic heteroarylpiperazine

ABSTRACT

Hypoglycemic agents with a mechanism of depeptidyl peptidase IV (DPP-4) inhibition are suitable for once daily oral dosing. It is difficult to strike a balance between inhibitory activity and duration of action in plasma for inhibitors bearing an electrophilic nitrile group. We explored fused bicyclic heteroarylpiperazine substituted at the γ-position of the proline structure in the investigation of L-prolylthiazolidines lacking the electrophilic nitrile. Among them, 2-trifluoroquinolyl compound **8g** is the most potent, long-lasting DPP-4 inhibitor ($IC_{50}$ = 0.37 nmol/L) with high selectivity against other related peptidases. X-ray crystal structure determination of **8g** indicates that CH-π interactions generated between the quinolyl ring and the guanidinyl group of Arg358 enhances the DPP-4 inhibitory activity and selectivity.

© 2012 Elsevier Ltd. All rights reserved.

## 1. Introduction

The number of patients with type 2 diabetes is increasing rapidly worldwide.[1] Diabetics may suffer debilitating cardiovascular, eye, kidney, and nerve damage and are at risk of premature handicap and death due to these and other diabetic complications, which are the result of glucose toxicity caused by hyperglycemia. Current treatment strategies include reducing insulin resistance, supplementing the insulin deficiency with exogenous insulin, enhancing endogenous insulin secretion, reducing hepatic glucose output, and limiting glucose absorption.[2] Among them, insulin secretagogues such as glinides (e.g., repaglinide), which are widely used, need to be taken before each meal for avoiding a risk of hypoglycemia. Therefore the antihyperglycemic agent which is orally available in a once-daily regimen without causing hypoglycemia had been much awaited in medical settings.

An incretin hormone glucagon-like peptide-1 (GLP-1) stimulates insulin biosynthesis and secretion in response to meal ingestion, inhibits glucagon secretion, and promotes proliferation of pancreatic β cells. Active GLP-1 (GLP-1[7-36]amide) is rapidly degraded in vivo by depeptidyl peptidase IV (DPP-4), which is a serine protease recognizing an amino acid sequence having proline or alanine at the N-terminal penultimate position to produce a dipeptide and the inactive GLP-1.[3] The DPP-4 inhibition increases the plasma concentration of active GLP-1, causes the secretion of insulin in response to increased blood glucose level;[4] therefore, DPP-4 inhibitors are expected to be safer and once daily agents.[5]

Several DPP-4 inhibitors have been already approved, including sitagliptin (**1**, first in class), vildagliptin (**2**), alogliptin (**3**) and saxagliptin (**4**) (Fig. 1).[6] Several other candidates have been reported to be in an advanced stage in clinical trials for type 2 diabetes.[5b]

Many known DPP-4 inhibitors have the P1–P2 fragment[7] and some of them possess the electrophilic trap such as a nitrile or boronic acid group on the P1 pyrrolidine to form a covalent bond with Ser630 of the catalytic triad in the active site.[8] These

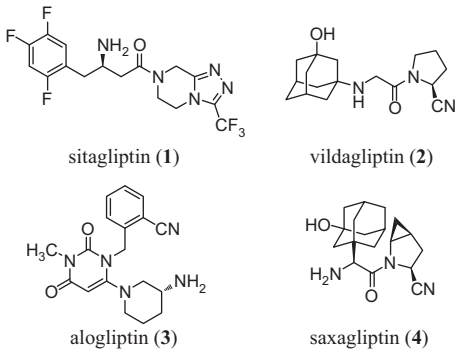



**Figure 1.** DPP-4 inhibitors.

* Corresponding author.
*E-mail address:* akahoshi.fumihiko@mk.mt-pharma.co.jp (F. Akahoshi).
† Present address: Development Division, Mitsubishi Tanabe Pharma Corporation, 17-10, Nihonbashi-Koamicho, Chuo-ku, Tokyo 103-8405, Japan.
‡ Present address: Tanabe R&D Service Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan.
§ Present address: CMC Division, Mitsubishi Tanabe Pharma Corporation, 3-16-89, Kashima, Yodogawa-ku, Osaka-shi, Osaka 532-8505, Japan.

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.06.033

compounds were chemically unstable due to intramolecular cyclization between the electrophilic nitrile and the amine of the P2 part,[9] and unable to be expected to control plasma glucose all day long for once-daily treatment. In this class, saxagliptin (**4**) improved chemical stability by introduction of a *cis*-4,5-methano bridge to the prolinenitrile.[10] On the other hand, we started research on novel DPP-4 inhibitors without the electrophilic nitrile moiety to increase chemical stability and focused on the substituent at the γ-position of the proline moiety of the prolylthiazolidine core structure to increase further affinity with the S2 subsite of DPP-4.[11] We previously reported that a program of optimizing γ-substituents by introducing 4-arylpiperazine resulted in highly potent and long-lasting inhibitors.[12] The SAR study revealed that the introduction of an electron-deficient 4-arylpiperazine scaffold was preferable to increase the inhibitory activity. In particular, substituted pyridylpiperazine compound **5a** proved exceptionally potent in vitro despite the lack of an electrophilic nitrile group (Fig. 2). Another important feature of DPP-4 inhibitors is their selectivity against other related prolyl peptidases such as DPP-8 and DPP-9.[13] DPP-8 and DPP-9 inhibition have been reported to be associated with multiorgan toxicities and profound immunotoxicity in rats and dogs,[14] and DPP-4 selectivity is one of the key issues for clinical use. Although phenyl analog **5b** showed potent DPP-4 inhibitory activity, it possessed moderate selectivity against DPP-8 and DPP-9 as described in a later section. We anticipated that potent, selective and long-lasting DPP-4 inhibitors would be designed by the optimization of the L-prolylthiazolidine moiety having extensive interactions with the S2 subsite.

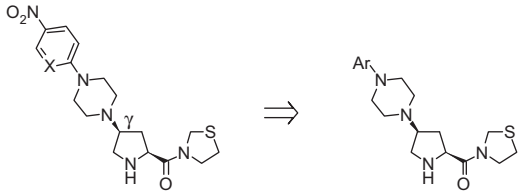
Here we addressed the introduction of a sterically more bulky aromatic ring such as a fused bicyclic heteroaryl group on the piperazine moiety to interact the extensive subsite and discovered a highly potent, selective and long-lasting DPP-4 inhibitor with a favorable profile as a potential once-daily therapeutic agent for type 2 diabetes.

## 2. Chemistry

The synthesis of a series of fused bicyclic heteroarylpiperazine compounds is shown in Scheme 1. The key intermediate **7a** was prepared by condensation with *N*-Boc-*trans*-4-hydroxyproline (**6a**) and thiazolidine, followed by oxidation with dimethyl sulfoxide and sulfur trioxide.[15] A rapid and efficient synthesis of **8** and **9** was performed using reductive amination of the key intermediate ketone **7a** with a fused bicyclic heteroarylpiperazine followed by removal of the Boc group, as previously reported.[11] This reductive amination afforded only cis-isomers. Functionalized benzimidazolylpiperazine derivatives **9b** and **9c** were synthesized through nucleophilic substitution of piperazine compound **10** and the corresponding 2-chloroimidazole **11**.

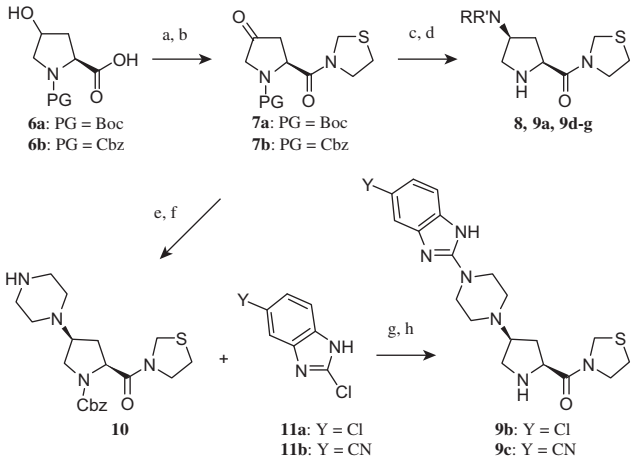
## 3. Results and discussion

A series of quinoline and isoquinoline derivatives was evaluated for human and rat DPP-4 inhibitory activity in vitro and these re-



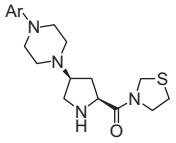

**5a**: X=N; $IC_{50}$ = 0.92 nmol/L
**5b**: X = CH; $IC_{50}$ = 1.6 nmol/L
**Ar**: fused bicyclic heteroaryl

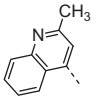
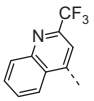
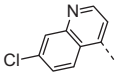
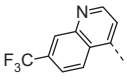
**Figure 2.** (*S*)-γ-Substituted L-prolythiazolidines as DPP-4 inhibitors.




**Scheme 1.** Reagents and conditions: (a) Thiazolidine, HOBt, EDC, DMF; (b) DMSO, $SO_3$-pyridine, $Et_3N$; (c) RR′NH, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane; (d) HCl, AcOEt; (e) *N*-Boc-piperazine, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane; (f) THF, $CH_2Cl_2$; (g) $iPr_2NEt$, *N*-methyl-2-pyrrolidone, 100 °C; (h) 30% HBr–AcOH or TFA, thioansole.

**Table 1**
Inhibition of quinoline and isoquinoline derivatives

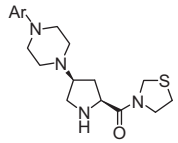


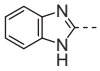
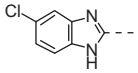
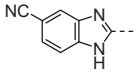
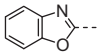
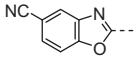
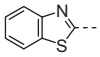
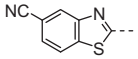

| Compound | Ar | DPP-4 inhibition, $IC_{50}$ (nmol/L) | |
|---|---|---|---|
| | | Human | Rat |
| **5a** |  | 0.92 | 1.1 |
| **8a** |  | 0.61 | 0.83 |
| **8b** |  | 0.73 | 0.79 |
| **8c** |  | 0.51 | 0.56 |
| **8d** |  | 2.2 | 3.0 |
| **8e** |  | 0.95 | 1.0 |
| **8f** |  | 0.56 | 1.5 |
| **8g** |  | 0.37 | 0.59 |
| **8h** |  | 0.61 | 0.62 |
| **8i** |  | 1.6 | 1.3 |

sults are listed in Table 1. The unsubstituted 1-isoquinolyl compound **8a** had a little more potent activity than the previously reported 5-nitro-2-pyridyl compound **5a**.[12] We already reported that introduction of a nitrile or nitro group on the phenyl or pyridyl ring led to an increase in activity.[12] Introduction of a chloro or nitrile group at the 4-position of isoquinoline resulted in compounds **8b** and **8c** with similar activities to compounds **8a**, suggesting that no further increase of activity would be expected by the substitution. 4-Quinolyl compound **8e** was more suitable than 2-quinolyl analog **8d**. The substituent effect of 4-quinolyl compounds was investigated to explore the extension of the S2 subsite and the introduction of a trifluoromethyl group led to the most potent compound **8g** among the reported DPP-4 inhibitors possessing the prolylthiazolidine core structure.[11,12] The introduction of a chlorine atom at the 7-position of 4-quinolyl maintained the activity (**8h**), whereas the introduction of a bulky group such as trifluoromethyl decreased the activity (**8i**). These results indicate that the S2 subsite of DPP-4 has extensive and shallow space and is responsible for increasing the activity.

X-ray crystal structure determination shows that **8g** made many interactions with the active site of DPP-4 (Fig. 3). The thiazolidine moiety fully occupies the S1 hydrophobic subsite. The secondary amino group of the proline moiety forms salt bridges to Glu205 and Glu206, and the carbonyl oxygen forms a hydrogen bond with Asn710. The quinolyl ring is stacked with the side chain of Phe357 in the S2 subsite. The trifluoromethyl group on the quinolyl ring also interacted with Tyr585[16] and this interaction explained that compound **8f** having a methyl group instead showed a 2-fold loss in activity. Since Tyr585 is exposed to solvent and the side chain forms hydrogen bonds with water molecules, the interaction between trifluoromethyl and Tyr585 is geometrically imperfect. Thus the interaction seems to work weaker than that in ideal hydrogen bonds. The distance from the carbon atom of the guanidine group of Arg358 to the quinolyl ring is 3.3 Å, which is slightly shorter than that of common CH-π interactions.[17] This suggests that CH-π interactions formed by induced fit between the quinolyl ring and the guanidinyl group of Arg358 increased the activity. We define the space consisting of Arg358 and surrounding amino acids as 'S2 extensive subsite' (Fig. 3).

**Table 2**
Inhibition of benzimidazole, benzoxazole and benzothiazole derivatives



| Compound | Ar | DPP-4 inhibition, $IC_{50}$ (nmol/L) | |
|---|---|---|---|
| | | Human | Rat |
| **9a** |  | 0.80 | 1.1 |
| **9b** |  | 0.59 | 1.03 |
| **9c** |  | 0.39 | 0.61 |
| **9d** |  | 1.2 | 1.5 |
| **9e** |  | 0.50 | 0.72 |
| **9f** |  | 0.55 | 0.77 |
| **9g** |  | 0.42 | 0.69 |

Kim et al., reported that the guanidine moiety of Arg358 interacts with the trifluoromethyl substituent of sitagliptin (**1**), which seems to increase the activity and that much attention has focused on the interaction with Arg358 in DPP-4 inhibitors recently.[18] This result suggests that the interaction with the S2 extensive subsite plays an important role for DPP-4 inhibition.

In vitro human and rat DPP-4 inhibitory activity of fused bicyclic heteroaryl, such as benzimidazole, benzoxazole and benzothiazole, are listed in Table 2. The activities of compounds **9a**, **9d**, and

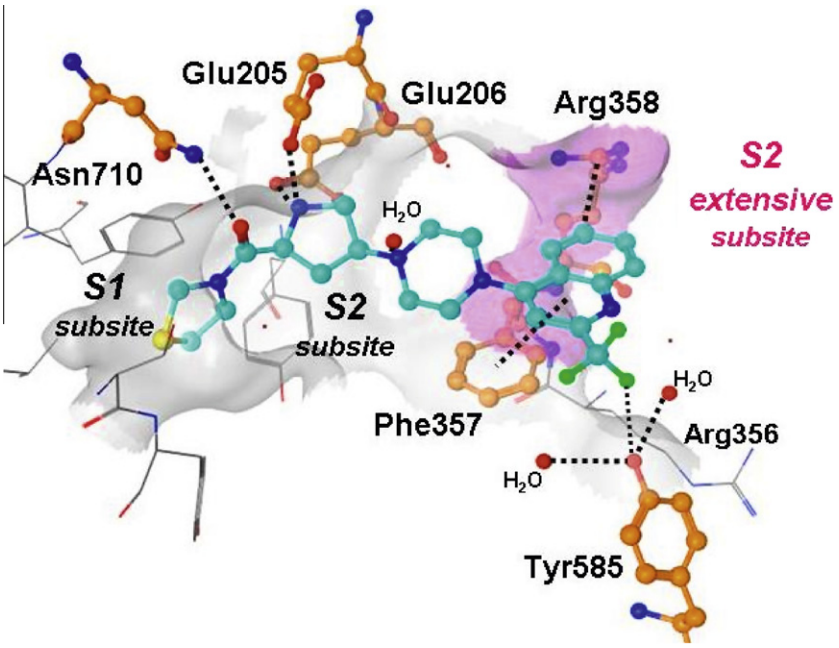



**Figure 3.** Binding interactions in active site. Co-crystallization of compound **8g** and human DPP-4. The surface in pink represents S2 extensive subsite.

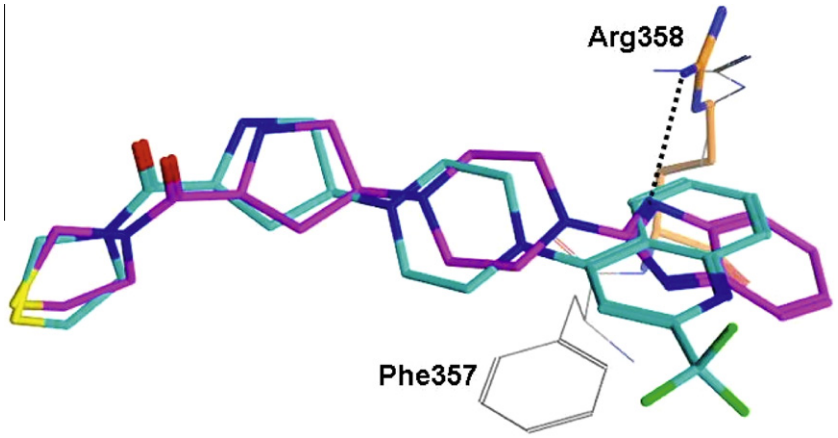



**Figure 4.** Docking model of compound **9a** and DPP-4. Docking model of compound **9a** (magenta carbon) and DPP-4 is superimposed on the X-ray structure of **8g** (cyan carbon). Phe357 and Arg358 are analyzed with compound **8g**. Arg358 (orange stick) is included in the X-ray structure '1NU8'[19] in Protein Data Bank.

**9f** were similar to those of **8a** and **8e**. Figure 4 shows a docking model of compound **9a** and DPP-4 which is superimposed on the X-ray structure of **8g**. In this model, compound **9a** lost the stacking interaction with Phe357. Meanwhile, benzimidazole of **9a** formed a weak hydrogen bond with the side chain of Arg358 known for multiple conformations by many DPP-4 X-ray structures. Since the lost interaction with Phe357 would be compensated by the flexibility of Arg358, compounds **9a**, **9d**, and **9f** seem to keep similar activities to **8a** and **8e**. Nitrile compounds **9c** and **9e** showed 2-fold higher activities than unsubstituted compounds **9a** and **9d**, respectively. The docking model of **9e** is shown in Figure 5. The nitrile group on the benzoxazole formed a direct hydrogen bond with the side chain of Arg356. It might be expected to work much more effectively, however, the penalty of the desolvation energy of Arg356 making the hydrogen bond with the nitrile group has to be taken into consideration. Moreover, Arg356 is observed to form hydrogen bonds with the side chain of Glu403 and the main chain of Val404 in the X-ray structure of DPP-4 complexed with compound **8g**, thus the conformation of Arg356 is rigid. Since the nitrile group of compound **9g** made no hydrogen bond with the side chain of Arg356 in the docking model (data not shown), **9g** is thought to have comparable activity to **9f**.

The enzymes most closely related to DPP-4 are the fibroblast activation protein (FAP), DPP-II, DPP-8 and DPP-9.[13] Although the precise physiological functions of the enzymes are not known, DPP-8 and DPP-9 are widely distributed cytosolic enzymes, and their inhibition would induce the toxicity of DPP-4 inhibitors identified to date, including alopecia, thrombocytopenia, anaemia, enlarged spleen, multiple histological pathologies, and animal mortality.[14] The (4-nitrophenyl)piperazine compound **5b** has moderate selectivity against DPP-8 and DPP-9 (8- and 19-fold, respectively), while the quinolyl compound **8g** showed at least 200-fold selectivity over them (Table 3). Kang et al., reported that Phe357 plays an important role in increasing the selectivity against DPP-9.[20] Since both **5b** and **8g** form interactions with Phe357, there would be additional interactions between **8g** and DPP-4 to explain the high selectivity of **8g**. Amino acid sequences in the S1 subsite (Y547, S630, Y631, V656, Y662, Y666, N710, V711 and H740 of DPP-4) are common between DPP-4, DPP-8 and DPP-9,[21] but those of the S2 extensive subsite are different (Table 4), suggesting that the interaction formed by induced fit between the quinolyl ring with the S2 extensive subsite would contribute to the high selectivity of **8g** against DPP-8 and DPP-9. In order to increase the selectivity, the interaction with the S2 extensive subsite should be carefully considered.

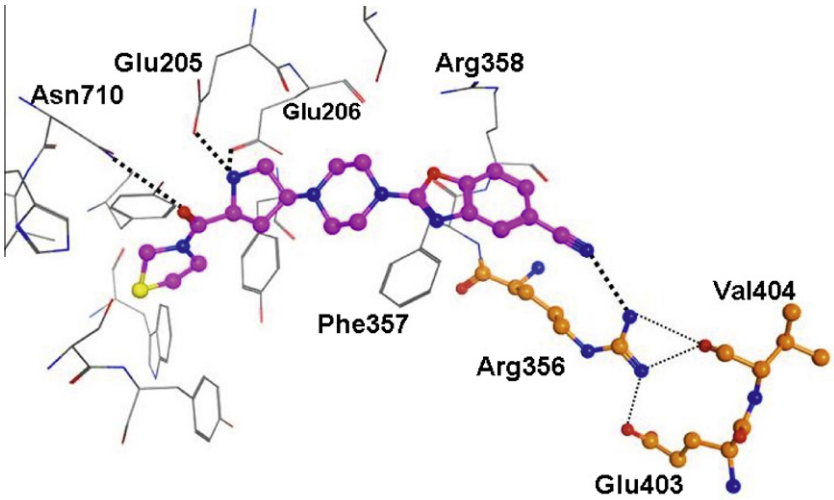



**Figure 5.** Docking model of compound **9e** and DPP-4. Compound **9e** (magenta carbon) forms a hydrogen bond with the side chain of Arg356 (orange carbon). Arg356 has hydrogen bonds with Glu403 and Val404 (orange stick carbon).

**Table 3**
Selectivity of DPP-4 inhibition against DPP-8 and DPP-9

| Compound | Inhibitory activity $IC_{50}$ (nmol/L) on human enzymes | | |
|---|---|---|---|
| | DPP-4 | DPP-8 | DPP-9 |
| **5b** | 1.6 | 12.2 | 30.2 |
| **8g** | 0.37 | 72.4 | 105 |

**Table 4**
The alignment of the amino acids in S2 extensive subsite of DPP-4, DPP-8 and DPP-9

| DPP-4 family | Corresponding amino acids in S2 extensive subsite | |
|---|---|---|
| DPP-4 | Arg358 | Tyr585 |
| DPP-8 | Asp435 | His693 |
| DPP-9 | Asp425 | Gln684 |

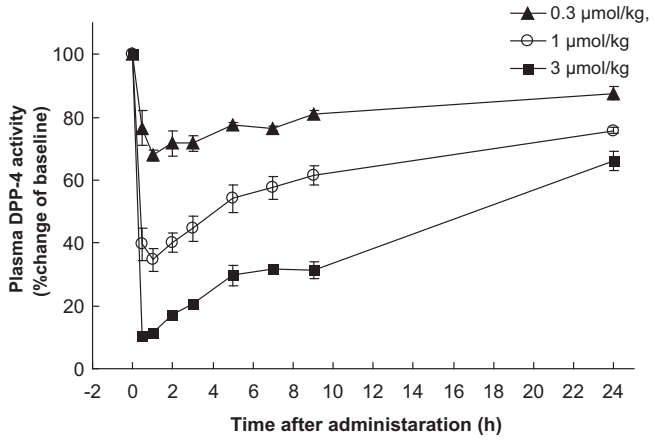



**Figure 6.** Effect of **8g** on plasma DPP-4 activity (% change of baseline) in Wistar rat. **8g** was orally administrated at a dose of 0.3, 1 or 3 μmol/kg at 0 h. Data are expressed as means ± SEM ($n = 3$).

Evaluation of plasma DPP-4 activity after oral dosing of the compound **8g** can be used to predict the efficacy of antihyperglycemic activity and pharmacokinetic profile. The representative compound **8g** was administered orally to Wistar rats at a dose of 0.3, 1 or 3 μmol/kg and the plasma DPP-4 activity was evaluated ex vivo. As shown in Figure 6, compound **8g** Indicated dose-dependent, fast-onset and long-lasting DPP-4 inhibitory activity. Most noteworthy, more than 40% inhibition of plasma DPP-4 was sustained at a single oral dose of 3 μmol/kg until 24 h.

## 4. Conclusion

A series of prolylthiazolidines with fused bicyclic heteroarylpiperazine substitution has been discovered as DPP-4 inhibitors, and the most potent compound **8g** showed high selectivity and in vivo efficacy. X-ray crystal structure determination indicates that CH-π interactions formed by induced fit between the quinolyl ring and the guanidinyl group of Arg358 enhances the activity and selectivity for DPP-4 inhibition.

## 5. Experimental

### 5.1. Chemistry

#### 5.1.1. General

$^1$H NMR spectra were measured on a Bruker DPX-300 instrument or on a Bruker AMX-500 with tetramethylsilane as the internal standard; chemical shifts are reported in parts per million (ppm, $\delta$ units). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet; dt, double triplet; br s, broad singlet. Electron analysis for carbon, hydrogen, and nitrogen was performed with a Yanagimoto CHN CORDER MT-6. LC–MS spectra were obtained on a PE-Sciex API 165 spectrometer with electrospray ionization (ESI) mode. Chromatography refers to flash chromatography conducted on silica gel BW-300 (Fuji Silysia). All chemicals and solvents were of reagent grade unless otherwise specified. For drying organic solutions in extraction, anhydrous sodium sulfate or anhydrous magnesium sulfate was used unless otherwise indicated. The following abbreviations are used: DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBT, 3-hydroxybenztriazole hydrate.

#### 5.1.2. 3-((*S*)-1-*tert*-Butoxycarbonyl-4-oxopyrrolidin-2-ylcarbonyl)thiazolidine (7a)

A mixture of *N*-*tert*-butoxycarbonyl-L-*trans*-hydroxyproline (**6a**) (69.4 g, 0.3 mol), thiazolidine (29.4 g, 0.33 mmol), HOBT (50.5 g, 0.33 mol) and EDC (63.3 g, 0.33 mol) in DMF (300 mL) was stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure. To the residue was added a saturated aqueous sodium hydrogen carbonate solution and the mixture was extracted with ethyl acetate. The extract was dried and concentrated under reduced pressure to give 3-[(2*S*,4*R*)-1-*tert*-butoxycarbonyl-4-hydroxypyrrolidin-2-ylcarbonyl]thiazolidine (56.3 g, 62%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.41–1.45 (9H, m), 1.95–2.34 (2H, m), 2.62–3.25 (2H, m), 3.40–3.98 (4H, m), 4.40–4.90 (4H, m).

To a solution of the above compound (55.4 g, 183 mmol) and triethylamine (46 mL, 330 mmol) in dichloromethane (350 mL) was added sulfur trioxide-pyridine complex (52.4 g, 329 mmol) in DMSO (150 mL) under ice cooling and the mixture was stirred for 2 h. The reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue purified by silica gel chromatography with n-hexane/ethyl acetate (1:1, v/v) to give the title compound (30.3 g, 55%) as a white powder. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 1.36, 1.40 (9H, s), 2.36–2.45 (1H, m), 2.97–3.12 (3H, m), 3.62–3.71 (2H, m), 3.74–3.94 (2H, m), 4.33–4.80 (2H, m), 4.91–5.04 (1H, m).

#### 5.1.3. 3-{(2*S*,4*S*)-4-[4-(Isoquinolin-1-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8a)

A mixture of **7a** (606 mg, 2.02 mmol), 1-(isoquinolin-1-yl)piperazine (692 mg, 2.42 mmol) and sodium cyanoborohydride (130 mg, 2.07 mmol) in methanol (10 mL) was stirred at room temperature for 14 h. The reaction mixture was concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with chloroform/methanol (20:1, v/v) to give 3-{(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-[4-(isoquinolin-1-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine (216 mg, 21%) as a pale-yellow powder.

To a solution of the above compound (215 mg, 0432 mmol) in methanol (3 mL) and chloroform (3 mL) was added 4 mol/L hydrochloric acid in 1,4-dioxane (1 mL) and the mixture stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and the residue was crystallized with ethanol to give the title compound (99 mg, 42%) as a pale-yellow powder. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 2.25–2.30 (1H, m), 3.00–3.17 (3H, m), 3.59–3.95 (12H, m), 4.13–4.18 (1H, m), 4.49–4.77 (3H, m), 7.59 (1H, d, $J = 6.1$ Hz), 7.71–7.74 (1H,

m), 7.86–7.89 (1H, m), 8.02 (1H, d, $J$ = 8.2 Hz), 8.08 (1H, d, $J$ = 6.1 Hz), 8,21 (1H, d, $J$ = 8.5 Hz), 9.25 (1H, br s), 10.89 (1H, br s); Anal. Calcd for $C_{21}H_{27}N_5OS{\cdot}3HCl{\cdot}0.3C_2H_6O{\cdot}1.5H_2O$: C, 47.36; H, 6.40; N, 12.79. Found: C, 47.26; H, 6.42; N, 12.60; LC–MS (ESI) $m/z$ 398.2 $[M+H]^+$.

#### 5.1.4. 3-{(2*S*,4*S*)-4-[4-(4-Chloroisoquinolin-1-yl)piperazin-1-yl]-pyrrolidin-2-ylcarbonyl}thiazolidine hemipentahydrochloride (8b)

To a solution of **7a** (450 mg, 1.50 mmol), 1-(4-chloroisoquinolin-1-yl)piperazine (446 mg, 1.80 mmol) and acetic acid (0.090 mL, 1.6 mmol) in 1,2-dichloroethane (8 mL) was added sodium triacetoxyborohydride (636 mg, 3.00 mmol) and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue purified by silica gel chromatography with chloroform/methanol (50:1, v/v) to give 3-{(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-[4-(4-chloroisoquinolin-1-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine (596 mg, 75%) as a white powder.

The above compound (592 mg, 1.11 mmol) was dissolved in 1.1 mol/L hydrogen chloride in methanol (10 mL), and the mixture was stirred at room temperature for 5 days. The reaction mixture was concentrated under reduced pressure, and the residue was crystallized with ethanol to give the title compound (318 mg, 52%) as a pale-yellow powder. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.32–2.46 (1H, m), 2.95–4.20 (16H, m), 4.43–4.78 (3H, m), 7.74–7.82 (1H, m), 7.90–7.97 (1H, m), 8.14 (1H, d, $J$ = 8.0 Hz), 8.23 (1H, d, $J$ = 8.3 Hz), 8.30 (1H, s), 9.17 (1H, br s), 10.83 (1H, br s), 12.53 (1H, br s); Anal. Calcd for $C_{21}H_{26}ClN_5OS{\cdot}2.5HCl{\cdot}1.5H_2O$: C, 45.85; H, 5.77; N, 12.73. Found: C, 45.59; H, 6.05; N, 12.82; LC–MS (ESI) $m/z$ 432.2 $[M+H]^+$.

#### 5.1.5. 3-{(2*S*,4*S*)-4-[4-(4-Cyanoisoquinolin-1-yl)piperazin-1-yl]-pyrrolidin-2-ylcarbonyl}thiazolidine Trihydrochloride (8c)

1-Chloro-4-cyanoisoquinoline (500 mg, 2.65 mmol) was added to piperazine (4.61 g, 53.5 mmol) at 140 °C. The mixture was stirred at 140 °C for 2 h, and then water was added to the reaction mixture. The mixture was extracted with chloroform, and the extract was washed with brine, dried and concentrated under reduced pressure to give 1-(4-cyanoisoquinolin-1-yl)piperazine (491 mg, 78%) as a dark brown solid. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.08–3.17 (4H, m), 3.62–3.68 (4H, m), 7.59 (1H, t, $J$ = 7.1 Hz), 7.77 (1H, t, $J$ = 7.1 Hz), 8.02–8.09 (2H, m), 8.47 (1H, s).

The title compound was prepared in 36% yield using the above compound in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.26–2.43 (1H, m), 2.93–4.20 (16H, m), 4.44–4.78 (3H, m), 7.74–7.82 (1H, m), 7.75–8.05 (2H, m), 8.22 (1H, d, $J$ = 8.4 Hz), 8.69 (1H, s), 9.16 (1H, br s), 10.85 (1H, br s), 12.65 (1H, br s); Anal. Calcd for $C_{22}H_{26}N_6OS{\cdot}3HCl{\cdot}0.4C_2H_6O{\cdot}H_2O$: C, 48.18; H, 5.92; N, 14.79. Found: C, 48.02; H, 6.16; N, 15.13; LC–MS (ESI) $m/z$ 423.2 $[M+H]^+$.

#### 5.1.6. 3-{(2*S*,4*S*)-4-[4-(Quinolin-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8d)

The title compound was prepared in 61% yield using 1-(quinolin-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.20–2.30 (1H, m), 2.96–3.17 (3H, m), 3.64–4.40 (13H, m), 4.47–4.76 (3H, m), 7.50 (1H, t, $J$ = 7.5 Hz), 7.60 (1H, d, $J$ = 9.6 Hz), 7.77 (1H, t, $J$ = 7.8 Hz), 7.93 (1H, d, $J$ = 7.5 Hz), 8.15–8.20 (1H, m), 8.44 (1H, d, $J$ = 9.6 Hz), 9.21 (1H, br s), 10.68 (1H, br s); Anal. Calcd for $C_{21}H_{27}N_5OS{\cdot}3HCl{\cdot}3.5H_2O$: C, 44.25; H, 6.54; N, 12.29. Found: C, 44.23; H, 6.34; N, 12.18; LC–MS (ESI) $m/z$ 398.2 $[M+H]^+$.

#### 5.1.7. 3-{(2*S*,4*S*)-4-[4-(Quinolin-4-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8e)

The title compound was prepared in 34% yield using 1-(quinolin-4-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.16–2.40 (1H, m), 2.70–4.30 (16H, m), 4.40–4.80 (3H, m), 7.37 (1H, d, $J$ = 6.9 Hz), 7.77 (1H, t, $J$ = 8.1 Hz), 8.04 (1H, t, $J$ = 8.4 Hz), 8.21 (2H, d, $J$ = 8.7 Hz), 8.85 (1H, d, $J$ = 6.9 Hz); Anal. Calcd for $C_{21}H_{27}N_5OS{\cdot}3HCl{\cdot}0.5C_2H_6O{\cdot}2.2H_2O$: C, 46.39; H, 6.62; N, 12.30. Found: C, 46.34; H, 6.54; N, 12.47; LC–MS (ESI) $m/z$ 398.2 $[M+H]^+$.

#### 5.1.8. 3-{(2*S*,4*S*)-4-[4-(2-Methylquinolin-4-yl)piperazin-1-yl]-pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8f)

The title compound was prepared in 75% yield using 1-(2-methylquinol-4-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.20–2.42 (1H, m), 2.81 (3H, s), 2.91–3.20 (3H, m), 3.30–4.26(13H, m), 4.44–4.87 (3H, m), 7.35 (1H, s), 7.73 (1H, t, $J$ = 7.6 Hz), 8.00 (1H, t, $J$ = 7.6 Hz), 8.16 (1H, d, $J$ = 8.4 Hz), 8.24 (1H, d, $J$ = 8.4 Hz); Anal. Calcd for $C_{22}H_{29}N_5OS{\cdot}3HCl{\cdot}0.6C_2H_6O{\cdot}1.4H_2O$: C, 48.56; H, 6.75; N, 12.21. Found: C, 48.64; H, 6.72; N, 12.11; LC–MS (ESI) $m/z$ 412.4 $[M+H]^+$.

#### 5.1.9. 3-{(2*S*,4*S*)-4-[4-(2-Trifluoromethylquinolin-4-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine dihydrochloride (8g)

The title compound was prepared in 47% yield using 1-(2-trifluoromethylquinolin-4-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 2.41–2.43 (1H, m), 3.08–3.18 (3H, m), 3.57–3.98 (12H, m), 4.20–4.24 (1H, m), 4.50–4.81 (3H, m), 7.39 (1H, s), 7.76 (1H, t, $J$ = 7.6 Hz), 7.88–7.91 (1H, m), 8.12–8.17 (2H, m), 9.26 (1H, br s), 11.10 (1H, br s); Anal. Calcd for $C_{22}H_{26}F_3N_5OS{\cdot}2HCl{\cdot}3H_2O$: C, 44.60; H, 5.78; N, 11.82. Found: C, 44.85; H, 5.73; N, 11.87; LC–MS (ESI) $m/z$ 466.4 $[M+H]^+$.

#### 5.1.10. 3-{(2*S*,4*S*)-4-[4-(7-Chloroquinolin-4-yl)piperazin-1-yl]-pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8h)

The title compound was prepared in 55% yield using 1-(7-chloroquinolin-4-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.10–2.37 (1H, m), 2.84–4.00 (16H, m), 4.41–4.82 (3H, m), 7.36 (1H, d, $J$ = 6.9 Hz), 7.77 (1H, dd, $J$ = 1.8, 9.0 Hz), 8.22 (1H, d, $J$ = 9.0 Hz), 8.27 (1H, d, $J$ = 1.8 Hz), 8.85 (1H, d, $J$ = 6.9 Hz), 9.18 (1H, br s), 10.82 (1H, br s); Anal. Calcd for $C_{21}H_{26}ClN_5OS{\cdot}3HCl{\cdot}1.5H_2O$: C, 44.38; H, 5.67; N, 12.32. Found: C, 44.24; H, 5.71; N, 12.23; LC–MS (ESI) $m/z$ 432.2 $[M+H]^+$.

#### 5.1.11. 3-{(2*S*,4*S*)-4-[4-(7-Trifluoromethylquinolin-4-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine Trihydrochloride (8i)

The title compound was prepared in 23% yield using 1-(7-trifluoromethylquinolin-4-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.20–2.47 (1H, m), 2.90–3.20 (3H, m), 3.30–4.30 (13H, m), 4.45–4.85 (3H, m), 7.46 (1H, d, $J$ = 6.7 Hz), 7.98 (1H, dd, $J$ = 1.5, 9.0 Hz), 8.43 (1H, d, $J$ = 8.9 Hz), 8.62 (1H, s), 8.96 (1H, d, $J$ = 6.7 Hz); Anal. Calcd for $C_{22}H_{26}F_3N_5OS{\cdot}3HCl{\cdot}0.5C_2H_6O{\cdot}H_2O$: C, 44.85; H, 5.56; N, 11.37. Found: C, 44.94; H, 5.51; N, 11.38; LC–MS (ESI) $m/z$ 466.4 $[M+H]^+$.

#### 5.1.12. 3-{(2*S*,4*S*)-4-[4-(Benzimidazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine dihydrochloride (9a)

The title compound was prepared in 43% yield using 1-(benzimidazol-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.72–2.16 (1H, m), 2.65–4.30 (16H, m), 4.40–4.80 (3H, m), 7.18–7.33 (2H, m), 7.36–7.51 (2H, m), 8.95 (1H, br s), 9.70 (1H, br s), 10.50 (1H, br s), 13.71 (1H, br s); Anal. Calcd for $C_{19}H_{26}N_6OS{\cdot}2HCl{\cdot}3H_2O$: C, 44.44; H, 6.67; N,

16.37. Found: C, 44.67; H, 6.52; N, 16.73; LC–MS (ESI) *m/z* 387.2 $[M+H]^+$.

#### 5.1.13. 3-[(2*S*,4*S*)-1-Benzyloxycarbonyl-4-(piperazin-1-yl)pyrrolidin-2-ylcarbonyl]thiazolidine (10)

To a solution of 3-((*S*)-1-benzyloxycarbonyl-4-oxopyrrolidin-2-ylcarbonyl)thiazolidine (**7b**) (1.36 g, 4.07 mmol), 1-(*tert*-butoxycarbonyl)piperazine (910 mg, 4.89 mmol) and acetic acid (0.24 mL, 4.2 mmol) in 1,2-dichloroethane (40 mL) was added sodium triacetoxyborohydride (1.72 g, 8.11 mmol) and the mixture was stirred at room temperature overnight. The reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with ethyl acetate to give 3-[(2*S*,4*S*)-1-benzyloxycarbonyl-4-(4-*tert*-butoxycarbonylpiperazin-1-yl)pyrrolidin-2-ylcarbonyl]thiazolidine (1.80 g, 88%) as a white powder.

A mixture of the above compound (1.79 g, 3.55 mmol) and trifluoroacetic acid (10 mL) in dichloromethane (100 mL) was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and the residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure to give the title compound (1.38 g, 96%) as a white powder. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.46–1.55 (1H, m), 1.90–3.14 (13H, m), 3.52–3.88 (3H, m), 4.33–4.72 (3H, m), 4.87–5.08 (2H, m), 7.27–7.43 (5H, m); LC–MS (ESI) *m/z* 405.4 $[M+H]^+$.

#### 5.1.14. 3-{(2*S*,4*S*)-4-[4-(5-Chlorobenzimidazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine Trihydrobromide (9b)

A mixture of **10** (345 mg, 0.853 mmol), 2,5-dichlorobenzimidazole (**11a**) (191 mg, 1.02 mmol) and *N*,*N*-diisopropylethylamine (0.18 mL, 1.0 mmol) in *N*-methyl-2-pyrrolidone (6 mL) was stirred at 100 °C overnight. The reaction mixture was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by high performance liquid chromatography to give 3-{(2*S*,4*S*)-1-benzyloxycarbonyl-4-[4-(5-chlorobenzimidazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine (122 mg, 26%) as a pale-yellow powder.

A mixture of the above compound (110 mg, 0.198 mmol) and 30% hydrogen bromide-acetic acid solution (10 mL) was stirred at room temperature for 6 h. Diethyl ether was added to the reaction mixture, and the precipitate was collected by filtration and recrystallized with ethanol to give the title compound (56 mg, 40%) as a white powder. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 1.76–1.96 (1H, m), 2.70–4.87 (16H, m), 4.46–4.74 (3H, m), 7.32 (1H, dd, *J* = 1.7, 8.5 Hz), 7.44 (1H, d, *J* = 8.5 Hz), 7.48 (1H, d, *J* = 1.7 Hz), 8.96 (1H, br s), 9.59 (1H, br s), 13.15 (1H, br s); Anal. Calcd for $C_{19}H_{25}ClN_6OS{\cdot}3HBr{\cdot}2H_2O$: C, 32.61; H, 4.61; N, 12.01. Found: C, 32.65; H, 4.38; N, 11.82; LC–MS (ESI) *m/z* 421.2 $[M+H]^+$.

#### 5.1.15. 2-Chlorobenzimidazole-5-carbonitrile (11b)

A solution of 3,4-diaminobenzonitrile (2.60 g, 18.2 mmol) and pyridine (2 mL, 25 mmol) in DMF (20 mL) was added a solution of bis(trichloromethyl)carbonate (2.12 g, 7.14 mmol) in tetrahydrofuran (20 mL) dropwise under ice-cooling. The mixture was stirred at room temperature for 18 h. Dilute hydrochloric acid was added to the reaction mixture, and the mixture was extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. To the residue was added ethyl acetate, and then the precipitate was collected by filtration to give 2-hydroxybenzimidazole-5-carbonitrile (896 mg, 29%) as a purple solid. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 7.07 (1H, d, *J* = 8.2 Hz), 7.31 (1H, s), 7.39 (1H, d, *J* = 8.2 Hz), 11.03 (1H, br s), 11.16 (1H, br s); LC–MS (ESI) *m/z* 160.0 $[M+H]^+$.

A mixture of the above compound (894 mg, 5.62 mmol) and phosphorus oxychloride (12 mL) was refluxed for 3 h. The reaction mixture was added to ice, and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with n-hexane/ethyl acetate (2:3, v/v) to give the title compound (322 mg, 32%) as a white powder. LC–MS (ESI) *m/z* 178.0 $[M+H]^+$.

#### 5.1.163-{(2*S*,4*S*)-4-[4-(5-Cyanobenzimidazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine Trihydrochloride (9c)

3-{(2*S*,4*S*)-1-Benzyloxycarbonyl-4-[4-(5-cyanobenzimidazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine was prepared in 54% yield using **11b** in the procedures outlined for **9b**.

A mixture of the above compound (200 mg, 0.367 mmol) and thioanisole (0.43 mL, 3.7 mmol) in trifluoroacetic acid (10 mL) was stirred at room temperature overnight. Diethyl ether was added to the reaction mixture, and the precipitate was collected by filtration and purified by high performance liquid chromatography and converted to hydrochloride salt with 4 mol/L hydrogen chloride in ethyl acetate to give the title compound (50 mg, 26%) as a white powder. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 1.89–2.09 (1H, m), 2.78–4.20 (16H, m), 4.47–4.82 (3H, m), 7.51 (1H, d, *J* = 8.2 Hz), 7.61 (1H, d, *J* = 8.2 Hz), 7.81 (1H, s), 8.97 (1H, br s), 10.28 (1H, br s); Anal. Calcd for $C_{20}H_{25}N_7OS{\cdot}3HCl{\cdot}0.4C_4H_8O_2{\cdot}2.7H_2O$: C, 42.90; H, 6.10; N, 16.21. Found: C, 42.92; H, 5.82; N, 16.12; LC–MS (ESI) *m/z* 412.4 $[M+H]^+$.

#### 5.1.17. 3-{(2*S*,4*S*)-4-[4-(Benzoxazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (9d)

The title compound was prepared in 34% yield using 1-(benzoxazol-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.20–2.42 (1H, m), 2.89–3.20 (3H, m), 3.25–4.35 (13H, m), 4.40–4.80 (3H, m), 7.10 (1H, dt, *J* = 1.2, 7.5 Hz), 7.22 (1H, dt, *J* = 1.2, 7.8 Hz), 7.37 (1H, dd, *J* = 0.6, 7.8 Hz), 7.47 (1H, d, *J* = 7.8 Hz), 9.25 (1H, br s), 11.00 (1H, br s); Anal. Calcd for $C_{19}H_{25}N_5O_2S{\cdot}3HCl{\cdot}0.3C_2H_6O{\cdot}1.5H_2O$: C, 43.78; H, 6.15; N, 13.02. Found: C, 43.74; H, 6.23; N, 13.18; LC–MS (ESI) *m/z* 388.4 $[M+H]^+$.

#### 5.1.18. 3-{(2*S*,4*S*)-4-[4-(5-Cyanobenzoxazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine Dihydrochloride (9e)

The title compound was prepared in 28% yield using 1-(5-cyanobenzoxazol-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.94–2.26 (1H, m), 2.80–3.00 (1H, m), 3.00–4.30 (15H, m), 4.45–4.78 (3H, m), 7.56 (1H, dd, *J* = 1.8, 8.4 Hz), 7.66 (1H, d, *J* = 8.4 Hz), 7.83 (1H, d, *J* = 1.5 Hz), 9.05 (1H, br s), 10.43 (1H, br s); Anal. Calcd for $C_{20}H_{24}N_6O_2S{\cdot}2HCl{\cdot}0.5H_2O$: C, 48.58; H, 5.50; N, 17.00. Found: C, 48.33; H, 5.58; N, 16.71; LC–MS (ESI) *m/z* 413.4 $[M+H]^+$.

#### 5.1.19. 3-{(2*S*,4*S*)-4-[4-(Benzothiazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (9f)

The title compound was prepared in 64% yield using 1-(benzothiazol-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.20–2.44 (1H, m), 2.90–3.20 (3H, m), 3.35–4.30 (13H, m), 4.42–4.82 (3H, m), 7.16 (1H, t, *J* = 7.2 Hz), 7.35 (1H, t, *J* = 7.2 Hz), 7.55 (1H, d, *J* = 7.8 Hz), 7.86 (1H, d, *J* = 7.5 Hz), 9.25 (1H, br s), 10.90 (1H, br s); Anal. Calcd for $C_{19}H_{25}N_5OS_2{\cdot}3HCl{\cdot}0.2C_2H_6O{\cdot}H_2O$: C, 43.14; H, 5.82; N, 12.96. Found: C, 43.07; H, 5.70; N, 12.99; LC–MS (ESI) *m/z* 404.4 $[M+H]^+$.

#### 5.1.20. 3-{(2*S*,4*S*)-4-[4-(5-Cyanobenzothiazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine Dihydrochloride (9g)

The title compound was prepared in 48% yield using 1-(5-cyanobenzothiazol-2-yl)piperazine in the procedures outlined for **8b**. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.04–2.28 (1H, m), 2.82–3.00 (1H, m), 3.00–4.30 (15H, m), 4.43–4.80 (3H, m), 7.53 (1H, dd, $J$ = 1.5, 8.2 Hz), 7.93 (1H, d, $J$ = 1.5 Hz), 8.07 (1H, d, $J$ = 8.2 Hz), 9.08 (1H, br s), 10.51 (1H, br s); Anal. Calcd for $C_{20}H_{24}N_6OS_2{\cdot}2HCl{\cdot}H_2O$: C, 46.24; H, 5.43; N, 16.18. Found: C, 46.05; H, 5.41; N, 16.04; LC–MS (ESI) $m/z$ 429.2 $[M+H]^+$.

### 5.2. X-Ray crystallographic study

The protein of human DPP-4 (33−766) secreted from insect cells was purified and crystallized according to the method reported by Hiramatsu et al.[22] The protein-inhibitor complex was obtained by soaking a preformed DPP-4 crystal in the presence of compound **8g** and preserved in liquid nitrogen for data collection at 100 K. X-ray diffraction data were collected at the high energy accelerator research organization (KEK) beam line BL5 and processed using the program HKL2000.[23] The structure of DPP-4 inhibitor complex was solved by molecular replacement with the program PHASER,[24] utilizing the previously determined coordinates of DPP-4 with Protein Data Bank accession code 1J2E. The structure was refined against all available data to 2.10 Å using Maximum likelihood (Refmac) to a crystallographic *R*-factor of 18.5% and free *R*-factor of 22.4%. Coordinates have been deposited with the Protein Data Bank, accession code 3VJM. Data collection and model refinement statistics are summarized in Table 5.

### 5.3. Docking studies in DPP-4

The x-ray crystal structure of DPP-4 complexed with compound **8g** was utilized in the docking calculations. The compounds were docked into DPP-4 using Glide 5.7.[25]

**Table 5**
DPP-4 complex with **8g** data collection and refinement statistics

| PDB entry code | 3VJM |
|---|---|
| *Crystal* | |
| Space group | $P2_12_12_1$ |
| Unit cell parameters: $a$ (Å) | 117.95 |
| $b$ (Å) | 125.94 |
| $c$ (Å) | 137.21 |
| *Data* | |
| Resolution (Å) | 50.00–2.10 (2.18–2.10) |
| Unique reflections | 119422 (10763) |
| Redundancy | 4.8 (4.5) |
| Completeness (%) | 91.0 (91.2) |
| $R_{merge}$[a] | 0.067 (0.399) |
| $I/\sigma$ | 11.7 |
| *Refinement* | |
| Resolution (Å) | 30.00–2.10 (2.15–2.10) |
| Unique reflections | 109644 (7466) |
| Completeness (%) | 91.8 (90.6) |
| Data in the test set | 5446 (411) |
| R-work | 0.187 (0.225) |
| R-free | 0.224 (0.277) |
| *Structure* | |
| Protein non-H atoms/B (Å$^2$) | 12180/28.4 |
| Ligand atoms/B (Å$^2$) | 64/27.6 |
| Water oxygen atoms/B (Å$^2$) | 987/31.3 |
| *Rmsd* | |
| Bond lengths (Å) | 0.011 |
| Bond angles (°) | 1.306 |
| *Ramachandran plot* | |
| Most favored regions (%) | 89.2 |
| Additionally allowed regions (%) | 10.2 |
| Generously allowed regions (%) | 0.6 |

Values in parentheses are for highest-resolution shell.
[a] $R_{merge} = \Sigma|(I-<I>)|/\Sigma(I)$, where I is the observed intensity.

### 5.4. Biological experiments

#### 5.4.1. DPP-4 inhibitory activity

The DPP-4 inhibitory activity of human and rat plasma was measured by fluorescence assay using Gly-Pro-MCA (Peptide Institute Inc.) as a DPP-4-specific fluorescent substrate. Reaction solutions containing 20 μL of human or rat plasma (10-fold diluted solution), 20 μL of fluorescent substrate (100 μmol/L), 140 μL of buffer (0.003% Brij-35 containing PBS), and 20 μL of test substrate (of various concentrations) were incubated at room temperature for 60 min using a 96-well flat-bottomed microtiter plate. The measured fluorescent intensity (excitation 360 nm/emission 465 nm, SPECTRA FLUOR, TECAN) was taken as the DPP-4 activity. The inhibitory rate relative to the solvent addition group was calculated and $IC_{50}$ values were determined by logistic analysis.

#### 5.4.2. DPP-8,9 inhibitory activity

DPP-8 and DPP-9 enzymes were prepared from cytoplasmic fractions of cells expressing recombinant human DPP-8 or DPP-9, respectively. Reaction solutions containing 20 μL of test compounds of various concentrations, 20 μL of enzyme preparations, 140 μL of buffer (0.003% Brij-35 containing PBS), and 20 μL of Gly-Pro-MCA (50 μmol/L, Peptide Institute Inc.) were incubated at 37 °C for 30 min using a 96-well flat-bottomed microtiter plate. The measured fluorescence intensity of 7-amino-4-methylcoumarin was taken as the enzyme.

#### 5.4.3. Plasma DPP-4 activity after oral administration of 8g to Wistar rats

Male Wistar rats (7–9 weeks of age) fasted overnight were used. Compound **8g** was dissolved in 0.5% hydroxypropylmethyl-cellulose and administered orally at a dose of 0.3, 1 or 3 μmol/kg. At pre-administration and at 0.5, 1, 2, 3, 5, 7, 9, and 24 h after administration, 0.1 mL of blood was collected from jugular vein. After centrifugation, 10 μL of plasma was diluted 10-fold using buffer (0.003% Brij-35 containing PBS). 20 μL of the diluted plasma was used instead of 20 μL of test substrate for the determination of DPP-4 inhibitory activity by fluorescence as described above.

## Acknowledgments

We thank Dr. Kazutoshi Watanabe and Dr. Kunitomo Adachi for helpful discussions. We also thank Dr. Tohru Nakajima and Dr. Takao Kondo for their insight and guidance for course of this work.

## References and notes

1. World Health Organization. 2011 <http://www.who.int/diabetes/en/>.
2. Bolen, S.; Feldman, L.; Vassy, J.; Wilson, L.; Yeh, H. C.; Marinpoulos, S.; Wiley, C.; Selvin, E.; Wilson, R.; Bass, E. B.; Brancati, F. L. *Ann. Intern. Med.* **2007**, *147*, 386.
3. (a) Kieffer, T. J.; McIntosh, C. H.; Pederson, R. A. *Endocrinology* **1995**, *136*, 3585; (b) Deacon, C. F.; Nauck, M. A.; Toft-Nielson, M.; Pridal, L.; Willms, B.; Holst, J. J. *Diabetes* **1995**, *44*, 1126.
4. Pospisilik, J. A.; Stafford, S. G.; Demuth, H.-U.; Brownsey, R.; Arkhouse, W.; Finegood, D. T.; McIntosh, C. H. S.; Pederson, R. A. *Diabetes* **2002**, *51*, 943.
5. (a) Idris, I.; Donnelly, R. *Diabetes Obes. Metab.* **2007**, *9*, 153; (b) von Geldern, T. W.; Trevillyan, J. M. *Drug Dev. Res.* **2006**, *67*, 627; (c) Weber, A. E. *J. Med. Chem.* **2004**, *47*, 4135.
6. Gallwitz, B. *Expert Opin. Invest. Drugs* **2011**, *20*, 723.
7. Havale, S. H.; Pal, M. *Bioorg. Med. Chem.* **2009**, *17*, 1783.
8. (a) Kelly, T. A.; Adams, J.; Bachovchin, W. W.; Barton, R. W.; Campbell, S. J.; Ciutts, S. J.; Kennedy, C. A.; Snow, R. J. *J. Am. Chem. Soc.* **1993**, *115*, 12637; (b) Peters, J.-U. *Curr. Top. Med. Chem.* **2007**, *7*, 579.
9. Ashworth, D. M.; Atrash, B.; Baker, G. R.; Baxter, A. J.; Jenkins, P. D. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1163.
10. (a) Magnin, D. R.; Robl, J. A.; Sulsky, R. B.; Augeri, D. J.; Huang, Y.; Simpkins, L. M.; Taunk, P. C.; Betebenner, D. A.; Robertson, J. G.; Abboa-Offei, B. E.; Wang, A.; Cap, M.; Xin, L.; Tao, L.; Sitkoff, D. F.; Malley, M. F.; Gougoutas, J. Z.; Khanna, A.; Huang, Q.; Han, S.-P.; Parker, R. A.; Hamann, L. G. *J. Med. Chem.* **2004**, *47*, 2587; (b) Augeri, D. J.; Robl, J. A.; Betebenner, D. A.; Magnin, D. R.; Khanna, A.; Robertson, J. S. G.; Wang, A.; Simpkins, L. M.; Taunk, P.; Huang, Q.; Han, S.-P.;

Abboa-Offei, B.; Cap, M.; Xin, L.; Tao, L.; Tozzo, E.; Welzel, G. E.; Egan, D. M.; Marcinkeviciene, J.; Chang, S. Y.; Biller, S. A.; Kirby, M. S.; Parker, R. A.; Hamann, L. G. *J. Med. Chem.* **2005**, *48*, 5025.
11. (a) Sakashita, H.; Akahoshi, F.; Kitajima, H.; Tsutsumiuchi, R.; Hayashi, Y. *Bioorg. Med. Chem.* **2006**, *14*, 3662; (b) Sakashita, H.; Akahoshi, F.; Yoshida, T.; Kitajima, H.; Hayashi, Y.; Ishii, S.; Takashina, Y.; Tsutsumiuchi, R.; Ono, S. *Bioorg. Med. Chem.* **2007**, *15*, 641.
12. Yoshida, T.; Sakashita, H.; Akahoshi, F.; Hayashi, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2618.
13. (a) Park, J. E.; Lenter, M. C.; Zimmermann, R. N.; Garin-Chesa, P.; Old, L. J.; Rettig, W. J. *J. Biol. Chem.* **1999**, *274*, 36505; (b) Abbott, C. A.; Yu, D. M.; Woollatt, E.; Sutherland, G. R.; McCaughan, G. W.; Gorrell, M. D. *Eur. J. Biochem.* **2000**, *267*, 6140; (c) Ajami, K.; Abbott, C. A.; McCaughan, G. W.; Gorrell, M. D. *Biochim. Biophys. Acta* **2004**, *1679*, 18.
14. Lankas, G. R.; Leiting, B.; Roy, R. S.; Eiermann, G. J.; Beconi, M. G.; Biftu, T.; Chan, C. C.; Edmondson, S.; Feeney, W. P.; He, H.; Ippolito, D. E.; Kim, D.; Lyons, K. A.; Ok, H. O.; Patel, R. A.; Petrov, A. N.; Pryor, K. A.; Qian, X.; Reigle, L.; Woods, A.; Wu, J. K.; Zaller, D.; Zhang, X.; Zhu, L.; Weber, A. E.; Thornberry, N. A. *Diabetes* **2005**, *54*, 2988.
15. Parikh, R.; Doering, W. V. E. *Arn. Soc.* **1967**, *89*, 5505.
16. Howard, J. A. K.; Hoy, V. J.; O'Hagan, D.; Smith, G. T. *Tetrahedron* **1996**, *52*, 12613.
17. Brandl, M.; Weiss, M. S.; Jabs, A.; Sühnel, J.; Hilgenfeld, R. *J. Mol. Biol.* **2001**, *307*, 357.
18. Kim, D.; Wang, L.; Beconi, M.; Eiermann, G. J.; Fisher, M. H.; He, H.; Hickey, G. J.; Kowalchick, J. E.; Leiting, B.; Lyons, K.; Marsilio, F.; McCann, M. E.; Patel, R. A.; Petrov, A.; Scapin, G.; Patel, S. B.; Roy, R. S.; Wu, J. K.; Wyvratt, M. J.; Zhang, B. B.; Thornberry, N. A.; Weber, A. E. *J. Med. Chem.* **2005**, *48*, 141.
19. Thoma, R.; Löffler, B.; Stihle, M.; Huber, W.; Ruf, A.; Hennig, M. *Structure* **2003**, *11*, 947.
20. Kang, N. S.; Ahn, J. H.; Kim, S. S.; Chae, C. H.; Yoo, S.-E. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3716.
21. Rummey, C.; Metz, G. *Proteins* **2007**, *66*, 160.
22. Hiramatsu, H.; Kyono, K.; Shima, H.; Fukushima, C.; Sugiyama, S.; Inaka, K.; Yamamoto, A.; Shimizu, R. *Acta Crystallogr.* **2003**, *D59*, 595.
23. Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307.
24. McCoy, A. J.; Grosse-Kunstleve, R. W.; Storoni, L. C.; Read, R. J. *Acta Crystallogr.* **2005**, *D61*, 458.
25. Glide; Schrodinger, LLC, New York, NY. 2011 <http://www.schrodinger.com/>.