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Original article

Design, synthesis and biological evaluation of hetero-aromatic moieties substituted pyrrole-2-carbonitrile derivatives as dipeptidyl peptidase IV inhibitors

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ABSTRACT

A series of novel hetero-aromatic moieties substituted α-amino pyrrole-2-carbonitrile derivatives was designed and synthesized based on structure–activity relationships (SARs) of pyrrole-2-carbonitrile inhibitors. All compounds demonstrated good dipeptidyl peptidase IV (DPP4) inhibitory activities (IC_{50} = 0.004–113.6 μM). Moreover, compounds **6h** (IC_{50} = 0.004 μM) and **6n** (IC_{50} = 0.01 μM) showed excellent inhibitory activities against DPP4, good selectivity (compound **6h**, selective ratio: DPP8/DPP4 = 450.0; DPP9/DPP4 = 375.0; compound **6n**, selective ratio: DPP8/DPP4 = 470.0; DPP9/DPP4 = 750.0) and good efficacy in an oral glucose tolerance test in ICR mice. Furthermore, compounds **6h** and **6n** demonstrated moderate PK properties (compound **6h**, F% = 37.8%, $t_{1/2}$ = 1.45 h; compound **6n**, F% = 16.8%, $t_{1/2}$ = 3.64 h).

1. Introduction

Inhibition of dipeptidyl peptidase IV (DPP IV, DPP4, also known as CD26) has become an attractive and potential therapeutic strategy for treatment of type 2 diabetes mellitus (T2DM) [1]. DPP4 is a multifunctional type II transmembrane serine protease glycoprotein [2] which is responsible for the rapid inactivation of glucagon-like peptide-1 (GLP-1). GLP-1 is an important incretin which regulates blood glucose levels [3], and is released from L-cells and stimulates insulin biosynthesis and secretion [4], inhibits glucose release [5], and delays gastric emptying resulting in reduced appetite [6]. The bioactivity of GLP-1 influences multiple aspects of glucose homeostasis and is important in the normalization of blood glucose levels in diabetic patients. To date, the therapeutic rationale for DPP4 inhibition has focused on prolonging the half-life of GLP-1 bioactivity [1].

Recently, several DPP4 inhibitors [7–14] including **1** (Sitagliptin, MK-0431) [15], **2** (Vildagliptin, LAF237) [16], **3** (Saxagliptin, BMS-477118) [17], **4** (Alogliptin, SYR-322) [18], and **5** (Linagliptin, BI1356) [19] (Fig. 1) have been approved for T2DM treatment. These agents have been demonstrated to be sensitive to lower glucose and HbA_{1c} levels and have successfully improved glucose tolerance in T2DM patients [20]. However, the issue of poor selectivity was still to focus according to peptidomimetic inhibitors [21–25]. Compared to peptidomimetic inhibitors, non-peptidomimetic inhibitors showed good selectivity [26–29]. In our group, a novel series of 1-(γ-1,2,3-triazol substituted prolyl)-(*S*)-3,3-difluoropyrrolidines derivatives [30] had been reported, which showed good selectivity. However, 1-(γ-1,2,3-triazol substituted prolyl)-(*S*)-3,3-difluoropyrrolidines derivatives showed moderate DPP4 inhibitory activities (micro-molar levels). Aim to discovery of compounds with excellent DPP4 inhibitory activities and selectivity, we designed and synthesized hetero-

Abbreviation: DPP4, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; T2DM, type 2 diabetes mellitus; SARs, structure–activity relationships; OGTTs, oral glucose tolerance tests; PK, pharmacokinetics; AUC, area under curve.

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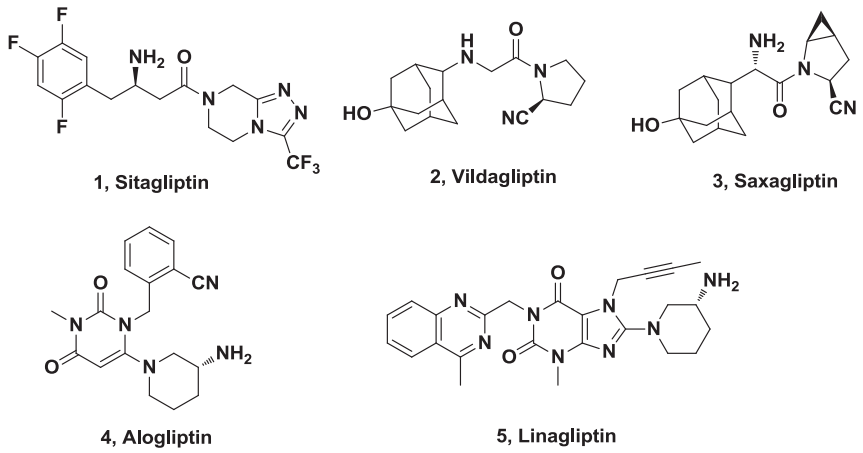


Fig. 1. Representative DPP4 inhibitors.

aromatic moieties substituted α-amino pyrrole-2-carbonitrile derivatives as a novel, potent, and selective DPP4 inhibitors for the treatment of T2DM.

2. Results and discussion

2.1. Chemistry

2.1.1. Design of compounds

Investigation of the SARs of the pyrrole-2-carbonitrile inhibitors previously reported [31], which showed that the nitrile group effectively formed a covalent adduct with the catalytic Ser630 residue and bind to DPP4 in the S1 pocket. Therefore, the pyrrole-2-carbonitrile moiety was retained in our design (Fig. 2). Furthermore, the P2 region has been mainly substituted with aromatic moieties [9,32,33] in previous studies. Aliphatic moieties with research involving hetero-aromatic moiety substituted compounds were limited [7,10,16,17,34,35]. Therefore, a novel series of compounds including penta- and hexa-hetero-aromatic systems (**6a**–**e**) was designed. Furthermore, introducing the fluorine atom in Denagliptin [32] demonstrated that the inhibitory activity was improvement compared to pyrrole-2-carbonitrile derivatives. The reason could be the fluorine atom interacted with certain amino acid residues *via* hydrogen bond. Accordingly, a series of novel hetero-aromatic moiety substituted α-amino 4-fluoropyrrole-2-carbonitrile derivatives (**6f**–**n**) was designed.

2.1.2. Synthesis of target compounds

Compounds **6a**–**e** were synthesized according to Scheme 1. The important intermediate compound **11** was obtained from commercially available compound **7** *via* protection, amidation, dehydration, and deprotection, followed by coupling reactions with *N*-Boc-α-amino acids (**12a**–**e**, commercially available) to yield the compounds **13a**–**e**. Deprotection of **13a**–**e** with trifluoroacetic acid (TFA) produced the target compounds **6a**–**e**.

The synthetic route of compounds **6f**–**n** was shown in Scheme 2. Commercially available compound **14** was protected and then fluorinated with diethylaminosulfur trifluoride (DAST) to generate compound **16**. Compound **16** produced compound **20** [36] *via* demethylation, amidation, dehydration, and deprotection, followed by coupling reactions with *N*-Boc-α-amino acids (**12f**–**n**) to yield the compounds **21f**–**n**. Deprotection of **21f**–**n** with TFA produced the target compounds **6f**–**n**.

2.2. Biological evaluation

2.2.1. In vitro enzyme inhibition studies

All the synthesized compounds (**6a**–**n**) were evaluated *in vitro* for their capacity to inhibit DPP4 (Table 1). Due to the diversity of serine proteases, the inhibitory activities of the other members of the serine protease family (DPP8 and DPP9) were also evaluated. Because inhibition of DPP8/9 was associated with toxicity in animal studies [31], selectivity against DPP8/9 was particularly important.

The SARs of synthesized pyrrole-2-carbonitrile derivatives were discussed as follows. Compound **6a**, which was furan-3-yl substituted, showed moderate inhibitory activity against DPP4 (IC_{50} = 113.6 μM). In order to improve DPP4 inhibitory activities, the furan group was replaced with thiophene, thiazole, and pyridine group. Compounds **6b** (IC_{50} = 0.17 μM) and **6c** (IC_{50} = 0.03 μM) which were thiophen-3-yl and benzo[*b*]thiophen-3-yl substituted demonstrated 668.2-fold and 3786.7-fold improvement in DPP4 inhibitory activities, respectively. While thiazol-4-yl and pyridin-2-yl group substituted compounds **6d** (IC_{50} = 0.33 μM) and **6e** (IC_{50} = 0.52 μM) showed 344.2-fold and 218.5-fold improvement in inhibitory activities. The discrepancy in inhibitory activities with compounds could be enhancement the liposolubility of compounds **6b** and **6c** when the oxygen atom was replaced with a sulfur atom. However, the pyridin-2-yl group substituted compound **6e** exhibited decreased inhibitory activity when compared to compound **6b**, by 3.1-fold. This could be the difference of the electronegativity of two compounds.

Investigation of the inhibitory activities and selectivity of the pyrrolidine-2-carbonitrile derivatives revealed that compound **6c** showed good DPP4 inhibition, however, the selectivity of compound **6c** was lower (SR: DPP8/DPP4 = 327.6; DPP9/DPP4 = 425.9) than **Sitagliptin** (SR: DPP8/DPP4 = 1680.0; DPP9/DPP4 = 5455.0).

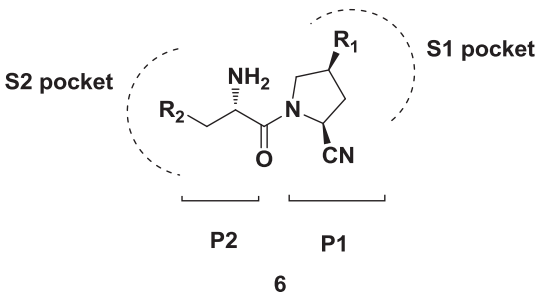
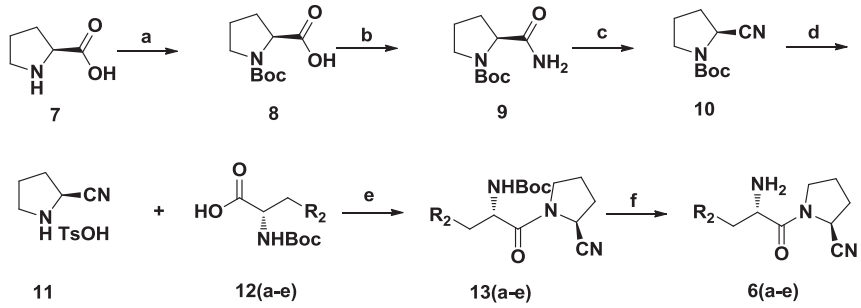


Fig. 2. Design of novel α-amino pyrrole-2-carbonitrile derivatives.



Scheme 1. Reagents and conditions: (a) $(Boc)_2O$, $NaHCO_3$, dioxane, 24 h; (b) $(Boc)_2O$, NH_4HCO_3, pyridine, dioxane, 6 h; (c) cyanuric chiloride, DMF, 1 h; (d) TsOH, CH_3CN, rt, 24 h; (e) HATU, DIPEA, DMF, 5 h; (f) CH_2Cl_2, TFA, 0 °C to rt, 1 h.

Therefore, to enhance the DPP4 inhibitory activities and selectivity, the fluorine atom was introduced to 4-position of the pyrrole-2-carbonitrile moiety, and the 4-fluoropyrrole-2-carbonitrile derivatives were synthesized.

From the SARs of 4-fluoropyrrole-2-carbonitrile derivatives, the inhibitory activities and selectivity were relatively improved. Compared to the pyrrole-2-carbonitrile compound **6c** (IC_{50} = 0.03 μM), compound **6f** (IC_{50} = 0.005 μM, SR: DPP8/DPP4 = 280.0; DPP9/DPP4 = 520.0) showed 6.0-fold improvement in DPP4 inhibitory activity. However, introduction of fluorine at the 4-position of pyrrole-2-carbonitrile moiety of compound **6c** resulted in a 1.7-fold decrease in inhibitory activity (compound **6g**, IC_{50} = 0.05 μM). To our pleasure, compound **6h** (IC_{50} = 0.004 μM, SR: DPP8/DPP4 = 450.0; DPP9/DPP4 = 375.0), which was thiazole-4-yl substituted, showed excellent DPP4 inhibitory activity and more prominent selectivity than compound **6f**. The DPP4 inhibitory activity of compound **6h** was 82.5-fold higher than that of compound **6d** (IC_{50} = 0.33 μM). This suggested that the fluorine atom was an important influence on improving inhibitory activities and selectivity. Considering compound **6h** was apt to metabolize, some groups including of methyl, aryl, and thiophen-2-yl substituted at the 2-position of the thiazole were synthesized and evaluated. However, these compounds **6i**–**m** decreased DPP4 inhibitory activities by 10.0- to 97.5-fold when compared to compound **6h**. Replacement of thiophene moiety with 6-bromobenzo[*d*][1,3]dioxol-5-yl yielded compound **6n** (IC_{50} = 0.01 μM, SR: DPP8/DPP4 = 470.0; DPP9/DPP4 = 750.0), which also possessed excellent DPP4 inhibitory activity and selectivity against related peptides.

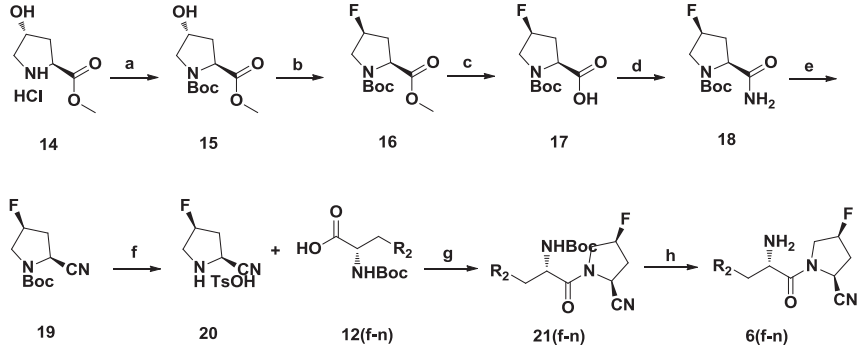
2.2.2. Inhibition mode and binding mode of compounds *6d*, *6h*, and *6n*

From the SARs of pyrrolidine-2-carbonitrile derivatives and 4-fluoropyrrolidine-2-carbonitrile derivatives, compounds **6h** and **6n** were found to be potent and selective DPP4 inhibitors. The inhibition mode of compounds **6h** and **6n** with DPP4 were rapid binding, evidenced by the stable inhibition rates from the first second incubation by 0.004 μM of compound **6h** and 0.01 μM of compound **6n** (Fig. 3A). The reversible dissociation was shown by recovery activity of DPP4 by the dialysis process (Fig. 3B). The further kinetic characterization of compounds **6h** and **6n** were determined, and shown as the competitive inhibitory mode by the same *Y*-intercept but with different slopes and X-intercepts between different concentration of inhibitor from the double-reciprocal plotting ($1/v$ vs $1/[S]$) (Fig. 3C–D), the K_i values of **6h** and **6n** were 0.0035 μM and 0.009 μM respectively, calculated by using the Michaelis–Menten equation of $1/v = (K_m/[V_{max}[S]])(1 + [I]/K_i) + 1/V_{max}$.

To gain structural information for further optimization, the 3D binding modes of compounds **6d**, **6h**, and **6n** to DPP4 (from 2AJL) were generated based on docking simulations (Fig. 4). The binding modes indicated that the pyrrole-2-carbonitrile moiety of compounds **6d**, **6h**, and **6n** occupied the S1 hydrophobic pocket, and the nitrile group interacted with the side chains of Ser630 and His740. The carbonyl group formed a hydrogen bond with residue Arg125, while the α-amino group formed two hydrogen bonds with two glutamate residues (Glu205 and Glu206). The simulations further indicated that the 6-bromobenzo[*d*][1,3]dioxole moiety of compound **6n** could stack against the side chain of Phe357. Introduction of the fluorine atom at the 4-position of pyrrole-2-carbonitrile, resulted in 82.5-fold and 33.0-fold improvement in inhibitory activity from compounds **6h** and **6n**, respectively in comparison of compound **6d**. The binding mode indicated that the fluorine atom may form a hydrogen bond with residue Tyr547, which could be the main reason for the inhibitory activity enhancement.

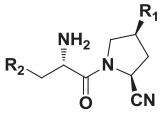
2.2.3. In vivo studies

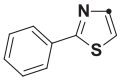
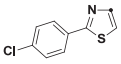
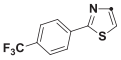
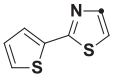
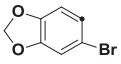
Based on *in vitro* potency and selectivity analysis, compounds **6h** and **6n** were selected for acute efficacy evaluation by the oral



Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, $NaHCO_3$, dioxane, 24 h; (b) DAST, CH_2Cl_2, −78 °C to rt, 24 h; (c) LiOH, dioxane, H_2O, overnight; (d) $(Boc)_2O$, NH_4HCO_3, pyridine, dioxane, 6 h; (e) cyanuric chiloride, DMF, 1 h; (f) TsOH, CH_3CN, rt, 24 h; (g) EDCI, HOBt, TEA, DMF, 20 h; (h) CH_2Cl_2, TFA, 0 °C to rt, 1 h.

Table 1
Potency and selectivity of pyrrolidine-2-carbonitrile derivatives.



Compd.	R_1	R_2	IC_{50} (μM)[a]			SR[b]	
			DPP4	DPP8	DPP9	DPP8/DPP4	DPP9/DPP4
6a	H		113.6 ± 17.6	>288.2	>288.2	>2.5	>2.5
6b	H		0.17 ± 0.02	55.7 ± 5.7	72.4 ± 11.8	327.6	425.9
6c	H		0.03 ± 0.02	12.3 ± 1.3	10.6 ± 1.1	410.0	353.3
6d	H		0.33 ± 0.02	11.5 ± 1.7	13.9 ± 1.2	34.8	42.1
6e	H		0.52 ± 0.02	125.1 ± 4.1	143.6 ± 15.1	240.6	276.1
6f	F		0.005 ± 0.001	1.4 ± 0.4	2.6 ± 0.4	280.0	520.0
6g	F		0.05 ± 0.00	3.4 ± 0.7	3.2 ± 0.5	68.0	64.0
6h	F		0.004 ± 0.004	1.8 ± 0.3	1.5 ± 0.3	450.0	375.0
6i	F		0.04 ± 0.00	3.3 ± 0.6	6.8 ± 0.9	82.5	170.0
6j	F		0.39 ± 0.03	0.9 ± 0.2	5.4 ± 0.7	2.3	13.8
6k	F		0.05 ± 0.01	1.0 ± 0.3	2.0 ± 0.2	20.0	40.0
6l	F		0.05 ± 0.00	1.3 ± 0.2	1.3 ± 0.1	26.0	26.0
6m	F		0.12 ± 0.02	0.7 ± 0.2	2.7 ± 0.4	5.8	22.5
6n	F		0.01 ± 0.00	4.7 ± 0.6	7.5 ± 0.3	470.0	750.0
Sitagliptin	/	/	0.02 ± 0.06	33.6 ± 5.1	109.1 ± 12.7	1680.0	5455.0

[a] Data represent the mean value ± SD with quadruplicate assay.
[b] Selectivity ratio.

glucose tolerance test (OGTT) in ICR mice. A single dose of either compound **6h** or **6n** (15 and 50 mg/kg) was administered to ICR mice. The OGTT produced a significant decrease in glucose level after 4 h compared with the vehicle group. **Vildagliptin** (**LAF237**), which was used as a positive control, reduced the area under curve from 0 to 120 min $(AUC)_{0-120\ min}$ to 19.9% (**LAF237**, 2516.4 ± 145.0; vehicle control, 3140.8 ± 104.5) at a dose of 30 mg/kg. Compound **6h** reduced the value to 10.7% (2804.1 ± 119.2) and 12.8% (2739.8 ± 211.3), respectively. Compound **6n** reduced the value to 12.2% (2756.3 ± 149.9) and 23.6% (2398.7 ± 284.6) at dose of 15 mg/kg and 50 mg/kg in a dose-dependent manner, respectively (see Fig. 5).

Based on the DPP4 inhibitory activities (compound **6h**, IC_{50} = 0.004 μM; compound **6n**, IC_{50} = 0.01 μM) and selectivity

(compound **6h**, selective ratio: DPP8/DPP4 = 450.0; DPP9/DPP4 = 375.0; compound **6n**, selective ratio: DPP8/DPP4 = 470.0; DPP9/DPP4 = 750.0), compound **6h** was selected for the chronic effects experiment.

The chronic effects of compound **6h** were investigated in diabetic BKS *db*/*db* mice with multiple doses (5 mg/kg/day to 50 mg/kg/day) for 5 weeks. **LAF237** (15 mg/kg/day) was included as a positive control. Table 2 showed that compound **6h** significantly decreased the fasting blood glucose level compared to vehicle control group (18.1 ± 1.9 of 50 mg/kg/day v.s. 25.0 ± 1.2, respectively). The OGTT revealed that the glucose tolerance capacity during 60–120 min was significant improved by treatment with compound **6h** in a dose-dependent manner, as demonstrated by the reduction of the AUC_{0-120} (3094.8 ± 261.7 of 15 mg/kg/day, 2958.5 ± 321.9 of 50 mg/kg/day group v.s. 3915.4 ± 166.9 of vehicle control, see Table 2).

2.3. Pharmacokinetic evaluation of compounds **6h** and **6n**

The pharmacokinetic (PK) profiles of the selected compounds **6h** and **6n** were assessed in Sprague–Dawley (SD) rats (Table 3). The C_{max} of compound **6h** at 0.3 h was 2711 ng/mL with an $AUC_{0-\infty}$ 2731 ng/mL*h at a dose of 50 mg/kg. Moreover, compound **6h** demonstrated high clearance in rats. The absolute oral bioavailability for compound **6h** was moderate (37.8%) and low for compound **6n** (16.8%), while compound **6h** had a lower half-life than compound **6n** (1.45 h v.s. 3.64 h, respectively).

2.4. hERG testing of compounds **6h** and **6n**

Blockade of the hERG channel is a significant hurdle encountered in drug discovery [37]. Based on *in vivo* results; compounds **6h** and **6n** were chosen for hERG testing (Table 4). The IC_{50} values of compounds **6h** and **6n** on hERG were 176.6 μM and 48.0 μM using FluxOR™ thallium assay, respectively.

2.5. Liver metabolic enzymes P450 testing of compounds **6h** and **6n**

Compound **6h** showed no inhibition of liver metabolic enzymes such as CYP3A4 and CYP2C9 (percentage inhibition, 7.4% and 11.2%, respectively, in 100 μM). On the other hand, compound **6n** demonstrated no inhibition of CYP2C9 (percentage inhibition, −20.3% in 100 μM) while the inhibitory activity of CYP3A4 was 2.2 μM (Table 4).

3. Conclusion

On the basis of SARs investigations of pyrrole-2-carbonitrile inhibitors, we have designed, synthesized, and evaluated a series of novel hetero-aromatic moieties substituted α-amino pyrrolidine-2-carbonitrile derivatives as potent and selective DPP4 inhibitors. Compounds **6h** and **6n** possessed an excellent DPP4 inhibitory activities, high selectivity, good pharmacokinetic profiles, and good *in vivo* efficacy in an OGTT in ICR mice. Moreover, compound **6h** showed no hERG binding and no inhibition to liver metabolic enzymes. Further investigation of hetero-aromatic moieties substituted α-amino 4-fluoropyrrolidine-2-carbonitrile derivatives is in progress.

4. Experimental section

4.1. Chemistry

The reagents (chemicals) were purchased and used without further purification. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 and AMX-300 NMR (IS as TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray, and matrix-assisted laser desorption ionization (EI, and ESI) produced by a Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec 4.7 T. Optical rotations were reported as follows: $[\alpha]_D$ [22] (c = g/100 mL, in solvent).

4.1.1. (2S)-1-(tert-Butoxycarbonyl)pyrrolidine-2-carboxylic acid (**8**)

A solution of (2*S*)-pyrrolidine-2-carboxylic acid (**7**) (0.5 g, 4.34 mmol) in dioxane (15 mL) was added $(Boc)_2O$ (1.14 g, 5.21 mmol) and saturated $NaHCO_3$ (10.5 mL). The reaction was stirred at room temperature for overnight. The solvent was removed *in vacuo* and CH_2Cl_2 was added. The organics were washed with H_2O and saturated NaCl, dried, filtered and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EA/PE (1:5, v/v) to afford **8** (0.8 g, 86%) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.37–4.24 (m, 1H), 3.58–3.32 (m, 2H), 2.41–1.85 (m, 4H), 1.46 (s, 9H). MS (ESI) m/z 216 $[M + H]^+$.

4.1.2. tert-Butyl (2S)-2-carbamoylpyrrolidine-1-carboxylate (**9**)

A mixture of compound **8** (1.00 g, 4.65 mmol), $(Boc)_2O$ (1.52 g, 6.97 mmol), NH_4HCO_3 (0.55 g, 6.97 mmol) and pyridine (1.0 mL) in dioxane (20 mL) was stirred at room temperature for 6 h the product was extracted with CH_2Cl_2, washed with 1 M HCl and saturated NaCl, dried, filtrated, and concentrated. *n*-hexane (100 mL) was added and the product **9** (0.85 g, 85%) began to precipitate using the ultrasound as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.37–4.34 (m, 1H), 3.47–3.36 (m, 2H), 2.07–1.85 (m, 4H), 1.49 (s, 9H). MS (ESI) m/z 215 $[M + H]^+$.

4.1.3. tert-Butyl (2S)-2-cyanopyrrolidine-1-carboxylate (**10**)

A mixture of compound **9** (5 g, 23.3 mmol) and cyanuric chloride (2.58 g, 14.0 mmol) in DMF (10 mL) was stirred at room temperature for 1 h (monitored by TLC). After the reaction completed, the solution was extracted with EtOAc, washed, dried, concentrated, and purified by flash chromatography on silica gel, eluted with a mixture of PE/EA (1/1, *v*/v) to afford **10** (3.48 g, 76%) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.76 (s, 1H), 3.51 (s, 2H), 2.34–2.31 (m, 1H), 2.17–2.10 (m, 2H), 1.90–1.87 (m, 1H), 1.49 (s, 9H). MS (ESI) m/z 197 $[M + H]^+$.

4.1.4. (2S)-Pyrrolidine-2-carbonitrile 4-methylbenzene-1-sulfonic acid (**11**)

A solution of compound **10** (10.0 g, 50.96 mmol) in CH_3CN (50 mL) was added 4-methylbenzenesulfonic acid hydrate (14.54 g, 76.43 mmol) and stirred at room temperature for 24 h. After the reaction completed, the solution was removed *in vacuo*. The residual white solid was dissolved in EtOAc (100 mL) and put into fridge overnight, the product **11** (10.3 g, 75%) was precipitated as a white needle crystal. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.77 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 8.1 Hz, 2H), 4.76–4.75 (m, 1H), 3.51–3.50 (m, 2H), 2.38 (s, 3H), 2.34–2.31 (m, 1H), 2.17–2.09 (m, 2H), 1.90–1.87 (m, 1H). MS (ESI) m/z 97 $[M + H]^+$.

4.1.5. tert-Butyl N-[(2S)-1-[(2S)-2-cyanopyrrolidin-1-yl]-3-(furan-3-yl)-1-oxopropan-2-yl]carbamate (**13a**)

A solution of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(furan-3-yl)propanoic acid (compound **12a**, 105 mg, 0.410 mmol) in DMF (5 mL) was added HATU (283 mg, 0.745 mmol) and DIPEA

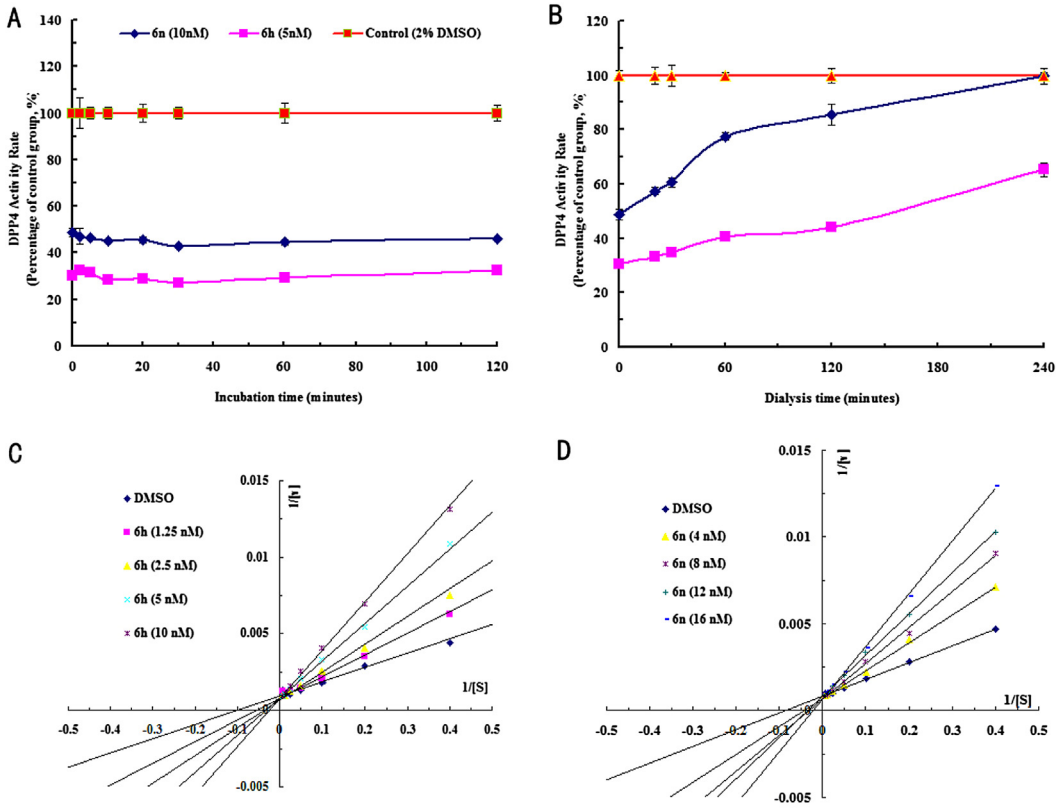


Fig. 3. Characterization of compounds **6h** and **6n** inhibitory effect on DPP4. (A) Time-independent inhibition of DPP4 by compounds **6n** and **6h**. (B) Reversibility of DPP4 inhibition by compounds **6n** and **6h**. (C) Kinetics of DPP4 inhibition by compound **6h**. (D) Kinetics of DPP4 inhibition by compound **6n**.

(96 mg, 0.745 mmol). After stirring for 30 min compound **11** (100 mg, 0.373 mmol) and additional DIPEA were added. This solution was allowed to stir at room temperature for 20 h and then the saturated $NaHCO_3$ was added. The mixture was extracted with EtOAc and washed with saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was purified with flash chromatography on silica gel, eluted with a mixture of PE/EA (4/1, *v*/v) to afford **13a** (105 mg, 85%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.33–7.32 (m, 1H), 6.28–6.27 (m, 1H), 6.14–6.13 (m, 1H), 5.46 (d, J = 8.0 Hz, 1H), 4.70–4.68 (m, 1H), 4.62–4.56 (m, 1H), 3.55–3.49 (m, 1H), 3.05–3.00 (m, 3H), 2.04–1.99 (m, 3H), 1.37 (s, 9H). MS (ESI) *m*/*z* 334 $[M + H]^+$.

*4.1.6. tert-Butyl N-[(2S)-1-[(2S)-2-cyanopyrrolidin-1-yl]-1-oxo-3-(thiophen-3-yl)propan-2-yl]carbamate (**13b**)*

In the same manner as described for **13a**, **13b** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(thiophen-3-yl)propanoic acid (**12b**). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.27–7.25 (m, 1H), 7.09 (s, 1H), 6.98–6.97 (m, 1H), 5.41 (d, J = 8.8 Hz, 1H), 4.68–4.65 (m, 1H), 4.52–4.46 (m, 1H), 3.44–3.38 (m, 1H), 3.04–2.99 (m, 4H), 2.80–2.75 (m, 2H), 1.39 (s, 9H). MS (ESI) *m*/*z* 350 $[M + H]^+$.

*4.1.7. tert-Butyl N-[(2S)-1-[(2S)-2-cyanopyrrolidin-1-yl]-1-oxo-3-(benzo[b]thiophen-3-yl)propan-2-yl]carbamate (**13c**)*

In the same manner as described for **13a**, **13c** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(benzo[*b*]thiophen-3-yl)propanoic acid (**12c**). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.93–7.82 (m, 2H), 7.44–7.32 (m, 2H), 7.28–7.26 (m, 1H), 5.54 (d, J = 8.0 Hz, 1H), 4.71–4.62 (m, 2H), 3.36–3.32 (m, 1H), 3.25–3.19 (m, 2H), 2.41–2.35 (m, 1H), 2.06–1.98 (m, 3H), 1.42 (s, 9H). MS (ESI) *m*/*z* 400 $[M + H]^+$.

*4.1.8. tert-Butyl N-[(2S)-1-[(2S)-2-cyanopyrrolidin-1-yl]-1-oxo-3-(thiazol-4-yl)propan-2-yl]carbamate (**13d**)*

In the same manner as described for **13a**, **13d** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(thiazol-4-yl)propanoic acid (**12d**). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.75–8.74 (m, 1H), 7.12 (s, 1H), 7.28–7.26 (m, 1H), 5.59 (d, J = 8.8 Hz, 1H), 4.77–4.66 (m, 2H), 3.62–3.56 (m, 1H), 3.32–3.27 (m, 1H), 3.19–3.17 (m, 2H), 2.92–2.82 (m, 1H), 2.15–2.07 (m, 2H), 1.34 (s, 9H). MS (ESI) *m*/*z* 351 $[M + Na]^+$.

*4.1.9. tert-Butyl N-[(2S)-1-[(2S)-2-cyanopyrrolidin-1-yl]-1-oxo-3-(pyridin-2-yl)propan-2-yl]carbamate (**13e**)*

In the same manner as described for **13a**, **13e** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(pyridin-2-yl)propanoic acid (**12e**). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.55–8.53 (m, 2H), 7.59–7.56 (m, 1H), 7.31–7.26 (m, 1H), 5.29 (d, J = 8.8 Hz, 1H), 4.74–4.71 (m, 1H), 4.61–4.55 (m, 1H), 3.57–3.50 (m, 1H), 3.05–2.94 (m, 3H), 2.19–2.11 (m, 3H), 1.40 (s, 9H). MS (ESI) *m*/*z* 345 $[M + H]^+$.

*4.1.10. 1-tert-Butyl 2-methyl (2S,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate (**15**)*

In the same manner as described for **8**, **15** was prepared from (2*S*,4*R*)-methyl 4-hydroxypyrrolidine-2-carboxylate hydrochloride (**14**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.35–4.48 (m, 2H), 3.72 (s, 3H), 3.46–3.65 (m, 2H), 2.03–2.29 (m, 2H), 1.39 (s, 9H). MS (ESI) *m*/*z* 246 $[M + H]^+$.

*4.1.11. 1-tert-Butyl 2-methyl (2S,4S)-4-fluoropyrrolidine-1,2-dicarboxylate (**16**)*

Under the Nitrogen protected, a solution of compound **15** (2 g, 8.2 mmol) in dry CH_2Cl_2 cooled to −78 °C, was added DAST (1.97 g,

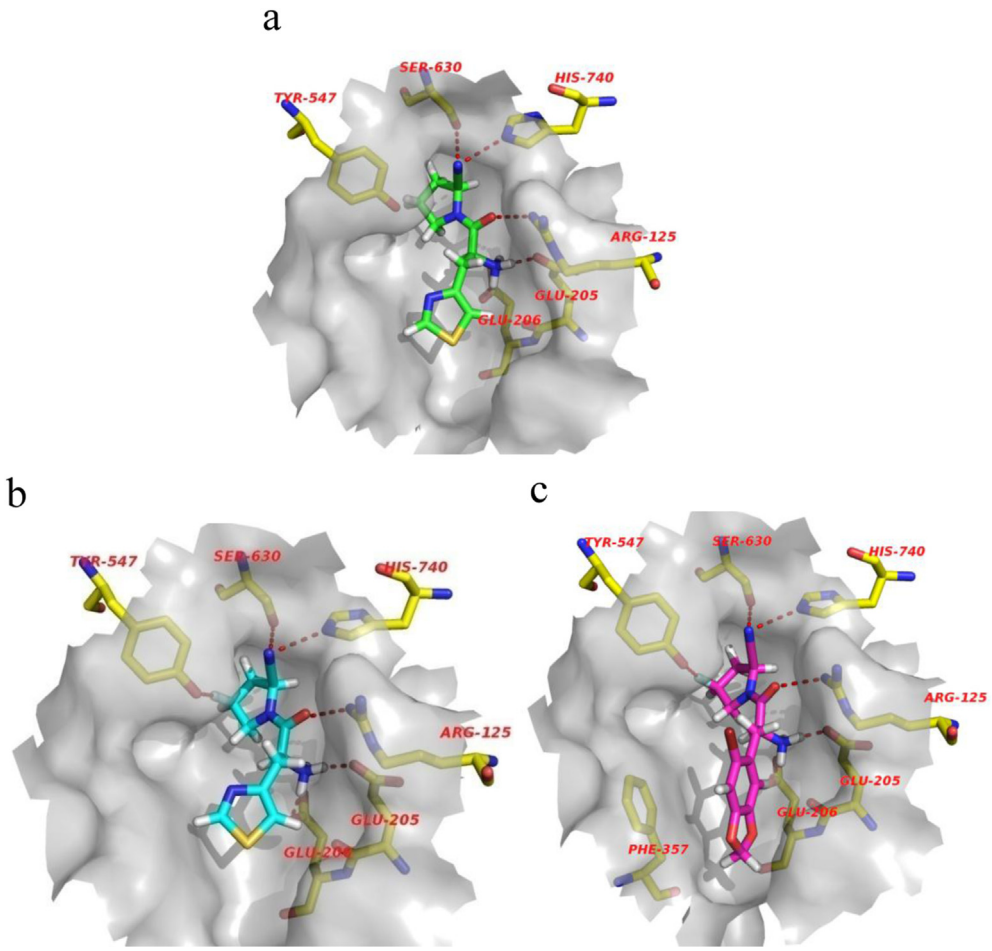


Fig. 4. Three-dimensional structural modes of inhibitors **6d** (a), **6h** (b), and **6n** (c) to DPP4 (PDB ID: 2AJL) derived from the docking simulations. These three images were generated using the Pymol program.

12.2 mmol). After stirring 3 h, the reaction slowly warmed to room temperature overnight. Then the reaction solution was poured into 200 mL ice and $NaHCO_3$ mixture solution and stirred acutely until no CO_2 evolution. The organic layer was separated and the aqueous layer extracted with CH_2Cl_2, dried, filtered and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EA/PE (1:4, *v*/v), to afford **16** (1.6 g, 78%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.18–5.34 (m, 2H), 3.72 (s,

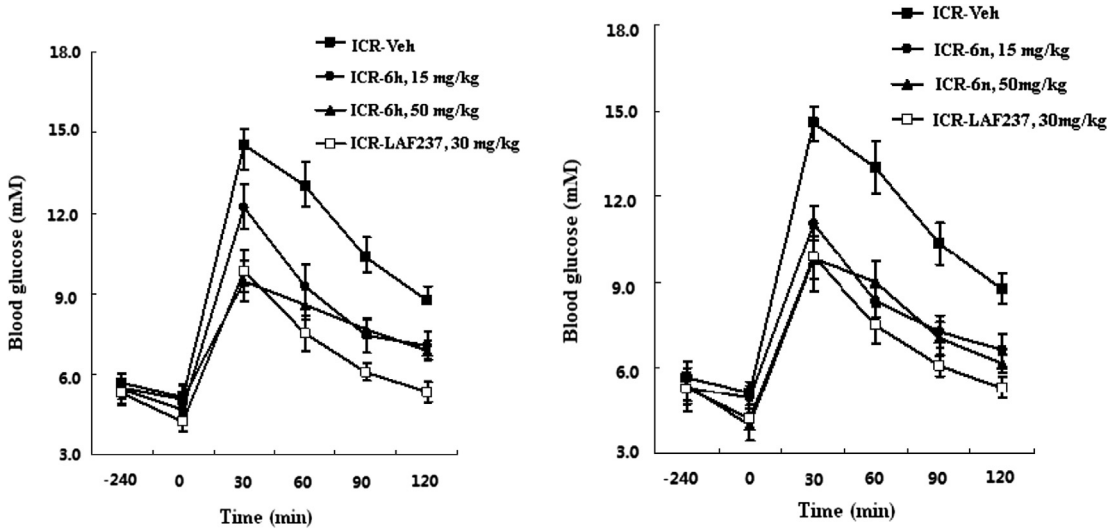


Fig. 5. Single dose of compounds **6h** and **6n** on OGTT in ICR mice. Compounds **6h** and **6n** were administered to ICR mice after 16-h starvation, then the oral glucose tolerance test (2.5 g/kg) was conducted after 4 h of the single dose, the blood glucose level at 0, 30, 60, 90 and 120 min were recorded for area under curve calculation. The results are presented as the mean ± SE.*, $p < 0.05$ compared to vehicle group; **, $p < 0.01$ compared to vehicle group ($n = 10$).

Table 2
Chronic effects of compound **6h** on OGTT of diabetic BKS *db/db* mice.

	Blood glucose (mmol/L) after orally glucose challenge					AUC 0–120 min
	0	30	60	90	120	
Veh	25.0 ± 1.2	46.6 ± 2.2	30.2 ± 1.5	27.2 ± 1.3	28.1 ± 1.3	3915.4 ± 166.9
6h-5	21.5 ± 1.7*	45.1 ± 2.4	27.5 ± 1.4	24.1 ± 2.6	24.5 ± 2.4*	3589.4 ± 218.1
6h-15	18.0 ± 1.7**	41.3 ± 2.2	21.8 ± 2.4**	20.8 ± 2.6*	20.6 ± 2.4**	3094.8 ± 261.7**
6h-50	18.1 ± 1.9**	38.0 ± 4.0*	19.9 ± 2.0**	20.5 ± 2.2**	20.4 ± 2.3**	2958.5 ± 321.9**
LAF237	17.9 ± 1.6**	31.3 ± 1.2**	21.0 ± 1.9**	20.7 ± 1.6**	21.4 ± 2.2**	2778.6 ± 183.2**

C57BKS *db*/*db* mice with **6h** (5 mg/kg/day to 50 mg/kg/day) treatment, oral glucose tolerance test (1.5 g/kg) was carried out after 6-hrs starvation of 5th-week treatment, the blood glucose level was recorded for the glucose tolerance capacity evaluation. The results are presented as the mean ± SE. *, $p < 0.05$ compared to vehicle group; **, $p < 0.01$ compared to vehicle group ($n = 8–11$).

3H), 3.46–3.65 (m, 2H), 2.03–2.29 (m, 2H), 1.40 (s, 9H). MS (ESI) *m/z* 248 $[M + H]^+$.

4.1.12. (2S,4S)-1-[(tert-Butoxy)carbonyl]-4-fluoropyrrolidine-2-carboxylic acid (**17**)

A solution of compound **16** (2.46 g, 9.96 mmol) in dioxane (20 mL), was added 10 mL of H_2O followed by lithium hydroxide hydrate (2.09 g, 49.8 mmol) at room temperature, the reaction was stirred for 3 h (monitored by TLC). Then the solution was filtered removing the excess lithium hydroxide, the filtrate was removed the solvent in *vacuo*, the residue was added 10 mL of H_2O and acidified with concentrated HCl to pH 3–4, the product began to precipitate, filtered and dried to afford **17** (2.2 g, 95%) as a white solid. ^{1}H NMR (CD_3OD, 300 MHz): δ 5.12–5.30 (m, 1H), 4.38–4.43 (m, 1H), 3.59–3.72 (m, 2H), 2.39–2.46 (m, 2H), 1.47 (s, 9H). MS (ESI) *m/z* 232 $[M - H]^-$.

4.1.13. tert-Butyl (2S,4S)-2-carbamoyl-4-fluoropyrrolidine-1-carboxylate (**18**)

In the same manner as described for compound **9**, compound **18** was prepared from (2*S*,4*S*)-1-[(*tert*-Butoxy)carbonyl]-4-fluoropyrrolidine-2-carboxylic acid (**17**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.59 (br, s, 1H), 5.13–5.31 (m, 1H), 4.36 (br, s, 1H), 3.52–3.81 (m, 2H), 2.31–2.78 (m, 2H), 1.48 (s, 9H). MS (ESI) *m/z* 233 $[M + H]^+$.

4.1.14. tert-Butyl (2S,4S)-2-cyano-4-fluoropyrrolidine-1-carboxylate (**19**)

In the same manner as described for compound **10**, compound **19** was prepared from *tert*-Butyl (2*S*,4*S*)-2-carbamoyl-4-fluoropyrrolidine-1-carboxylate (**18**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.21–5.41 (m, 1H), 4.62–4.76 (m, 1H), 3.49–3.93 (m, 2H), 2.63 (dd, $J_1 = 15.0$ Hz, $J_2 = 15.3$ Hz, 1H), 2.30–2.44 (m, 1H), 1.49 (s, 9H). MS (ESI) *m/z* 215 $[M + H]^+$.

4.1.15. (2S,4S)-4-Fluoropyrrolidine-2-carbonitrile: 4-methylbenzene-1-sulfonic acid (**20**)

In the same manner as described for compound **11**, compound **20** was prepared from *tert*-butyl (2*S*,4*S*)-2-cyano-4-fluoropy rrolidine-1-carboxylate (**19**). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.51 (dd, $J_1 = 12.0$ Hz, $J_2 = 6.0$ Hz, 2H), 7.11–7.16 (m, 2H), 5.45–5.62 (m, 1H), 4.99–5.04 (m, 1H), 3.42–3.67 (m, 2H), 2.30–2.70 (m, 3H), 3.17 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 143.3, 142.0, 130.0, 127.0, 116.4, 93.9, 92.5, 53.8 (d, $J = 19.0$ Hz), 46.8, 38.4 (d, $J = 17.0$ Hz), 21.4. MS (EI) *m/z* 114 $[M]^+$. HRMS (EI) *m/z* calcd $C_5H_7FN_2$ 114.0593 $[M]^+$, found 114.0668.

4.1.16. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxo-3-(thiophen-2-yl)propan-2-yl]carbamate (**21f**)

A solution of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(thiophen-2-yl)propanoic acid (compound **12f**, 104.2 mg, 0.384 mmol) in DMF (5 mL) was added HOBt (141.6 mg, 1.05 mmol) and EDCI (133.9 mg, 0.698 mmol). After stirring for 30 min compound **20** (100 mg, 0.349 mmol) and additional TEA (0.15 mL, 1.05 mmol) were added. This solution was allowed to stir at room temperature for 20 h and then the saturated $NaHCO_3$ was added. The mixture was extracted with EtOAc and washed with saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was purified with flash chromatography on silica gel, eluted with a mixture of PE/EA (4/1, *v*/v) to afford **21f** (112 mg, 86%) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.20 (dd, $J_1 = 6.0$ Hz, $J_2 = 6.0$ Hz, 1H), 6.93–6.97 (m, 1H), 6.92 (dd, $J_1 = 6.0$ Hz, $J_2 = 6.0$ Hz, 1H), 5.13–5.18 (m, 1H), 4.93 (d, $J = 9.0$ Hz, 1H), 4.45–4.52 (m, 1H), 3.74–3.92 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.16–3.35 (m, 3H), 2.56 (t, 1H, $J = 15.0$ Hz), 2.14–2.36 (m, 1H), 1.41 (s, 9H). MS (ESI) *m/z* 390 $[M + Na]^+$.

4.1.17. tert-Butyl N-[(2S)-3-(benzo[b]thiophen-3-yl)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxopropan-2-yl]carbamate (**21g**)

In the same manner as described for compound **21f**, compound **21g** was prepared from (2*S*)-3-(benzo[*b*]thiophen-3-yl)-2-[(*tert*-butoxycarbonyl)amino]propanoic acid (**12g**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.88–7.92 (m, 2H), 7.40–7.45 (m, 2H), 7.32 (s, 1H), 5.09–5.14 (m, 1H), 4.91 (d, $J = 9.0$ Hz, 1H), 4.60–4.68 (m, 1H), 3.50–3.67 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.24–3.39 (m, 2H), 3.75–3.87 (m, 1H), 2.51 (t, 1H, $J = 15.0$ Hz), 2.06–2.29 (m, 1H), 1.43 (s, 9H). MS (ESI) *m/z* 440 $[M + Na]^+$.

4.1.18. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxo-3-(thiazol-4-yl)propan-2-yl]carbamate (**21h**)

In the same manner as described for compound **21f**, compound **21h** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(thiazol-4-yl)propanoic acid (**12h**). ^{1}H NMR ($CDCl_3$, 300 MHz):

Table 3
Selected PK parameters for compounds **6h** and **6n** in SD rats.

Compd.	Admini.	Dose mg/kg	T_{max} h	C_{max} ng/mL	AUC_{0-t} ng/mL*h	$AUC_{0-\infty}$ ng/mL*h	MRT h	$t_{1/2}$ h	CLz L/h/kg	F %
6h	p.o.	50	0.3	2711	2731	2838	1.47	1.45	/	37.8
	i.v.	20	0.3	2957	2889	2896	1.48	2.27	6.96	/
6n	p.o.	20	0.3	2204	1291	1581	3.15	3.64	/	16.8
	i.v.	5	0.3	2027	1918	1918	0.55	0.63	2.61	/

p.o., oral administration; i.v., intravenous injection.

Table 4
hERG and liver metabolic enzymes P450 testing of compounds **6h** and **6n**.

Compd.	hERG[a] (IC_{50}, μM)	CYP450 (IC_{50}, μM)	
		CYP3A4	CYP2C9
6h	176.6	NI[b]	NI
6n	48.0	2.2	NI

[a] using FluxOR™ thallium assay.
[b] NI: no inhibition (100 μM).

δ 7.77 (d, J = 3.0 Hz, 1H), 7.15 (d, J = 3.0 Hz, 1H), 5.50 (d, J = 6.0 Hz, 1H), 5.19–5.24 (m, 1H), 4.88 (d, J = 6.0 Hz, 1H), 4.69–4.75 (m, 1H), 3.82–3.95 (dd, J_1 = 9.0 Hz, J_2 = 9.0 Hz, 1H), 3.63–3.72 (m, 1H), 3.22 (d, J = 6.0 Hz, 2H), 2.53 (t, 1H, J = 12.0 Hz), 2.18–2.33 (m, 1H), 1.38 (s, 9H). MS (ESI) m/z 391 $[M + Na]^+$.

*4.1.19. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2-methylthiazol-4-yl)-1-oxopropan-2-yl]carbamate (**21i**)*

In the same manner as described for compound **21f**, compound **21i** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(2-methylthiazol-4-yl)propanoic acid (**12j**). ^1H NMR ($CDCl_3$, 300 MHz): δ 6.90 (s, 1H), 5.18–5.23 (m, 1H), 4.90 (d, J = 9.0 Hz, 1H), 4.68–4.74 (m, 1H), 3.86–3.99 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.73–3.83 (m, 1H), 3.12 (d, J = 6.0 Hz, 2H), 2.68 (s, 3H), 2.55 (t, 1H, J = 12.0 Hz), 2.17–2.40 (m, 1H), 1.40 (s, 9H). MS (ESI) m/z 405 $[M + Na]^+$.

*4.1.20. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxo-3-(2-phenylthiazol-4-yl)propan-2-yl]carbamate (**21j**)*

In the same manner as described for compound **21f**, compound **21j** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(2-phenylthiazol-4-yl)propanoic acid (**12j**). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.90–7.97 (m, 2H), 7.40–7.45 (m, 3H), 7.08 (s, 1H), 5.46 (d, J = 6.0 Hz, 1H), 5.23–5.28 (m, 1H), 4.90 (d, J = 9.0 Hz, 1H), 4.75–4.83 (m, 1H), 3.80–3.98 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.68–3.80 (m, 1H), 3.21–3.26 (m, 2H), 2.52 (t, 1H, J = 15.0 Hz), 2.17–2.36 (m, 1H), 1.39 (s, 9H). MS (ESI) m/z 467 $[M + Na]^+$.

*4.1.21. tert-Butyl N-[(2S)-3-(2-(4-chlorophenyl)thiazol-4-yl)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxopropan-2-yl] carbamate (**21k**)*

In the same manner as described for compound **21f**, compound **21k** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl) amino]-3-(2-(4-chlorophenyl)thiazol-4-yl)propanoic acid (**12l**). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.88 (d, J = 3.0 Hz, 2H), 7.40 (d, J = 9.0 Hz, 2H), 7.09 (s, 1H), 5.47 (d, J = 9.0 Hz, 1H), 5.24–5.32 (m, 1H), 4.88 (d, J = 9.0 Hz, 1H), 4.75–4.90 (m, 1H), 3.80–3.97 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.69–3.80 (m, 1H), 3.18–3.24 (m, 2H), 2.52 (t, 1H, J = 15.0 Hz), 2.11–2.36 (m, 1H), 1.40 (s, 9H). MS (ESI) m/z 501 $[M + Na]^+$.

*4.1.22. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxo-3-(2-(4-(trifluoromethyl)phenyl)thiazol-4-yl)propan-2-yl]carbamate (**21l**)*

In the same manner as described for compound **21f**, compound **21l** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(2-(4-(trifluoromethyl)phenyl)thiazol-4-yl)propanoic acid (**12l**). ^1H NMR ($CDCl_3$, 300 MHz): δ 8.04 (d, J = 9.0 Hz, 2H), 7.67 (d, J = 9.0 Hz, 2H), 7.17 (s, 1H), 5.50 (d, J = 9.0 Hz, 1H), 5.21–5.26 (m, 1H), 4.84 (d, J = 9.0 Hz, 1H), 4.77–4.85 (m, 1H), 3.81–3.98 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.69–3.81 (m, 1H), 3.20–3.32 (m, 2H), 2.51 (t, 1H, J = 15.0 Hz), 2.13–2.34 (m, 1H), 1.40 (s, 9H). MS (ESI) m/z 535 $[M + Na]^+$.

*4.1.23. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxo-3-(2-(thiophen-2-yl)thiazol-4-yl)propan-2-yl]carbamate (**21m**)*

In the same manner as described for compound **21f**, compound **21m** was prepared from (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(2-(thiophen-2-yl)thiazol-4-yl)propanoic acid (**12m**). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.49 (d, J = 3.0 Hz, 1H), 7.38 (d, J = 6.0 Hz, 1H), 7.04–7.10 (m, 1H), 7.01 (s, 1H), 5.40 (d, J = 9.0 Hz, 1H), 5.20–5.28 (m, 1H), 4.88 (d, J = 9.0 Hz, 1H), 4.75–4.83 (m, 1H), 3.81–3.99 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.75–3.87 (m, 1H), 3.09–3.24 (m, 2H), 2.51 (t, 1H, J = 15.0 Hz), 2.11–2.36 (m, 1H), 1.41 (s, 9H). MS (ESI) m/z 409 $[M + Na]^+$.

*4.1.24. tert-Butyl N-[(2S)-3-(6-bromobenzo[d][1,3]dioxol-5-yl)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-1-oxopropan-2-yl] carbamate (**21n**)*

In the same manner as described for compound **21f**, compound **21n** was prepared from (2*S*)-3-(6-bromobenzo[*d*][1,3]dioxol-5-yl)-2-[(*tert*-butoxycarbonyl)amino]propanoic acid (**12n**). ^1H NMR ($CDCl_3$, 300 MHz): δ 6.96 (s, 1H), 6.69 (s, 1H), 5.93 (s, 1H), 5.87 (s, 1H), 5.30–5.36 (m, 1H), 4.87 (d, J = 9.0 Hz, 1H), 4.45–4.61 (m, 1H), 3.74–3.90 (dd, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 1H), 3.31–3.45 (m, 1H), 3.08–3.14 (m, 1H), 2.85–2.94 (m, 2H), 2.56 (t, 1H, J = 15.0 Hz), 2.21–2.35 (m, 1H), 1.35 (s, 9H). MS (ESI) m/z 507 $[M + Na]^+$.

*4.1.25. (2S)-1-[(2S)-2-amino-3-(Furan-3-yl)propanoyl]pyrrolidine-2-carbonitrile (**6a**)*

A solution of **13a** (120 mg) in dry CH_2Cl_2 (2 mL) was added CF_3COOH (1 mL) at ice-bathe and warmed to room temperature. After 1 h, the mixture was concentrated, and the residue was added 10 mL Et_2O. The white solid was precipitated, filtered and to afford **6a** as TFA salt (72 mg, 85%). HPLC: 96.12%, t_R = 1.32 min ^1H NMR (400 MHz, DMSO-d_6): δ 8.19 (br, s, 2H), 7.60 (s, 1H), 6.44–6.43 (m, 1H), 6.25 (d, J = 3.2 Hz, 1H), 4.80 (q, J = 4.0 Hz, 1H), 4.33 (t, J = 6.4 Hz, 1H), 3.53 (q, J = 9.2 Hz, 2H), 3.11–3.09 (m, 1H), 2.93–2.89 (m, 1H), 2.17–2.08 (m, 2H), 1.94–1.83 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.9, 134.8, 129.3, 127.1, 124.7, 118.9, 52.5, 51.9, 49.0, 46.7, 29.2, 24.4. MS (ESI) m/z 234 $[M + H]^+$. HRMS (ESI) calcd for $C_{11}H_{15}N_4OS$ 234.1243 $[M + H]^+$, found 234.1265.

*4.1.26. (2S)-1-[(2S)-2-Amino-3-(thiophen-3-yl)propanoyl] pyrrolidine-2-carbonitrile (**6b**)*

In the same manner as described for compound **6a**, compound **6b** was prepared from compound **13b**. HPLC: 100.00%, t_R = 1.31 min ^1H NMR (400 MHz, DMSO-d_6): δ 8.19 (br, s, 2H), 7.50–7.49 (m, 1H), 7.03–7.01 (m, 1H), 6.95 (d, J = 3.2 Hz, 1H), 4.80 (q, J = 4.8 Hz, 1H), 4.32 (t, J = 7.6 Hz, 1H), 3.52 (q, J = 7.2 Hz, 1H), 3.34–3.19 (m, 2H), 2.93–2.88 (m, 1H), 2.16–2.05 (m, 2H), 1.90–1.78 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.5, 159.4, 147.3, 145.4, 142.7, 118.9, 51.8, 46.9, 46.8, 33.3, 29.8, 25.2. MS (ESI) m/z 250 $[M + H]^+$. HRMS (ESI) calcd for $C_{11}H_{15}N_4OS$ 250.1014 $[M + H]^+$, found 250.1004.

*4.1.27. (2S)-1-[(2S)-2-Amino-3-(benzo[b]thiophen-3-yl)propanoyl] pyrrolidine-2-carbonitrile (**6c**)*

In the same manner as described for compound **6a**, compound **6c** was prepared from compound **13c**. HPLC: 96.16%, t_R = 1.36 min ^1H NMR (400 MHz, DMSO-d_6): δ 8.28 (br, s, 2H), 8.05 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 7.2 Hz, 1H), 7.50–7.41 (m, 3H), 4.80 (q, J = 4.8 Hz, 1H), 4.38 (t, J = 8.0 Hz, 1H), 3.45–3.36 (m, 2H), 2.68–2.64 (m, 1H), 2.15–2.10 (m, 2H), 2.05–2.00 (m, 1H), 1.83–1.80 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.1, 140.0, 138.4, 129.2, 126.5, 125.0, 123.6, 122.0, 118.8, 67.4, 50.8, 46.8, 40.2, 29.8, 25.7, 25.2. MS (ESI) m/z 300 $[M + H]^+$. HRMS (ESI) calcd for $C_{11}H_{15}N_4OS$ 300.1171 $[M + H]^+$, found 300.1176.

*4.1.28. (2S)-1-[(2S)-2-Amino-3-(thiazol-4-yl)propanoyl] pyrrolidine-2-carbonitrile (**6d**)*

In the same manner as described for compound **6a**, compound **6d** was prepared from compound **13d**. HPLC: 98.73%, t_R = 1.31 min ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.13 (br, s, 2H), 7.56–7.54 (m, 1H), 7.30–7.29 (m, 1H), 7.03–7.01 (m, 1H), 4.79 (q, J = 4.8 Hz, 1H), 4.29 (t, J = 8.0 Hz, 1H), 3.48 (q, J = 6.8 Hz, 1H), 3.12–3.05 (m, 2H), 2.88–2.84 (m, 1H), 2.15–2.07 (m, 2H), 1.91–1.88 (m, 1H), 1.78–1.75 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.1, 165.3, 155.0, 150.2, 118.9, 60.2, 56.5, 55.1, 28.9, 25.2, 22.3. MS (ESI) m/z 251 [M + H]$^+$. HRMS (ESI) calcd for $C_{11}H_{15}N_4OS$ 251.0967 [M + H]$^+$, found 251.0943.

*4.1.29. (2S)-1-[(2S)-2-Amino-3-(pyridin-2-yl)propanoyl] pyrrolidine-2-carbonitrile (**6e**)*

In the same manner as described for compound **6a**, compound **6e** was prepared from compound **13e**. HPLC: 95.91%, t_R = 1.30 min ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.17 (br, s, 2H), 7.16–7.14 (m, 2H), 6.89–6.87 (m, 2H), 4.81–4.78 (m, 1H), 4.26 (t, J = 8.0 Hz, 1H), 3.46–3.40 (m, 1H), 3.05–2.91 (m, 2H), 2.74–2.67 (m, 1H), 2.17–2.03 (m, 2H), 1.88–1.83 (m, 1H), 1.72–1.67 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.4, 148.5, 143.2, 118.8, 111.3, 109.3, 52.4, 50.4, 49.1, 46.7, 29.1, 24.3. MS (ESI) m/z 245 [M + H]$^+$. HRMS (ESI) calcd $C_{13}H_{17}N_4O$ 245.1402 [M + H]$^+$, found 245.1412.

*4.1.30. (2S,4S)-1-[(2S)-2-Amino-3-(thiophen-2-yl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6f**)*

In the same manner as described for compound **6a**, compound **6f** was prepared from compound **21f**. HPLC: 100.00%, t_R = 1.29 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.30–7.38 (m, 1H), 6.99–7.05 (m, 2H), 5.23–5.26 (m, 1H), 5.04 (d, J = 8.0 Hz, 1H), 4.28–4.35 (m, 1H), 3.65–3.78 (m, 1H), 3.39–3.44 (m, 2H), 3.14–3.29 (m, 1H), 2.53 (t, 1H, J = 12.0 Hz), 2.29–2.44 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 167.6, 134.2, 128.1, 127.5, 125.5, 117.0, 92.0 (d, J = 142.0 Hz), 52.7, 52.6 (d, J = 20.0 Hz), 45.1, 35.5 (d, J = 17.0 Hz), 30.8. MS (ESI) m/z 268 [M + H]$^+$. HRMS (ESI) calcd $C_{12}H_{14}FN_3OS$ 268.0920 [M + H]$^+$, found 268.0909.

*4.1.31. (2S,4S)-1-[(2S)-2-Amino-3-(benzo[b]thiophen-3-yl) propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6g**)*

In the same manner as described for compound **6a**, compound **6g** was prepared from compound **21g**. HPLC: 100.00%, t_R = 1.32 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.89–7.98 (m, 2H), 7.39–7.50 (m, 3H), 5.04–5.17 (m, 1H), 5.00 (d, J = 8.0 Hz, 1H), 4.39–4.43 (m, 1H), 3.60 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 3.42–3.54 (m, 2H), 2.67–2.75 (m, 1H), 2.45 (t, 1H, J = 12.0 Hz), 2.21–2.37 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 168.0, 140.7, 137.9, 127.4, 126.4, 124.5, 122.8, 120.7, 116.9, 91.6 (d, J = 143.0 Hz), 52.5 (d, J = 19.0 Hz), 51.0, 45.0, 35.3 (d, J = 17.0 Hz), 29.8. MS (ESI) m/z 318 [M + H]$^+$. HRMS (ESI) calcd $C_{16}H_{16}FN_3OS$ 318.1076 [M + H]$^+$, found 318.1083.

*4.1.32. (2S,4S)-1-[(2S)-2-Amino-3-(thiazol-4-yl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6h**)*

In the same manner as described for compound **6a**, compound **6h** was prepared from compound **21h**. HPLC: 100.00%, t_R = 1.29 min ^{1}H NMR (CD_3OD, 400 MHz), δ 9.31 (s, 1H), 7.65 (s, 1H), 5.31-5.45 (m, 1H), 5.03 (d, J = 8.0 Hz, 1H), 4.55 (t, 1H, J = 12.0 Hz), 3.78–3.91 (m, 1H), 3.61–3.70 (m, 1H), 3.42–3.47 (m, 2H), 2.88 (s, 1H), 2.39–2.53 (m, 3H), 2.04–2.11 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 167.0, 156.7, 146.2, 120.4, 117.2, 92.3 (d, J = 141.0 Hz), 53.1 (d, J = 19.0 Hz), 50.9, 45.1, 35.5 (d, J = 17.0 Hz), 30.2. MS (ESI) m/z 269 [M + H]$^+$. HRMS (ESI) calcd $C_{11}H_{13}FN_4OS$ 269.0872 [M + H]$^+$, found 269.0887.

*4.1.33. (2S,4S)-1-[(2S)-2-Amino-3-(2-methylthiazol-4-yl) propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6i**)*

In the same manner as described for compound **6a**, compound **6i** was prepared from compound **21i**. HPLC: 95.77%, t_R = 1.30 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.20 (s, 1H), 5.30–5.44 (m, 1H), 4.99 (d, J = 8.0 Hz, 1H), 4.43 (t, 1H, J = 6.0 Hz), 3.61–3.80 (m, 2H), 3.34 (s, 1H), 3.26–3.29 (m, 2H), 2.69 (s, 3H), 2.54 (t, 1H, J = 12.0 Hz), 2.31–2.47 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 168.1, 167.5, 147.9, 117.2, 117.0, 92.2 (d, J = 142.0 Hz), 52.7 (d, J = 19.0 Hz), 51.3, 44.9, 35.5 (d, J = 17.0 Hz), 31.9, 17.4. MS (ESI) m/z 283 [M + H]$^+$. HRMS (ESI) calcd $C_{12}H_{15}FN_4OS$ 283.1029 [M + H]$^+$, found 283.1025.

*4.1.34. (2S,4S)-1-[(2S)-2-amino-3-(2-phenylthiazol-4-yl) propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6j**)*

In the same manner as described for compound **6a**, compound **6j** was prepared from compound **21j**. HPLC: 96.34%, t_R = 1.32 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.90–7.96 (m, 2H), 7.40–7.50 (m, 3H), 7.37 (s, 1H), 5.27–5.42 (m, 1H), 4.58 (t, 1H, J = 6.0 Hz), 4.11 (q, J = 12.0 Hz, 1H), 3.87–4.01 (m, 1H), 3.61–3.72 (m, 1H), 3.34–3.49 (m, 2H), 2.66 (m, 1H), 2.12–2.29 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 169.0, 164.3, 164.1, 150.9, 133.0, 130.2, 128.9, 126.0, 117.5, 89.5 (d, J = 175.0 Hz), 56.6, 54.2, 52.1 (d, J = 15.0 Hz), 35.8 (d, J = 21.0 Hz), 33.7. MS (ESI) m/z 345 [M + H]$^+$. HRMS (ESI) calcd $C_{17}H_{17}FN_4OS$ 345.1185 [M + H]$^+$, found 345.1192.

*4.1.35. (2S,4S)-1-[(2S)-2-Amino-3-(2-(4-chlorophenyl)thiazol-4-yl) propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6k**)*

In the same manner as described for compound **6a**, compound **6k** was prepared from compound **21k**. HPLC: 100.00%, t_R = 1.35 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.98 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 12.0 Hz, 2H), 7.40 (s, 1H), 5.25–5.39 (m, 1H), 5.02 (d, J = 8.0 Hz, 1H), 4.56 (t, 1H, J = 6.0 Hz), 3.74–3.83 (m, 2H), 3.39 (d, J = 8.0 Hz, 2H), 2.53 (t, 1H, J = 12.0 Hz), 2.34–2.49 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 168.1, 167.5, 161.8, 149.7, 134.0, 131.8, 128.9, 127.5, 118.3, 117.2, 92.2 (d, J = 177.0 Hz), 52.7 (d, J = 23.0 Hz), 51.1, 45.0, 35.4 (d, J = 21.0 Hz), 32.1. MS (ESI) m/z 379 [M + H]$^+$. HRMS (ESI) calcd $C_{17}H_{16}ClFN_4OS$ 379.0796 [M + H]$^+$, found 379.0801.

*4.1.36. (2S,4S)-1-[(2S)-2-Amino-3-(2-(4-(trifluoromethyl)phenyl) thiazol-4-yl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6l**)*

In the same manner as described for compound **6a**, compound **6l** was prepared from compound **21l**. HPLC: 100.00%, t_R = 1.35 min ^{1}H NMR (CD_3OD, 400 MHz), δ 8.19 (d, J = 8.0 Hz, 2H), 7.77 (d, J = 12.0 Hz, 2H), 7.49 (s, 1H), 5.25–5.39 (m, 1H), 5.02 (d, J = 8.0 Hz, 1H), 4.58 (t, 1H, J = 6.0 Hz), 3.74–3.83 (m, 2H), 3.42 (d, J = 8.0 Hz, 2H), 2.53 (t, 1H, J = 12.0 Hz), 2.34–2.48 (m, 1H). ^{13}C NMR (CD_3OD , 100 MHz): δ 167.5, 167.4, 161.5, 150.1, 136.5, 131.4 (q, J_1 = 26.0 Hz, J_2 = 26.0 Hz), 126.8, 125.7, 119.2, 117.2, 92.2 (d, J = 22.0 Hz), 52.7 (d, J = 19.0 Hz), 51.1, 45.0, 35.4 (d, J = 17.0 Hz), 32.1. MS (ESI) m/z 413 [M + H]$^+$. HRMS (ESI) calcd $C_{18}H_{16}F_4N_4OS$ 413.1059 [M + H]$^+$, found 413.1047.

*4.1.37. (2S,4S)-1-[(2S)-2-Amino-3-(2-(thiophen-2-yl)thiazol-4-yl) propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6m**)*

In the same manner as described for compound **6a**, compound **6m** was prepared from compound **21m**. HPLC: 100.00%, t_R = 1.30 min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.57–7.63 (m, 2H), 7.29 (s, 1H), 7.09–7.15 (m, 1H), 5.27–5.39 (m, 1H), 5.02 (d, J = 12.0 Hz, 1H), 4.55 (t, 1H, J = 6.0 Hz), 3.75–3.82 (m, 2H), 3.33 (d, J = 8.0 Hz, 2H), 2.57 (t, 1H, J = 12.0 Hz), 2.31–2.49 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 167.6, 163.2, 148.9, 136.4, 128.1, 127.8, 127.1, 117.3, 117.2, 92.2 (d, J = 178.0 Hz), 52.7 (d, J = 24.0 Hz), 51.0, 44.9, 35.4 (d, J = 21.0 Hz), 32.1. MS (ESI) m/z 351 [M + H]$^+$. HRMS (ESI) calcd $C_{15}H_{15}FN_4OS_2$ 351.0750 [M + H]$^+$, found 351.0756.

*4.1.38. (2S,4S)-1-[(2S)-2-Amino-3-(6-bromobenzo[d][1,3]dioxol-5-yl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**6n**)*

In the same manner as described for compound **6a**, compound **6n** was prepared from compound **21n**. HPLC: 100.00%, $t_R = 1.30$ min ^{1}H NMR (CD_3OD, 400 MHz), δ 7.10 (s, 1H), 6.74 (s, 1H), 5.98–6.05 (m, 2H), 5.89 (s, 1H), 5.17–5.31 (m, 1H), 4.99 (d, $J = 8.0$ Hz, 1H), 4.28–4.32 (m, 1H), 3.61–3.74 (m, 1H), 2.92–3.12 (m, 3H), 2.50 (t, 1H, $J = 12.0$ Hz), 2.25–2.43 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 167.5, 161.6, 149.2, 148.6, 125.3, 116.9, 115.6, 114.2, 112.2, 111.1, 102.2, 92.0 (d, $J = 142.0$ Hz), 52.5 (d, $J = 19.0$ Hz), 50.2, 44.9, 37.5, 35.3 (d, $J = 17.0$ Hz). MS (ESI) m/z 384 $[M + H]^+$. HRMS (ESI) calcd $C_{15}H_{15}BrFN_3O_3$ 384.0359 $[M + H]^+$, found 384.0352.

4.2. In vitro DPP4, FAP, DPP7, DPP8, and DPP9 enzyme assay

4.2.1. Preparation of the DPPs enzyme

The DPP4, FAP, DPP7, DPP8 and DPP9 enzymes were expressed in high five cells using a baculoviral system (Bac-To-Bac; Life Technologies) according to the literature [38], and his6-tagged recombinant proteins were purified by Ni-NTA resin individually.

4.2.2. Enzyme-based assay of DPP4

To measure the activity of DPP4, a continuous fluorometric assay was employed using Ala-Pro-AMC, which is cleaved by the enzyme to release the fluorescent aminomethylcoumarin (AMC). Liberation of AMC was monitored using an excitation wavelength of 355 nm and an emission wavelength of 460 nm using Envision microplate reader (PerkinElmer). A typical reaction contained 50 pmol/L enzyme, 10 μmol/L Ala-Pro-AMC, different concentrations of the compounds synthesized in this work, and assay buffer (100 mmol/L HEPES, pH 7.5, 0.1 mg/mL BSA) in a total reaction volume of 50 μL. The dose response of inhibition test was carried out in quadruplicate. And the IC_{50} data was calculated using the software GraphPad Prism 5, and chosen the equation "sigmoidal dose–response (variable slope)" for curve fitting.

4.2.3. Enzyme-based assay of DPPs inhibition selectivity

The inhibitory effect of each compounds on DPPs were assayed by continuous fluorometric method. We used Nle-Pro-AMC as substrate to measure the activities of DPP7 and FAP, and Ala-Pro-AMC for DPP8 and DPP9 in the optimized pH (5.5 for DPP7 and 8.0 for other members) assay system. The selective dose response of inhibition test on DPPs and data analysis is the same as DPP4 assay system in quadruplicate.

*4.2.4. Characterization of compound **6h** and **6n** on DPP4*

In the time-independent inhibition experiment, 50 pmol/L DPP4 were incubated by 0.004 μM of compound **6h** or 0.01 μM of compound **6n** (2% DMSO as blank) for different times, and then add 45 μL mixture of enzyme and compounds to a final 50 μL assay system to initiate the enzyme reaction. And reversibility of DPP4 inhibition by compounds was demonstrated by dialysis experiments. In brief, mixture of 50 pmol/L DPP4 and compounds (0.004 μM compound **6h**, 0.01 μM compound **6n**, and 2% DMSO as blank) were dialyzed for different times, and then add 5 μL substrate solution to 45 μL dialysis samples to start the enzymatic assay. The dissociation of the enzyme–inhibitor complex was monitored by substrate hydrolysis by measuring the fluorescence using Envision microplate reader.

For DPP4 kinetic study, the assay was carried out in a 50 μL system containing 100 mmol/L HEPES, pH 7.5, 0.1 mg/mL BSA, 50 pmol/L DPP4, substrate Ala-Pro-AMC in 2-fold dilution from 160 μmol/L to 1.25 μmol/L, and different concentrations of the inhibitors. In the presence of the competitive inhibitor, the Michaelis–Menten equation is described as $1/v = (K_m/[V_{max}[S]])(1+[I]/K_i)+1/V_{max}$, where K_m is the Michaelis constant, v is the initial rate, V_{max} is the maximum rate, and $[S]$ is the substrate concentration.

4.2.5. Pharmacokinetic profile in SD rats

Compounds **6h** and **6n** were administered to SD rats. After oral and intravenous administration, blood samples were collected. The blood samples were centrifuged to obtain the plasma fraction. The plasma samples were deproteinized with methanol containing an internal standard. After centrifugation, the supernatant was diluted with methanol and centrifuged again. The compound concentrations in the supernatant were measured by LC/MS/MS.

4.2.6. Oral glucose tolerance test in ICR mice and chronic study in C57BKS db/db mice

All animal experiments were approved by the Animal Care and Use Committee of Shanghai Institute of Materia Medica. For the acute single dose study, vehicle (0.5% methycellulose, 10 mL/kg), compounds **6h** (15 and 50 mg/kg), **6n** (15 and 50 mg/kg) and **LAF237** (30 mg/kg) were administered to ICR mice after 16-hrs starvation, then the oral glucose tolerance test (2.5 g/kg) was conducted after 30 min of the single dose, the blood glucose level at 0, 15, 30, 60, 90, and 120 min were recorded for area under curve calculation. For the chronic study in C57BKS *db*/*db* mice with compound **6h** (5, 15 and 50 mg/kg/day) treatment, oral glucose tolerance test (1.5 g/kg) was carried out after 6 h starvation of 5th-week treatment, the blood glucose level was recorded for the glucose tolerance capacity evaluation. Vehicle (0.5% methycellulose, 10 mL/kg/day) and **LAF237** (15 mg/kg/day) were included as negative and positive control, respectively.

The results are presented as the mean ± SE. Differences between the groups were analyzed with the Student's t-test. *, $p < 0.05$ or **, $p < 0.01$ was regarded as statistically significant.

4.2.7. hERG testing using FluxOR™ thallium assay

Step1: Growing cells. CHO-hERG-ZG cells are grown in 75 cm [2] flask with complete medium with 100 μg/mL G418 and 100 μg/mL HygromycinB until 80–90% confluency. Wash cells with PBS once. Incubate cells with 1 mL 0.25% Trypsin until all cells are rounded and can be easily dislodged from the surface. Add 10 mL complete medium to stop Trypsin activity. Disassociate cells by thoroughly, repetitively pipetting. Transfer them to 50 mL Falcon tube and spin down at 1000 rpm for 5 min. Aspirate medium and resuspend cells using a small volume of complete medium, like 0.5 mL. Count cell density. Step 2: Cell seeding. CHO-hERG-ZG cells are plated into 96-well plates and after plating, tap plates on sides to separate cells and let plates sit in the dark at RT for 30 min before incubation at 37 °C for 16–18 h. Cells will reach 80% confluency. After overnight incubation the cells are media changed in loading buffer (old media is tapped out) and incubated in the dark at RT for 90 min. Remove the loading buffer and replace with assay buffer. Compounds **6h** and **6n** were added to the cell plate. The cell plate is incubated with compounds for 20 min in the dark at RT. Load the cell plates on FDSS. Fluorescent signals will be recorded every 2 s till 10 s. At 10 s, stimulus buffer will be added to cells. Then fluorescent signals will be recorded every second till 180 s, data QC on FDSS.

4.3. Binding studies

The DPP4 protein was extracted from RCSB Protein Data Bank (PDB ID: 2AJL). Compounds were generated using Sybyl program. Gasteiger–Hückel charge was used and the conformation of each compound was minimized using default parameters. Docking studies were performed using Glide program. The DPP4 protein was processed by minimal minimization with OPLS2005 force field. The grid was sized to 15 Å in each direction at the center of the binding

pocket. Compounds were prepared for docking using Ligprep. Ligand docking was performed in XP mode and flexible option, with up to 100 poses saved per molecule. Glide score was consulted for results analyzing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.021.

References

[1] K.L. Longenecker, K.D. Stewart, D.J. Madar, C.G. Jakob, E.H. Fry, S. Wilk, C.W. Lin, S.J. Ballaron, M.A. Stashko, T.H. Lubben, H. Yong, D. Pireh, Z. Pei, F. Basha, P.E. Wiedeman, T.W. von Geldern, J.M. Trevillyan, V.S. Stoll, Biochemistry 45 (24) (2006) 7474–7482.
[2] (a) A. Yaron, F. Nadier, Crit. Rev. Biochem. Mol. Biol. 28 (1) (1993) 31–81;
(b) A.M. Lambeir, C. Durnx, S. Scharpe, I. Meester, Crit. Rev. Clin. Lab. Sci. 40 (3) (2003) 209–294;
(c) R. Mentlein, Regul. Pept. 85 (1) (1999) 9–24;
(d) C. Oefner, A. D'Arcy, A. Mac Sweeney, S. Pierau, R. Gardiner, G.E. Dale, Acta Crystallogr. D. Biol. Crystallogr. 59 (Pt. 7) (2003) 1206–1212.
[3] (a) J.F. Gautier, S. Fetita, E. Sobngwi, C. Salaün-Martin, Diabetes Metab. 31 (2005) 233–242;
(b) A.E. Weber, J. Med. Chem. 47 (17) (2004) 4135–4141;
(c) C.F. Deacon, M.A. Nauck, J. Meier, K. Hücking, J.J. Holst, J. Clin. Endocrinol. Metab. 85 (10) (2000) 3575–3581.
[4] S. Mojsov, G.C. Weir, J.F. Habener, J. Clin. Invest. 79 (2) (1987) 616–619.
[5] M.A. Nauck, M.M. Heimesaat, K. Behle, J.J. Holst, M.S. Nauck, R. Ritzel, M. Hufner, W.H. Schmiegel, J. Clin. Endocrinol. Metab. 87 (3) (2002) 1239–1246.
[6] A. Wettergren, B. Schjoldager, P.E. Mortensen, J. Myhre, J. Christiansen, J. Holst, Dig. Dis. Sci. 38 (4) (1993) 665–673.
[7] S.W. Wright, M.J. Ammirati, K.M. Andrews, A.M. Brodeur, D.E. Danley, S.D. Doran, J.S. Lillquist, L.D. McClure, R.K. McPherson, S.J. Orena, J.C. Parker, J. Polivkova, X. Qiu, W.C. Soeller, C.B. Soglia, J.L. Treadway, M.A. VanVolkenburg, H. Wang, D.C. Wilder, T.V. Olson, J. Med. Chem. 49 (11) (2006) 3068–3076.
[8] D.J. Madar, H. Kopecka, D. Pireh, H. Yong, Z. Pei, X. Li, P.E. Wiedeman, S.W. Djuric, T.W. Von Geldern, M.G. Fickes, L. Bhagavatula, T. McDermott, S. Wittenberger, S.J. Richards, K.L. Longenecker, K.D. Stewart, T.H. Lubben, S.J. Ballaron, M.A. Stashko, M.A. Long, H. Wells, B.A. Zinker, A.K. Mika, D.W. Beno, A.J. Kempf-Grote, J. Polakowski, J. Segreti, G.A. Reinhart, R.M. Fryer, H.L. Sham, J.M. Trevillyan, J. Med. Chem. 49 (21) (2006) 6416–6420.
[9] H. Sakashita, H. Kitajima, M. Nakamura, F. Akahoshi, Y. Hayashi, Bioorg. Med. Chem. Lett. 15 (10) (2005) 2441–2445.
[10] H. Fukushima, A. Hiratate, M. Takahashi, A. Mikami, M. Saito-Hori, E. Munetomo, K. Kitano, S. Chonan, H. Saito, A. Suzuki, Y. Takaoka, K. Yamamoto, Bioorg. Med. Chem. 16 (7) (2008) 4093–4106.
[11] P. Mattei, M. Boehringer, P. Di Giorgio, H. Fischer, M. Hennig, J. Huwyler, B. Kocer, B. Kuhn, B.M. Loeffler, A. Macdonald, R. Narquizian, E. Rauber, E. Sebokova, U. Sprecher, Bioorg. Med. Chem. Lett. 20 (3) (2010) 1109–1113.
[12] H.M. Pattzi, S. Pitale, M. Alpizar, C. Bennett, A.M. O'Farrell, J. Li, J.M. Cherrington, H.P. Guler, PHX1149-PROT202 Study Group, Diabetes Obes. Metab. 12 (4) (2010) 348–355.
[13] T.P. Cho, Y.F. Long, L.Z. Gang, W. Yang, L.H. Jun, S.G. Yuan, F.J. Hong, W. Lin, G.D. Liang, Z. Lei, L.J. Jing, G.A. Shen, S.G. Hong, W. Dan, F. Ying, Y.P. Ke, L. Ying, F. Jun, M.X. Tai, Bioorg. Med. Chem. Lett. 20 (12) (2010) 3565–3568.
[14] Y. Nishio, H. Kimura, N. Sawada, E. Sugaru, M. Horiguchi, M. Ono, Y. Furuta, M. Sakai, Y. Masui, M. Otani, T. Hashizuka, Y. Honda, J. Deguchi, T. Nakagawa, H. Nakahira, Bioorg. Med. Chem. 19 (18) (2011) 5490–5499.
[15] D. Kim, L. Wang, M. Beconi, G.J. Eiermann, M.H. Fisher, H. He, G.J. Hickey, J.E. Kowalchick, B. Leiting, K. Lyons, F. Marsilio, M.E. McCann, R.A. Patel, A. Petrov, G. Scapin, S.B. Patel, R.S. Roy, J.K. Wu, M.J. Wyvratt, B.B. Zhang, L. Zhu, N.A. Thornberry, A.E. Weber, J. Med. Chem. 48 (1) (2005) 141–151.
[16] E.B. Villhauer, J.A. Brinkman, G.B. Naderi, B.F. Burkey, B.E. Dunning, K. Prasad, B.L. Mangold, M.E. Russell, T.E. Hughes, J. Med. Chem. 46 (13) (2003) 2774–2789.
[17] D.J. Augeri, J.A. Robl, D.A. Betebenner, D.R. Magnin, A. Khanna, J.G. Robertson, A. Wang, L.M. Simpkins, P. Taunk, Q. Huang, S.P. Han, B. Abboa-Offei, M. Cap, L. Xin, L. Tao, E. Tozzo, G.E. Welzel, D.M. Egan, J. Marcinkeviciene, S.Y. Chang, S.A. Biller, M.S. Kirby, R.A. Parker, L.G. Hamann, J. Med. Chem. 48 (15) (2005) 5025–5037.
[18] J. Feng, Z. Zhang, M.B. Wallace, J.A. Stafford, S.W. Kaldor, D.B. Kassel, M. Navre, L. Shi, R.J. Skene, T. Asakawa, K. Takeuchi, R. Xu, D.R. Webb, S.L. Gwaltney 2nd, J. Med. Chem. 50 (10) (2007) 2297–2300.
[19] M. Eckhardt, E. Langkopf, M. Mark, M. Tadayyon, L. Thomas, H. Nar, W. Pfrengle, B. Guth, R. Lotz, P. Sieger, H. Fuchs, F. Himmelsbach, J. Med. Chem. 50 (26) (2007) 6450–6453.
[20] (a) B. Ahren, E. Simonsson, H. Larsson, M. Landin-Olsson, H. Torgeirsson, P.A. Jansson, M. Sandqvist, P. Bavenholm, S. Efendic, J.W. Eriksson, S. Dickinson, D. Holmes, Diabetes Care 25 (5) (2002) 869–875;
(b) B. Ahren, R. Gomis, E. Standl, D. Mills, A. Schweizer, Diabetes Care 27 (12) (2004) 2874–2880;
(c) B. Ahren, L. Landing-Olsson, P.A. Jansson, M. Sevensson, D. Holmes, A. Schweizer, J. Clin. Endocrinol. Metab. 89 (5) (2004) 2078–2084;
(d) B. Ahren, G. Pacini, J.E. Foley, A. Schweizer, Diabetes Care 28 (8) (2005) 1936–1940.
[21] B.A. Connolly, D.G. Sanford, A.K. Chiluwal, S.E. Healey, D.E. Peters, M.T. Dimare, W. Wu, Y. Liu, H. Maw, Y. Zhou, Y. Li, Z. Jin, J.L. Sudmeier, J.H. Lai, W.W. Bachovchin, J. Med. Chem. 51 (19) (2008) 6005–6013.
[22] H. Tsu, X. Chen, C.T. Chen, S.J. Lee, C.N. Chang, K.H. Kao, M.S. Coumar, Y.T. Yeh, C.H. Chien, H.S. Wang, K.T. Lin, Y.Y. Chang, S.H. Wu, Y.S. Chen, I.L. Lu, S.Y. Wu, T.Y. Tsai, W.C. Chen, H.P. Hsieh, Y.S. Chao, W.T. Jiaang, J. Med. Chem. 49 (1) (2006) 373–380.
[23] K.D. Koo, M.J. Kim, S. Kim, K.H. Kim, S.Y. Hong, G.C. Hur, H.J. Yim, G.T. Kim, H.O. Han, O.H. Kwon, T.S. Kwon, J.S. Koh, C.S. Lee, Bioorg. Med. Chem. Lett. 17 (15) (2007) 4167–4172.
[24] H. Sakashita, F. Akahoshi, T. Yoshida, H. Kitajima, Y. Hayashi, S. Ishii, Y. Takashina, R. Tsutsumiuchi, S. Ono, Bioorg. Med. Chem. 15 (2) (2007) 641–655.
[25] T.Y. Tsai, M.S. Coumar, T. Hsu, H.P. Hsieh, C.H. Chien, C.T. Chen, C.N. Chang, Y.K. Lo, S.H. Wu, C.Y. Huang, Y.W. Huang, M.H. Wang, H.Y. Wu, H.J. Lee, X. Chen, Y.S. Chao, W.T. Jiaang, Bioorg. Med. Chem. Lett. 16 (12) (2006) 3268–3272.
[26] R.P. Brigance, W. Meng, A. Fura, T. Harrity, A. Wang, R. Zahler, M.S. Kirby, L.G. Hamann, Bioorg. Med. Chem. Lett. 20 (15) (2010) 4395–4398.
[27] K.M. Andrews, D.A. Beebe, J.W. Benbow, D.A. Boyer, S.D. Doran, Y. Hui, S. Liu, R.K. McPherson, C. Neagu, J.C. Parker, D.W. Piotrowski, S.R. Schneider, J.L. Treadway, M.A. VanVolkenberg, W.J. Zembrowski, Bioorg. Med. Chem. Lett. 21 (6) (2011) 1810–1814.
[28] W. Meng, R.P. Brigance, H.J. Chao, A. Fura, T. Harrity, J. Marcinkeviciene, S.P. O'Connor, J.K. Tamura, D. Xie, Y. Zhang, H.E. Klei, K. Kish, C.A. Weigelt, H. Turdi, A. Wang, R. Zahler, M.S. Kirby, L.G. Hamann, J. Med. Chem. 53 (15) (2010) 5620–5628.
[29] Y. Miyamoto, Y. Banno, T. Yamashita, T. Fujimoto, S. Oi, Y. Moritoh, T. Asakawa, O. Kataoka, H. Yashiro, K. Takeuchi, N. Suzuki, K. Ikedo, T. Kosaka, S. Tsubotani, A. Tani, M. Sasaki, M. Funami, M. Amano, Y. Yamamoto, K. Aertgeerts, J. Yano, H. Maezaki, J. Med. Chem. 54 (3) (2011) 831–850.
[30] L. Zhang, M.B. Su, J.Y. Li, X. Ji, J. Wang, Z. Li, J. Li, H. Liu, Chem. Biol. Drug Des. 81 (2) (2013) 198–207.
[31] D.R. Magnin, J.A. Robl, R.B. Sulsky, D.J. Augeri, Y. Huang, L.M. Simpkins, P.C. Taunk, D.A. Betebenner, J.G. Robertson, B.E. Abboa-Offei, A. Wang, M. Cap, L. Xin, L. Tao, D.F. Sitkoff, M.F. Malley, J.Z. Gougoutas, A. Khanna, Q. Huang, S.P. Han, R.A. Parker, L.G. Hamann, J. Med. Chem. 47 (10) (2004) 2587–2598.
[32] C.D. Haffner, D.L. McDougald, S.M. Reister, B.D. Thompson, C. Conlee, J. Fang, J. Bass, J.M. Lenhard, D. Croom, M.B. Secosky-Chang, T. Tomaszek, D. McConn, K. Wells-Knecht, P.R. Johnson, Bioorg. Med. Chem. Lett. 15 (23) (2005) 5257–5261.
[33] (a) H. Sakashita, F. Akahoshi, H. Kitajima, R. Tsutsumiuchi, Y. Hayashi, Bioorg. Med. Chem. 14 (11) (2006) 3662–3671;
(b) T. Kondo, I. Sugimoto, T. Nekado, K. Ochi, T. Ohtani, Y. Tajima, S. Yamamoto, K. Kawabata, H. Nakai, M. Toda, Bioorg. Med. Chem. 15 (7) (2007) 2715–2735.
[34] S.J. Coutts, T.A. Kelly, R.J. Snow, C.A. Kennedy, R.W. Barton, J. Adams, D.A. Krolikowski, D.M. Freeman, S.J. Campbell, J.F. Ksiazek, W.W. Bachovchin, J. Med. Chem. 39 (10) (1996) 2087–2094.
[35] (a) E.B. Villhauer, J.A. Brinkman, G.B. Naderi, B.E. Dunning, B.L. Mangold, M.D. Mone, M.E. Russell, S.C. Weldon, T.E. Hughes, J. Med. Chem. 45 (12) (2002) 2362–2365;
(b) T. Kondo, T. Nekado, I. Sugimoto, K. Ochi, S. Takai, A. Kinoshita, A. Hatayama, S. Yamamoto, K. Kishikawa, H. Nakai, M. Toda, Bioorg. Med. Chem. 16 (4) (2008) 1613–1631.
[36] C.D. Haffner, D.L. McDougald, A.S. Randhawa, S.M. Reister, J.M.Lenhard, U.S. Patent 0, 171848 A1, 2004.
[37] C. Jamieson, E.M. Moir, Z. Rankovic, G. Wishart, J. Med. Chem. 49 (17) (2006) 5029–5046.
[38] J. Dobers, M. Zimmermann-Kordmann, M. Leddermann, T. Schewe, W. Reutter, H. Fan, Protein Expr. Purif. 25 (3) (2002) 527–532.