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Original article

The highly potent and selective dipeptidyl peptidase IV inhibitors bearing a thienopyrimidine scaffold effectively treat type 2 diabetes

Jifeng Deng [a,1], Li Peng [a,1], Guicheng Zhang [a], Xiaobing Lan [a], Chufang Li [a], Fuxin Chen [a], Yayao Zhou [a], Zuoxian Lin [a], Ling Chen [a], Renke Dai [a], Hongjiang Xu [b], Ling Yang [b], Xiquan Zhang [b], Wenhui Hu [a,*]

[a] Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Avenue, Guangzhou Science Park, Guangzhou 510530, China
[b] Jiangsu Chia-Tai Tianqing Pharmaceutical Co. Ltd, No. 8 Julong North Rd., Xinpu Lianyungang Jiangsu 222006, China

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ABSTRACT

New dipeptidyl peptidase IV inhibitors were designed based on Alogliptin using a scaffold-hopping strategy. All of the compounds constructed on a thienopyrimidine scaffold demonstrated good inhibition and selectivity for DPP-IV. Compound **10d** exhibited subnanomolar ($IC_{50} = 0.33$ nM) DPP-IV inhibitory activity, good *in vivo* efficacy and an acceptable pharmacokinetic profile. A pharmacokinetic-driven optimization of **10d** may lead to a new class of clinical candidate DPP-IV inhibitors.

1. Introduction

The high prevalence of type 2 diabetes (T2D) is a major global public health concern and affects approximately 180 million patients worldwide, with yet more people still undiagnosed or at the pre-diabetes stage. T2D, known as non-insulin-dependent diabetes mellitus, is a chronic, severe and increasingly prevalent disease [1,2]. Although many standard therapies for T2D are available in the market, none of them can stop disease progression. Therefore, new therapeutic agents are still needed to combat diabetes. Dipeptidyl peptidase IV (DPP-IV) inhibitors have emerged as a major breakthrough in anti-diabetic drug discovery. By blocking the DPP-IV enzyme, these inhibitors can prolong the half-life of active glucagons like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) and thus stimulate insulin biosynthesis and secretion. These inhibitors finally represent a way to effectively and safely control blood glucose in diabetic patients [3–5].

DPP-IV inhibitors possess several advantages over traditional anti-diabetic drugs, such as not leading to increased bodyweight or causing hypoglycemia, and are generally well-tolerated [6–8]. DPP-IV inhibitors may be disease-modifying therapies because they can delay or prevent the loss of functional β-cell mass and, therefore, slow disease progression [9].

Based on these observations, the inhibition of DPP-IV is an attractive strategy for the treatment of diabetes, and three DPP-IV inhibitors have been launched in the US or EU: Sitagliptin **1** [10,11], Vildagliptin **2** [12–14] and Saxagliptin **3** [15] (Fig. 1). Alogliptin **4** [16] has twice the potency of Sitagliptin as a DPP-IV inhibitor and is still in the FDA review process due to new guidelines for cardiac safety data [17–19].

For a chronic disease, a drug that can produce similar beneficial effects with less toxicity at low doses is always welcome. Though there are several new drugs been marketed such as Sitagliptin, Vildagliptin and Saxagliptin, and highly potent DPP-IV inhibitors are still in great need. While many novel inhibitors have been reported based on **1**, **2** and **3**, fewer analogs of Alogliptin have been disclosed, leaving a large amount of chemical space unexplored. Thus, our objective was to discover more inhibitors by modifying this drug candidate using a scaffold-hopping strategy [20]. Alogliptin was previously obtained by replacing the scaffold of quinazolinone compound with a pyrimidinedione. Herein, we report the discovery of highly potent DPP-IV inhibitors by incorporating a thienyl ring into Alogliptin's structure.

2. Chemistry

The synthesis of compounds **10a–e** is outlined in Scheme 1 which is similar to the synthetic protocol of Alogliptin [16].

* Corresponding author. Tel.: +86 20 32015211; fax: +86 20 32015299.
E-mail address: hu_wenhui@gibh.ac.cn (W. Hu).
[1] Joint first authors: these two authors contribute equally to this work.

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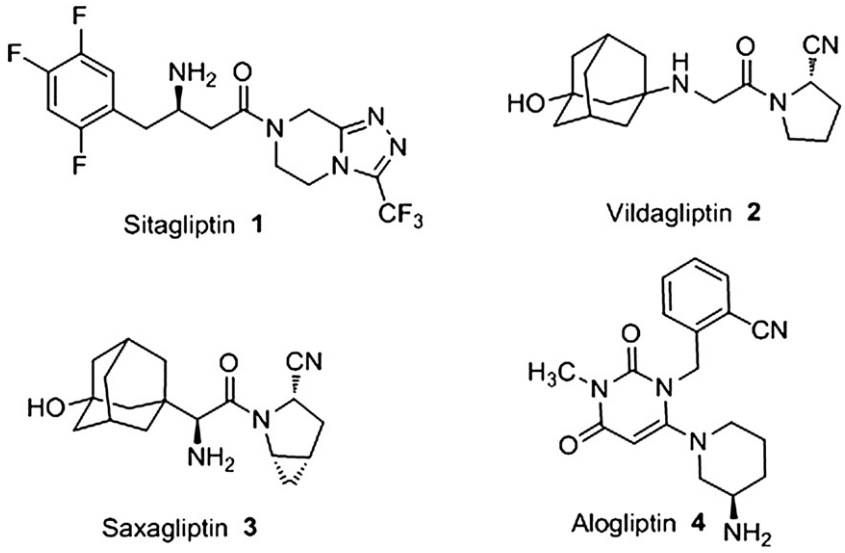


Fig. 1. Important DPP-IV inhibitors.

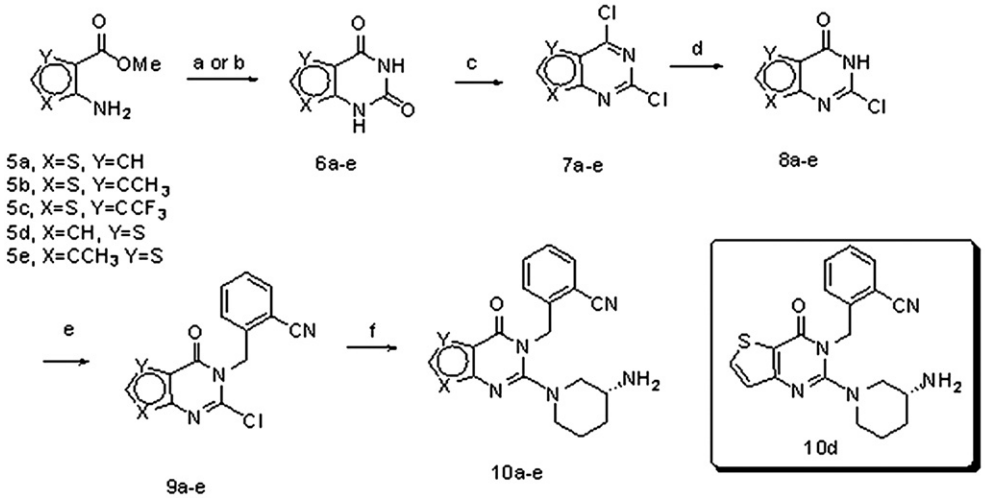
Briefly, the commercially available starting materials **5a**–**c** were either heated with urea [9] between 190 °C and 200 °C, or reacted with chlorosulfonyl isocyanate at −60 °C to generate the corresponding thienopyrimidines [21] **6a**–**e**. Chlorination of **6a**–**e** with phosphoryl trichloride yielded **7a**–**e**, which were hydrolyzed with aqueous sodium hydroxide to give key intermediates **8a**–**e**. Selective *N*-alkylation was performed using a previously published method [22] to produce compounds **9a**–**e**. The final compounds, **10a**–**e**, were obtained in high yields by the amination of the chloro precursors **9a**–**e** with 3-(*R*)-aminopiperidine.

3. Results and discussion

All the compounds listed in Table 1 were evaluated for activity against the DPP-IV enzyme and for selectivity against DPP-8 and DPP-9 [10,23]. We also conducted the human Ether-à-go-go Related Gene (hERG) study in accordance with the new FDA guidelines for evaluating the cardiovascular risk of new therapies for the treatment of T2D [24,25].

Remarkably, all the compounds except **10c** were more active than Alogliptin *in vitro*. Among them, compound **10d** was 10 times more active than Alogliptin. Additionally, all of the compounds in the series demonstrated good selectivity, while none of them inhibited DPP-8 or DPP-9. These compounds did not inhibit the hERG ion channel and may have less risk for cardiac side effect, which is a major concern for anti-diabetic drugs.

Compound **10a**, which has no substituents on the scaffold, showed good subnanomolar inhibition (0.87 nM, *in vitro*). The addition of a methyl to the 3-position (**10b**) decreased the activity slightly (1.32 nM). However, simply switching the methyl group (**10c**) to a trifluoromethyl group greatly decreased the activity (88.82 nM). Rotating the thiophenyl ring on the scaffold of **10a** led to compound **10d**, which had a 2-fold increased activity (0.33 nM) and was the most active compound in this study. Adding a 1-methyl group onto compound **10d** resulted in compound **10e**, which was still more active than Alogliptin *in vitro*. This simple investigation demonstrates that the orientation of the thiophenyl moiety is not important for inhibition and that functional groups on the ring do



Scheme 1. Synthesis of compounds **10a**–**e**. Reagents: (a) urea, 190–200 °C; (b) $ClSO_2NCO$, CH_2Cl_2, −60 °C; (c) $POCl_3$, DIEA, reflux; (d) 1 N NaOH; (e) 2-$CNPhCH_2Br$, NaH, LiBr; (f) 3-(*R*)-aminopiperidine, $NaHCO_3$, 150 °C.

Table 1
Inhibitory properties of compounds **10a**–**e**.

No.	X	Y	DPP-IV IC_{50} (nM)	DPP-8 IC_{50} (nM)	DPP-9 IC_{50} (nM)	hERG (μM)
10a	S	CH	0.87	>25,000	>25,000	484.6
10b	S	CCH_3	1.32	>25,000	>25,000	376.3
10c	S	CCF_3	88.82	>25,000	>25,000	NT[a]
10d	CH	S	0.33	>25,000	>25,000	517.4
10e	CCH_3	S	1.72	>25,000	>25,000	203.5
Alogliptin			3.4	>25,000	>25,000	>30[b]

[a] NT = not tested.
[b] Reported data, see Ref. [26].

exert some influence on activity. The IC_{50} value for Alogliptin under our experimental conditions was 3.4 nM, which is in agreement with the literature value (<10 nM) [26].

The most active compound in Table 1, **10d**, was chosen for *in vivo* evaluation (Figs. 2 and 3). Oral administration of **10d** in rats significantly reduced both blood glucose levels in an oral glucose tolerance test (OGTT) and serum DPP-IV activity in ICR male mice. The initial results showed that the *in vivo* efficacy of compound **10d** is as good as Alogliptin.

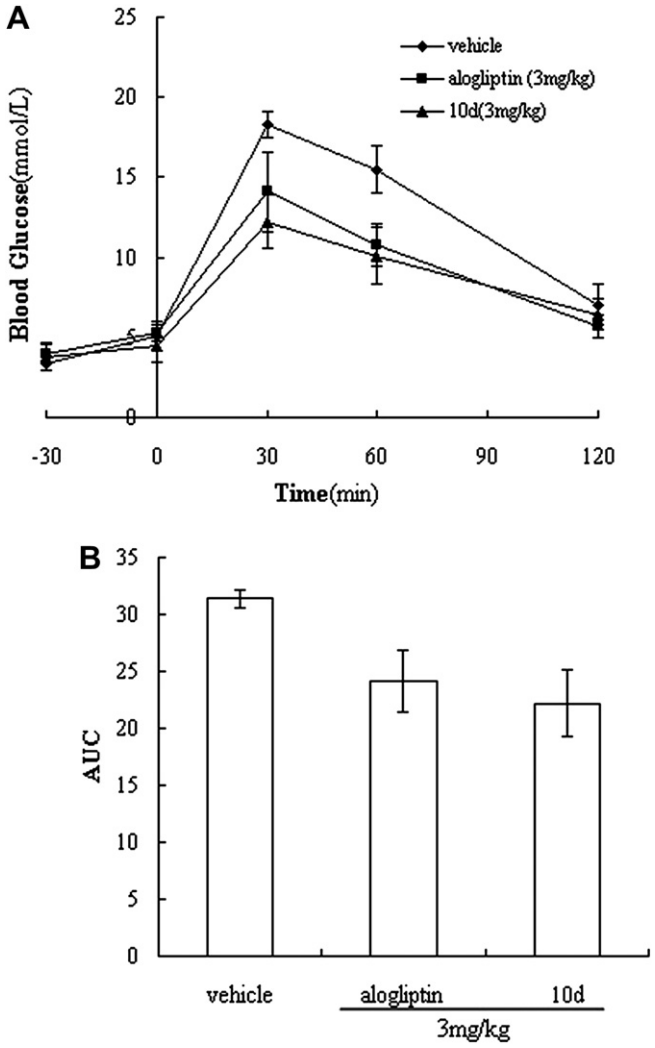


Fig. 2. Effect of compounds in the oral glucose tolerance test. Changes of blood glucose levels (A) and AUC values of delta blood glucose between 0 and 120 min (B) in an oral glucose tolerance test in male C57BL/6J mice fed a high-fat diet. Compound **10d** (3 mg/kg), Alogliptin (3 mg/kg), or vehicle was orally administered 30 min prior to an oral glucose challenge (2.5 g/kg). Data are mean ± S.E.M (n = 5/group).

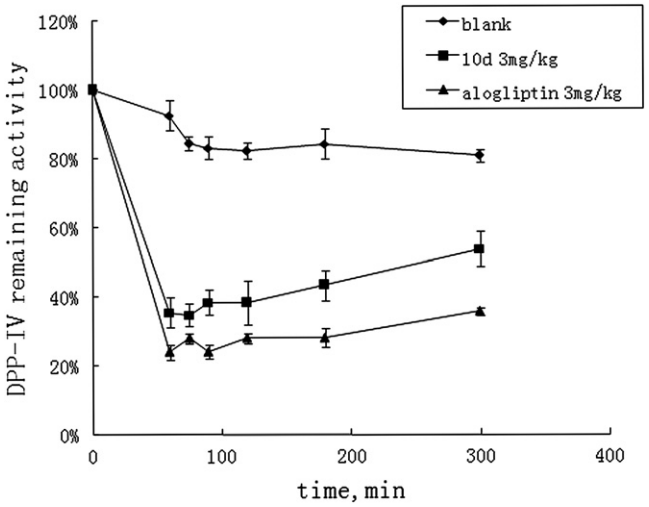


Fig. 3. Effect of compounds on serum DPP-IV activity in ICR male mice. Specific activity of serum DPP-IV tested in male C57BL/6J mice fed a high-fat diet. Compound **10d** (3 mg/kg), Alogliptin (3 mg/kg), or vehicle was orally administered. Data are mean ± S.E.M (n = 5/group).

Table 2 shows the pharmacokinetic profile of compound **10d** in Sprague Dawley Rats and Beagle Dogs. Both the $T_{1/2}$ and the oral bioavailability are acceptable. In our experiment, the oral bioavailability (F) in Sprague Dawley Rats of **10d** was 23.7% while in beagle dogs was 42.3%, which was less than the reported value (F = 43% in rats, F = 68% in dogs) for Alogliptin [16]. Thus, compound **10d** afforded desirable pharmacokinetic (PK) and pharmacodynamic (PD) profiles and is worthy of further evaluation as a drug candidate.

Even though compound **10d** could be considered as a potential drug candidate, there are still significant opportunities to improve its drug-like properties. For example, there is a disconnect between the *in vitro* and *in vivo* activity: compound **10d** was more than 10 times more active than Alogliptin *in vitro*, but its *in vivo* activity was a little lower than that of Alogliptin. The unexpectedly low *in vivo* activity could be the result of moderate PK properties. Thus, a PK-driven optimization to improve oral bioavailability of compounds in this series will be required to generate new drug candidates in the DPP-IV inhibitor class of therapeutics.

4. Conclusions

In summary, we have successfully generated highly potent DPP-IV inhibitors **10a** and **10d** with IC_{50} values in the subnanomolar range by using a scaffold replacement technique and by using Alogliptin as a starting point. All of the compounds investigated in the study showed good selectivity for DPP-IV over DPP-8 and DPP-9 and did not block the hERG ion channel. Compound **10d** met some of the requirements for being a drug candidate in terms of efficacy, selectivity and acceptable pharmacokinetic profile. Most importantly, we provided a novel series of compounds with the potential for PK-driven optimization to facilitate the discovery of anti-diabetic drugs.

5. Experimental

5.1. General chemistry procedures

^{1}H NMR spectra were recorded on a Bruker Avance 400 and ^{13}C NMR spectra on a Bruker Avance 500. Chemical shifts are expressed

Table 2
Selected PK parameters for compound **10d** in rats and dogs.

Species	No.	$T_{1/2}$ (h)	$AUC_{0\text{-}t}$ (μg h mL^{-1})	T_{max} (h)	C_{max} (μg mL^{-1})	MRT (h)	CLp (mL min^{-1} kg^{-1})	F (%)
Sprague Dawley rats	**10d** i.v. (10 mg/kg)	5.9 ± 3.2	1.48 ± 0.41	0.04 ± 0.02	1.23 ± 0.35	2.7 ± 0.32	6.97 ± 1.58	
Sprague Dawley rats	**10d** p.o. (20 mg/kg)	7.0 ± 3.9	0.69 ± 0.19	0.59 ± 0.71	0.17 ± 0.08	6.7 ± 1.0	6.40 ± 2.98	23.7
Beagle dogs	**10d** i.v. (10 mg/kg)	10.14 ± 5.29	13.26 ± 3.18	0.10 ± 0.04	5.75 ± 1.14	6.0 ± 1.1	22.18 ± 8.83	
Beagle dogs	**10d** p.o. (20 mg/kg)	11.29 ± 5.53	11.21 ± 1.93	0.91 ± 0.20	2.07 ± 0.85	9.0 ± 2.1	11.5 ± 3.17	42.3

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

in parts per million (ppm), and coupling constants are expressed in Hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br (broad). Low-resolution mass spectra (MS) and compound purity data were acquired on a Waters ZQ LC/MS single quadrupole system equipped with an electrospray ionization (ESI) source, a UV detector (220 nm and 254 nm), and an evaporative light scattering detector (ELSD). Preparative HPLC was conducted on the same system using mixtures of TFA (0.05%) buffered water and acetonitrile. Thin-layer chromatography was performed on 0.25 mm Merck silica gel plates (60F-254) and visualized with UV light, 5% ethanolic phosphomolybdic acid, ninhydrin or *p*-anisaldehyde solution. Flash column chromatography was performed on silica gel (230–400 mesh, Merck).

5.2. Synthesis of compounds ***5a–c***

5.2.1. Methyl 2-aminothiophene-3-carboxylate (***5a***)

The title compound was prepared according to the previously reported procedure [27]. A total of 132 g of crude product was obtained and used for the next reaction without further purification.

5.2.2. Methyl-2-amino-4-methylthiophene-3-carboxylate (***5b***)

The title compound was prepared according to the previously reported procedure [28]. A total of 21.0 g of **5b** was obtained in 60.5% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.06 (s, 2H), 5.81 (s, 1H), 3.81 (s, 1H), 2.26 (s, 3H).

5.2.3. Methyl-2-amino-4-(trifluoromethyl)thiophene-3-carboxylate (***5c***)

The title compound was prepared according to the reported procedure [29] and was isolated as a brown solid (6.0 g, yield 30.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.75 (s, 1H), 6.26 (s, 2H), 3.84 (s, 3H); MS (ESI) $C_7H_6NO_2F_3$ $[M-H]^-$ 224.0.

5.3. Synthesis of compounds ***6a–e***

5.3.1. Thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione (***6a***)

A mixture of methyl 2-aminothiophene-3-carboxylate **5a** (20.0 g, 0.13 mol) and urea (60 g, 1.0 mol) was heated at 200 °C for 2 h. The reaction mixture was cooled and poured into a sodium hydroxide solution, and any insoluble material was removed by filtration. The filtrate was then acidified with 2 N HCl to give a white precipitate, which was collected by filtration, washed with water and dried on a funnel to provide the title compound **6a** (13.7 g, yield 64%). MS (ESI): $C_6H_4N_2O_2S$ $[M-H]^-$ 167.1.

5.3.2. 5-Methylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione (***6b***)

The title compound was prepared from methyl-2-amino-4-methylthiophene-3-carboxylate **5b** according to the previously reported procedure [28] in 57.0% yield. ^{1}H NMR (400 MHz, DMSO-d6): δ 6.07 (s, 2H), 5.82 (s, 1H), 3.82 (s, 1H), 2.26 (s, 3H); MS (ESI) $C_7H_6N_2O_2S$ $[M-H]^-$ 181.1.

5.3.3. 5-(Trifluoromethyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione (***6c***)

The title compound was prepared according to the reported procedure [29] and was isolated as a white solid (4.7 g, yield 99.0%). ^{1}H NMR (400 MHz, DMSO-d6): δ 12.17 (s, 1H), 11.28 (s, 1H), 7.75 (s, 1H); MS (ESI) $C_7H_3N_2O_2SF_3$ $[M-H]^-$ 234.9.

5.3.4. Thieno[3,2-d]pyrimidine-2,4(1H,3H)-dione (***6d***)

Yield: 59%. ^{1}H NMR (400 MHz, DMSO-d6): δ 11.60–11.10 (br, s, 2H), 8.10 (d, $J = 5.2$ Hz, 1H), 6.9 (d, $J = 5.2$ Hz, 1H); MS: (ESI) $C_6H_4N_2O_2S$ $[M+H]^+$ 169.1.

5.3.5. 7-Methylthieno[3,2-d]pyrimidine-2,4(1H,3H)-dione (***6e***)

Yield: 50.0%. ^{1}H NMR (400 MHz, DMSO-d6): δ 11.42 (s, 1H), 11.22 (s, 1H), 7.69 (s, 1H), 2.20 (s, 3H); MS (ESI) $C_7H_6N_2O_2S$ $[M+H]^+$ 182.9.

5.4. Synthesis of compounds ***7a–d***

5.4.1. 2,4-Dichlorothieno[2,3-d]pyrimidine (***7a***)

A total of 0.8 mL N,N-dimethylaniline was added to 3.0 g of thieno[2,3-d]pyrimidine-2,4-(1H,3H)-dione **6a** in 20 mL $POCl_3$. The mixture was then heated under reflux for 16 h. Excess $POCl_3$ was removed *in vacuo*, and the resulting residue was treated with ice water to yield a precipitate. The solid was collected by filtration, washed with water and dried over a funnel to afford solid **7a** (1.3 g, yield 35.5%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.62 (d, $J = 6.4$ Hz, 1H), 7.43 (d, $J = 6.4$ Hz, 1H).

5.4.2. 2,4-Dichloro-5-methylthieno[2,3-d]pyrimidine (***7b***)

Yield: 31.0%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.20 (s, 1H), 2.93 (s, 1H), 2.66 (s, 3H).

5.4.3. 2,4-Dichloro-5-(trifluoromethyl)thieno[2,3-d]pyrimidine (***7c***)

Yield: 53.0%. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.88 (s, 1H).

5.4.4. 2,4-Dichlorothieno[3,2-d]pyrimidine (***7d***)

Yield: 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (d, $J = 5.5$ Hz, 1H), 7.55 (d, $J = 5.5$ Hz, 1H); MS: (ESI) $C_6H_2Cl_2N_2S$ $[M+H]^+$ 206.9.

5.4.5. 2,4-Dichloro-7-methylthieno[3,2-d]pyrimidine (***7e***)

Yield: 43.4%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (s, 1H), 2.49 (s, 3H); MS (ESI) $C_7H_4N_2Cl_2S$ $[M+H]^+$ 218.9.

5.5. Synthesis of compounds ***8a–d***

5.5.1. 2-Chlorothieno-[2,3-d]-pyrimidin-4(3H)-one (***8a***)

A mixture of 10.2 g of 2,4-dichlorothieno-[2,3-d]-pyrimidine **7a**, 120 mL 1 N NaOH and 20 mL of THF was stirred at room temperature under N_2 for 4 h. The solution was then chilled and adjusted to pH 5 with AcOH. The resulting precipitate was collected, washed with water and dried to afford **8a** as a solid (8.5 g, yield 92%). MS (ESI) $C_6H_3ClN_2OS$ $[M-H]^-$ 185.1.

5.5.2. 2-Chloro-5-methylthieno[2,3-d]pyrimidin-4(3H)-one (***8b***)

Yield: 90.3%. MS (ESI) $C_7H_5ClN_2OS$ $[M-H]^-$ 201.1.

*5.5.3. 2-Chloro-5-(trifluoromethyl)thieno[2,3-d]pyrimidin-4(3H)-one (**8c**)*

Yield: 96.0%. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.29 (s, 1H); MS (ESI) $C_7H_2N_2F_3ClOS$ $[M-H]^-$ 253.0.

*5.5.4. 2-Chlorothieno[3,2-d]pyrimidin-4(3H)-one (**8d**)*

Yield: 90%. ^{1}H NMR (400 MHz, DMSO-d6): δ 13.47 (s, 1H), 8.2 (d, $J = 5.5$ Hz, 1H), 7.55 (d, $J = 5.5$ Hz, 1H); MS: (ESI) $C_6H_3ClN_2OS$ $[M+H]^+$ 187.0.

*5.5.5. 2-Chloro-7-methylthieno[3,2-d]pyrimidin-4(3H)-one (**8e**)*

Yield: 57%. ^{1}H NMR (400 MHz, DMSO-d6): δ 7.83 (s, 1H), 2.25 (s, 3H); MS (ESI) $C_7H_5N_2ClOS$ $[M-H]^-$ 199.1.

*5.6. Synthesis of compounds **9a–d***

*5.6.1. 2-((2-Chloro-4-oxothieno-[2,3-d]pyrimidin-3-(4H)-yl)-methyl)benzonitrile (**9a**)*

NaH (2.1 g, 51.6 mmol) was added to a stirred solution of **8a** (8.4 g, 44.9 mmol) in DME (120 mL) and DMF (30 mL) at 0 °C. Twenty minutes later, LiBr (7.9 g, 89.7 mmol) was added, and the mixture was allowed to warm to room temperature. After 15 min, α-bromo-*o*-tolunitrile (10.15 g, 51.6 mmol) was then added, and the mixture was heated at 65 °C overnight. After cooling, the mixture was poured into water (1000 mL) with stirring to yield a precipitate. This solid was filtered and dried to give the title compound **9a** (11 g, yield 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78–7.70 (m, 1H), 7.58–7.49 (m, 2H), 7.45–7.40 (m, 1H), 7.33 (d, $J = 6.4$ Hz, 1H), 6.87 (d, $J = 6.4$ Hz, 1H), 5.75 (s, 2H).

*5.6.2. 2-((2-Chloro-5-methyl-4-oxothieno[2,3-d]pyrimidin-3(4H)-yl)methyl)benzonitrile (**9b**)*

Yield: 68.7%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (s, 1H), 7.61–7.59 (m, 2H), 7.48–7.44 (m, 1H), 6.86 (s, 1H), 5.48 (s, 2H), 2.56 (s, 3H); MS (ESI) $C_{15}H_{10}ClN_3OS$ $[M+H]^+$ 316.0.

*5.6.3. 2-((2-Chloro-4-oxo-5-(trifluoromethyl)thieno[2,3-d]pyrimidin-3(4H)-yl)methyl)benzonitrile (**9c**)*

Yield: 70.0%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (s, 1H), 7.74–7.72 (m, 2H), 7.63–7.54 (m, 1H), 7.46–7.41 (m, 1H), 7.15 (d, $J = 8$ Hz, 1H), 5.75 (s, 2H); MS (ESI) $C_{15}H_7N_3F_3ClOS$ $[M+H]^+$ 370.0.

*5.6.4. 2-((2-Chloro-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl)methyl) benzonitrile (**9d**)*

The title compound was prepared from 2-chlorothieno[3,2-d] pyrimidin-4(3H)-one **8d** in 96.8% yield by a method analogous to that used to make **9a**. ^{1}H NMR (400 MHz, DMSO-d6): δ 7.88 (d, $J = 5.2$ Hz, 1H), 7.73 (d, $J = 7.6$ Hz, 1H), 7.55 (t, $J = 7.6$ Hz, 1H), 7.43 (t, $J = 7.6$ Hz, 1H), 7.32 (d, $J = 5.2$ Hz, 1H), 7.16 (d, $J = 8$ Hz, 1H), 5.77 (s, 2H); MS (ESI) $C_{14}H_8ClN_3OS$ $[M+H]^+$ 302.0.

*5.6.5. 2-((2-Chloro-7-methyl-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl)methyl)benzonitrile (**9e**)*

Yield: 83%. MS (ESI) $C_{15}H_{10}N_3ClOS$ $[M+H]^+$ 316.0.

*5.7. Synthesis of compounds **10a–e***

*5.7.1. (R)-2-((2-(3-Aminopiperidin-1-yl)-4-oxothieno-[2,3-d]-pyrimidin-3(4H)-yl)methyl) benzonitrile (**10a**)*

A mixture of **9a** (13.1 g, 43.4 mmol), 3-(*R*)-aminopiperidine dihydrochloride (11.5 g, 66.0 mmol) and $NaHCO_3$ (17.4 g, 173.6 mmol) in 300 mL of ethanol in a sealed tube was heated at 150 °C for 6 h. The reaction mixture was then cooled to room temperature and filtered. The resulting filtrate was concentrated *in vacuo* and then purified by flash chromatography to give the title compound **10a** (12.35 g, yield 77.8%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71–7.65 (m, 1H), 7.51–7.47 (m, 1H), 7.36–7.32 (m, 2H), 7.08–7.04 (m, 2H), 5.56–5.47 (m, 2H), 3.27–3.24 (m, 1H), 3.14–3.11 (m, 1H), 2.97–2.91 (m, 1H), 2.85–2.78 (m, 1H), 2.64–2.59 (m, 1H), 1.96–1.91 (m, 1H), 1.78–1.73 (m, 1H), 1.68–1.61 (m, 1H), 1.25–1.21 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d6) δ 163.87, 159.06, 157.22, 141.25, 133.88, 133.44, 128.32, 127.56, 122.19, 122.14, 120.22, 117.58, 110.51, 59.07, 51.01, 47.69, 46.74, 33.60, 23.51; MS (ESI) $C_{19}H_{19}N_5OS$ $[M+H]^+$ 366.1.

*5.7.2. (R)-2-((2-(3-Aminopiperidin-1-yl)-5-methyl-4-oxothieno[2,3-d]pyrimidin-3(4H)yl)methyl) benzonitrile (**10b**)*

Yield: 45%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.5–7.564 (m, 2H), 7.50–7.48 (m, 1H), 7.44–7.40 (m, 1H), 6.66 (s, 1H), 5.31 (s, 2H), 3.26–3.23 (m, 1H), 3.15–3.11 (m, 1H), 2.99–2.93 (m, 1H), 286–2.79 (m, 1H), 2.66–2.61 (m, 1H), 2.51 (s, 3H), 1.99–1.95 (m, 1H), 1.82–1.76 (m, 1H), 1.68–1.57 (m, 1H), 1.42–1.26 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d6) δ 164.24, 159.54, 156.99, 141.45, 135.82, 133.95, 133.89, 133.43, 127.61, 118.87, 117.67, 116.84, 110.53, 55.85, 51.29, 46.99, 46.46, 30.77, 22.84, 16.39; MS (ESI) $C_{20}H_{21}N_5OS$, $[M-H]^-$ 380.1.

*5.7.3. (R)-2-((2-(3-Aminopiperidin-1-yl)-4-oxo-5-(trifluoromethyl) thieno[2,3-d]pyrimidin-3(4H)-yl)methyl)benzonitrile (**10c**)*

Yield: 82.0%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68–7.65 (m, 1H), 7.55–7.51 (m, 2H), 7.36 (t, $J = 7.6$ Hz, 1H), 7.14 (d, $J = 8.0$ Hz, 1H), 5.50 (s, 2H), 3.35–3.31 (m, 1H), 3.22–3.19 (m, 1H), 3.02–2.95 (m, 1H), 2.90–2.83 (m, 1H), 2.67–2.62 (m, 1H), 1.98–1.94 (m, 1H), 1.80–1.75 (m, 1H), 1.70–1.64 (m, 1H), 1.31–1.20 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d6) δ 166.14, 158.25, 157.48, 140.95, 133.89, 133.52, 128.36, 127.70, 125.10(d, $J_{C\text{-}F} = 7.0$ Hz),124.45(q, $J_{C\text{-}F} = 37.5$ Hz), 122.65, 120.50, 117.56, 114.98, 110.25, 58.55, 50.74, 47.57, 33.41, 23.38; MS (ESI) $C_{20}H_{18}F_3N_5OS$ $[M+H]^+$ 434.0.

*5.7.4. (R)-2-((2-(3-Aminopiperidin-1-yl)-4-oxothieno[3,2-d] pyrimidin-3(4H)-yl)methyl)benzonitrile (**10d**)*

The title compound was prepared from 2-((2-chloro-4-oxo-thieno[3,2-d]pyrimidin-3(4H)-yl) methyl)benzonitrile (**9d**) in 77.8% yield by a method analogous to that used to make **10a**. ^{1}H NMR (400 MHz, CD_3OD): δ 7.98 (dd, $J = 2$ Hz, 2 Hz, 1H), 7.73 (d, $J = 8$ Hz, 1H), 7.57 (t, $J = 7.6$ Hz, 1H), 7.42 (t, $J = 7.6$ Hz, 1H), 7.25 (d, $J = 4.8$ Hz, 1H), 7.12 (d, $J = 8$ Hz, 1H), 5.56 (s, 2H), 3.39 (m, 1H), 3.21 (m, 1H), 2.88 (m, 2H), 2.67 (m, 1H), 1.96 (m, 1H), 1.76 (m, 1H), 1.67 (m, 1H), 1.25 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d6) δ 158.52, 158.08, 155.97, 140.86, 135.58, 133.50, 133.03, 127.90, 126.98, 124.70, 118.10, 117.16, 110.06, 58.77, 50.77, 47.29, 46.04, 33.12, 23.11; MS (ESI) $C_{19}H_{19}N_5OS$ $[M+H]^+$ 366.1.

*5.7.5. (R)-2-((2-(3-Aminopiperidin-1-yl)-7-methyl-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl)methyl) benzonitrile (**10e**)*

Yield: 55.5%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.69 (d, $J = 12.8$ Hz, 1H), 7.59–7.53 (m, 2H), 7.40 (t, $J = 7.6$ Hz, 1H), 7.08(d, $J = 8.0$ Hz, 1H), 5.55 (s, 2H), 3.45–3.39 (m, 1H), 3.15 (d, $J = 12.4$ Hz, 1H), 2.95–2.81 (m, 2H), 2.74–2.69 (m, 1H), 2.34 (s, 3H), 1.97–1.94 (m, 1H), 1.78–1.74 (m, 2H), 1.35–1.26 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d6) δ 158.47, 157.63, 154.47, 140.95, 136.04, 133.43, 133.01, 127.88, 127.71, 127.28, 118.32, 117.27, 110.30, 54.27, 51.17, 46.33, 46.07, 29.22, 22.12, 12.37; MS (ESI) $C_{20}H_{21}N_5OS$ $[M+H]^+$ 380.1.

5.8. Biological assays for DPP-IV, DPP-8 and DPP-9 in vitro

DPP inhibition assay was performed according to a slightly modified method of Kim et al. [10]. An enzyme and chemicals (diluted in an assay buffer: 50 mM Tris, pH 7.5 and 0.1% bovine

serum albumin, pH 7.4) were mixed in a black 96-well plate, and then the plate was incubated for 10 min at room temperature to allow inhibitor/enzyme interaction. The enzyme reaction was started by the addition of 100 μmol/L Gly-Pro-AMC (in assay buffer) and incubated for 20 min at room temperature. The final concentration of Gly-Pro-AMC was 50 μmol/L. Gly-Pro-AMC is cleaved by the enzyme to release the fluorescent aminomethylcoumarin (AMC). Liberation of AMC was monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm with a microplate reader (synergy HT, BIO-TEK, USA). Three separate experiments were performed and means of IC_{50} values were calculated by a curve-fitting program (GraphPad Software Inc., San Diego, CA) [10,23].

5.9. Nonradioactive Rb^+ efflux assay procedure and analysis

The rubidium efflux assay was used to evaluate the ability to block hERG potassium channel. Forty thousand to fifty thousand HEK 293 cells were seeded into noncoated 96-well cell culture microplates and incubated for 24 h at 37 °C. After discarding the medium, 198 μL of open channel buffer and 2 μL of test compound solution (stocks 30 nM to 300 mM) were added to each well except for the control wells. Then the media were replaced by a mixture of 198 μL Rb^+ load buffer and 2 μL of test compound and incubated for 3 h at 37 °C. Cell layers were then quickly washed 3 times in order to remove extracellular Rb^+. Subsequently, a mixture of 198 μL channel opening buffer and 2 μL of test compound was added to the wells except for the control wells in order to activate the hERG channels. After incubation for 5 min, the supernatant was carefully removed and collected. Cells were lysed by addition of 200 μL of lysis-buffer. Samples were then stored at 4 °C until analysis on the Ion Channel Reader 8000.

5.10. Oral glucose tolerance test

Male mice were randomly assigned into three groups ($n = 6$ each group) and fasted 5 h before the treatment of **10d**. Each group of mice was respectively administered with **10d**, Alogliptin, or water at 60 min prior to an oral dose of 2.5 g/kg glucose. Approximately 300 μL blood samples were collected into sodium heparin containing tubes before drug administration, and another blood samples were collected just before glucose administration. Blood samples were sequentially collected after glucose administration at the time period of 15, 30, 60 and 120 min. The concentration of glucose in serum was measured, and the blood glucose level at time points of 15 and 30 min was compared between oral dosing with **10d** and water. $AUC_{0-120\ min\ Glu}$ was also calculated.

The concentration of glucose was measured as following. Work solution was prepared by mixing 100 mL 0.1% phenol solution and 100 mL glucose oxidase enzyme solution. 200 μL work solution was employed in each sampling tube. 5 μL serum sample, 5 μL deionized water, 5 μL of a set of different concentrations of glucose standard solution were added into each tube, respectively. The resulting solution was mixed and incubated at 37 °C for 20 min. The concentrations of glucose in these samples were then determined according to their absorbance at 505 nm.

5.11. Measurement of DPP-IV activity in ICR mice plasma

Each of approximately 300 μL blood samples from glucose tolerance measurements was collected and the sample collection time points were extended to the time period of 240 min after glucose administration. Plasma DPP-IV activity was measured using a continuous fluorometric assay with the substrate Gly-Pro-AMC, which is cleaved by DPP-IV to release the fluorescent AMC leaving group. 5 μL rat serum mixing with 35 μL 80 mM $MgCl_2$ was incubated at room temperature for 5 min. 10 μL 0.1 mM Gly-Pro-AMC and 20 μL buffer were then added and mixed. Fluorescence at 460 nm with excitation of 380 nm was measured over 18 min with 3-min interval. These measurements were used to calculate the specific activity of serum DPP-IV.

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