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Optimization of the benzamide fragment targeting the S_2' site leads to potent dipeptidyl peptidase-IV inhibitors

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ABSTRACT

Our recently successful identification of benzoic acid-based DPP-4 inhibitors spurs the further quest for in-depth structure-activity relationships (SAR) study in S_2' site DPP-4. Thus novel benzamide fragments were designed to target the S_2' site to compromise lipophilicity and improve oral activity. Exploring SAR by introduction of a variety of amide and halogen on benzene ring led to identification of several compounds, exerting moderated to excellent DPP-4 activities, in which 4′-chlorine substituted methyl amide **17g** showed most potent DPP-4 activity with the IC_{50} value of 1.6 nM. Its activity was superior to reference alogliptin. Docking study ideally verified and interpreted the obtained SAR of designed compounds. As a continuation, DPP-8/9 assays revealed the designed compounds exhibited good selectivity over DPP-8 and DPP-9. Subsequent cell-based test indicated compound **17g** displayed low toxicity toward the LO2 cell line up to 100 μM. *In vivo* evaluation showed compound **17g** robustly improved the glucose tolerance in normal mice. Importantly, **17g** exhibited reasonable pharmacokinetic (PK) profiles for oral delivery. Overall, compound **17g** has the potential to a safe and efficacious DPP-4 inhibitor for T2DM treatment.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a major public health menace leading to increasing worldwide morbidity at an alarming rate [1,2]. It is a chronic metabolic disease characterized by hyperglycaemia due to impaired β-cell function and insulin resistance. Currently used oral antidiabetic agents, such as metformin, sulfonylureas, thiazolidinediones, and insulin *etc*, produce beneficial effects on T2DM by reducing plasma glucose. However, long-term efficacy of the current antidiabetic drugs is still unsatisfactory [2,3]. As the progressive deterioration in β-cell function in T2DM patients, patients have to be generally subjected to an increasing polypharmacy to maintain appropriate glucose homeostasis, eventually, most of them need insulin treatment [2–4]. Moreover, undesirable side effects caused by those antidiabetic therapy, such as weight gain, hypoglycemia, and gastric symptoms, also raise concerns of the safety in T2DM patients [5]. Thus it is necessary to discover novel oral anti-diabetic therapeutics with enhanced safety and efficacy profiles [5].

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from the intestine in response to energy intake and glucose, which has a range of physiological effects to maintain glucose homeostasis, such as increasing insulin secretion, decreasing food intake, and promoting insulin sensitivity [6]. However, the active GLP-1 is cleaved and inactivated by a protease, dipeptidyl peptidase-IV (DPP-4), leading to a half-life of only 1–2 min [7]. Inhibition of DPP-4 prevents degradation of GLP-1, represents a promising approach for the treatment of T2DM [8]. Recently, several DPP-4 inhibitors, including sitagliptin [9], vildagliptin [10], saxagliptin [11], linagliptin [12], and alogliptin [13], anagliptin [14], and teneligliptin [15] are approved (Fig. 1). Clinical studies demonstrated that DPP-4 inhibitors are efficient and safe antidiabetic agents with low risk for hypoglycemia and weight gain [16]. However, some safety concerns were reported in clinical use of DPP-4 inhibitor, such as saxagliptin had an increased risk for hospitalization for heart failure [17], and sitagliptin was associated with severe and persistent joint pain [16]. In addition, different pharmacokinetic and pharmacodynamic profiles exist among the different members of the class of DPP-4 inhibitors, and this difference generated benefits to a specific segment of the population, **especially for old and children patients** [18]. Novel agents with significant structural heterogeneity may translate into different pharmacological properties, and will

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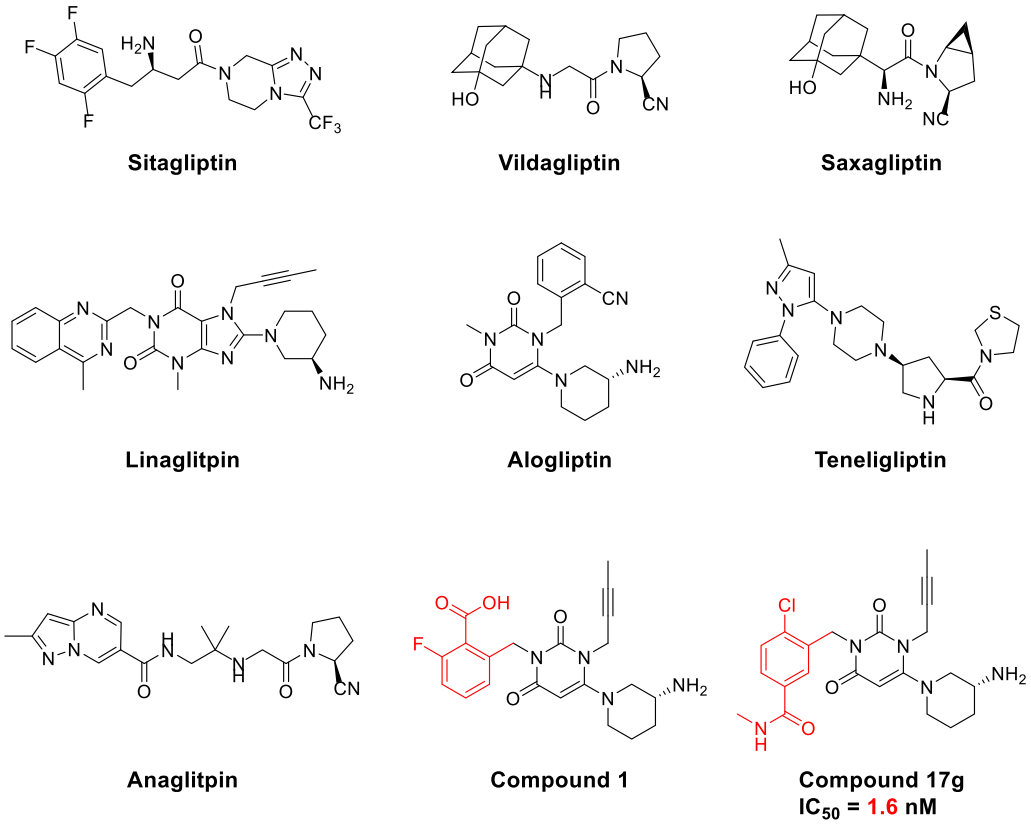
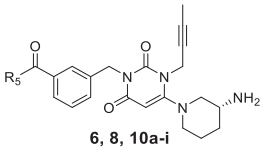


Fig. 1. The structures of marketed DPP-4 inhibitors, compound **1** and **17g**.

Table 1
In vitro DPP-4 inhibitory activities of compounds **10a–i**.



Compounds	R_5	%Inhibition at 200 nM[a]	%Inhibition at 40 nM[a]	IC_{50} (nM)[a,b]	cLogP[b,c]
6		95.6 ± 2.5	86.5 ± 1.0	5.9 ± 0.8	−0.48
8		96.8 ± 1.7	88.1 ± 2.7	7.5 ± 1.2	2.01
10a		96.0 ± 0.2	83.3 ± 0.9	8.9 ± 1.4	0.56
10b		95.0 ± 0.3	86.5 ± 0.7	3.6 ± 0.2	0.76
10c		86.8 ± 0.5	70.9 ± 0.6	11.8 ± 2.3	0.51
10d		96.0 ± 0.7	82.1 ± 0.3	4.6 ± 0.7	1.29
10e		72.8 ± 0.7	58.7 ± 2.3	27.0 ± 3.6	1.57
10f		93.4 ± 0.6	82.9 ± 0.5	13.2 ± 1.5	1.82
10g		90.0 ± 0.5	78.9 ± 0.6	19.8 ± 3.3	2.35
10h		74.6 ± 0.4	62.2 ± 0.8	27.2 ± 4.8	2.00
10i		93.1 ± 0.2	72.7 ± 0.9	8.2 ± 1.9	1.73
alogliptin		96.7 ± 1.8	85.1 ± 0.1	2.6 ± 0.9	NT

[a] Measured in two independent experiments.
[b] clogP were calculated by ChemBioDraw software Ultra 14.0.
[c] NT: not tested.

ultimately promote their uses in clinic [19]. Therefore, it is of great significant to discover novel and safer DPP-4 inhibitors [19].

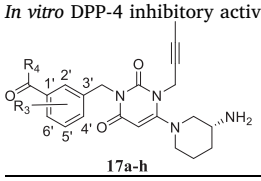
In spite of their diverse chemical structures of DPP-4 inhibitors reported in the literature [20,21], all DPP-4 inhibitors occupy S_1 and S_2 pocket [22,23], which have vital roles in the inhibitory activity. Specifically, the aromatic rings of sitagliptin and alogliptin, the cyanopyrrolidine rings of vildagliptin and saxagliptin, and alkyne of linagliptin bind to S_1 pocket, the primary or secondary amines of these inhibitors form salt bridges with Glu–Glu motif in S_2 pocket [22]. In addition to S_1 and S_2 pocket, there are other three binding sites, including S_1' site, S_2' site and S_2 extensive pocket, that parts of DPP-4 inhibitors selectively bind to improve the activity and/or selectivity against other DPP-4 homologues. Among those three binding site, S_2' site is made up of Trp629, Gly628, Trp627, His740, Lys554, Val546 and Tyr447 [8], in which only the characteristic methylquinazoline group of linagliptin binds, resulting in linagliptin as one of the most potent DPP-4 inhibitors [24]. We have explored several series of building blocks targeting the S_2' site, including triazole rings [25,26], benzoic acids [27], leading to discovery of several potent DPP-4 inhibitors. These evidences suggested that the S_2' site is a promising area to be explored. In our recent study, the o-benzoic acids were incorporated to N_3-position of uracil to interact with both Trp629 and Lys554 in S_2' site, and the compound **1** bearing 6-fluorobenzoic acid was identified as one of the most potent DPP-4 inhibitor [27]. The successful exploration spurs the further quest for in-depth SAR study in S_2' site. Therefore, we herein further designed and optimized the benzamide fragment targeting the S_2' site. Our efforts culminated in the discovery of (*R*)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-4-chloro-*N*-methylbenzamide (compound **17g**) as a potent (IC_{50} = 1.6 nM), selective, *in vivo* active DPP-4 inhibitor.

2. Results and discussion

2.1. Chemistry

The structures of the target compounds obtained in this study are shown in Tables 1 and 2. The synthetic routes are outlined in Schemes 1 and 2. The compounds **6**, **8**, **10a-i** were prepared as described in Scheme 1. The selective alkylation of the starting material 6-

Table 2
In vitro DPP-4 inhibitory activities of compounds **17a–i.**



17a-h

Compounds	R_3	R_4	DPP-4 IC_{50} (nM)[a,c]	DPP-8 IC_{50} (μM)[a]	DPP-9 IC_{50} (μM)[a]	cLogP[b,c]
17a	2′-F		16.6 ± 3.6	> 100	> 100	0.70
17b	2′-F		24.0 ± 4.9	> 100	> 100	1.23
17c	4′-F		2.2 ± 0.8	> 100	> 100	1.11
17d	4′-F		6.1 ± 1.3	> 100	> 100	1.64
17e	6′-F		5.7 ± 1.2	> 100	> 100	0.70
17f	6′-F		29.0 ± 4.8	> 100	> 100	1.23
17g	4′-Cl		1.6 ± 0.3	> 100	> 100	1.68
17h	4′-Cl		3.2 ± 0.7	> 100	> 100	2.21
alogliptin	–	–	2.6 ± 0.9	> 100	> 100	NT
vildagliptin	–	–	NT	2.3	0.14	NT

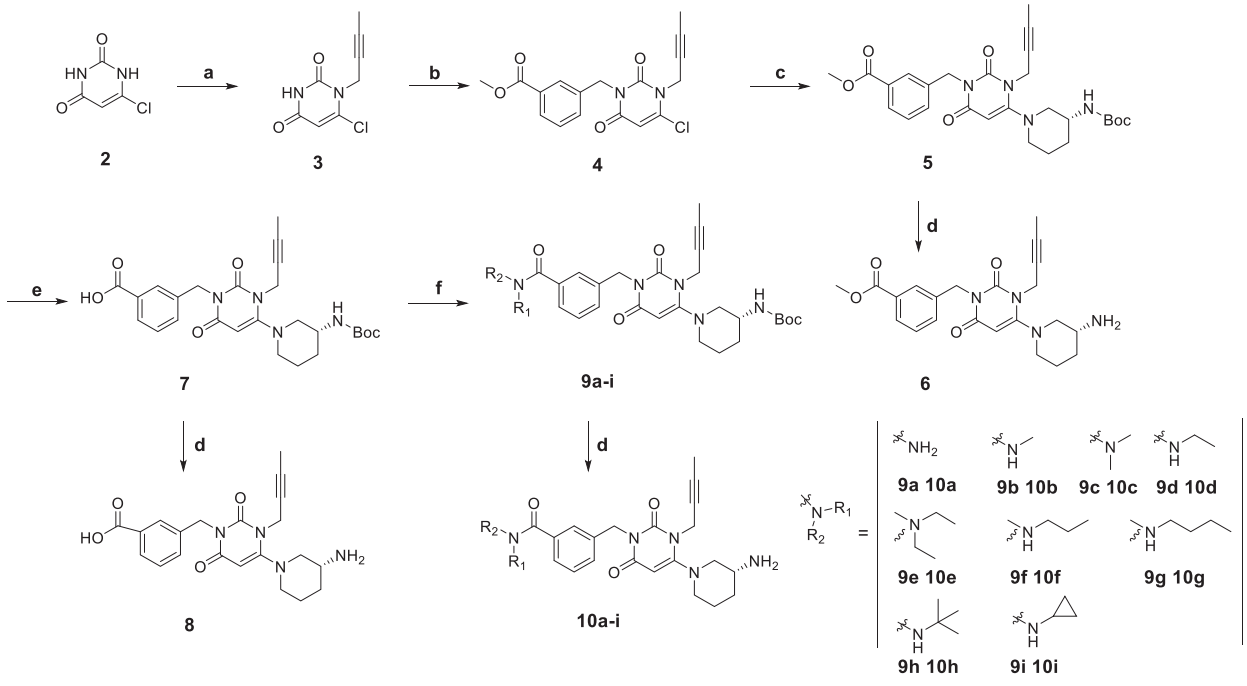
[a] Measured at least in two independent experiments.
[b] clogP were calculated by ChemBioDraw software Ultra 14.0.
[c] NT: not tested.

chlorouracil **2** with 1-bromo-but-2-yne provided product **3**, which was further alkylated with methyl 3-(bromomethyl)benzoate to yield intermediate **4**. **4** was treated with (*R*)-3-*N*-Boc-aminopiperidine to afford the desired compound **5**. Hydrolysis of **5** with LiOH provided benzoic acid **7**. The Boc groups of compounds **5** and **7** were removed to give target compounds **6** and **8**, respectively. Condensing the obtained **7** with a variety of amines via classical amidation reaction, followed by deprotection of Boc group, furnished the target compounds **10a–i**.

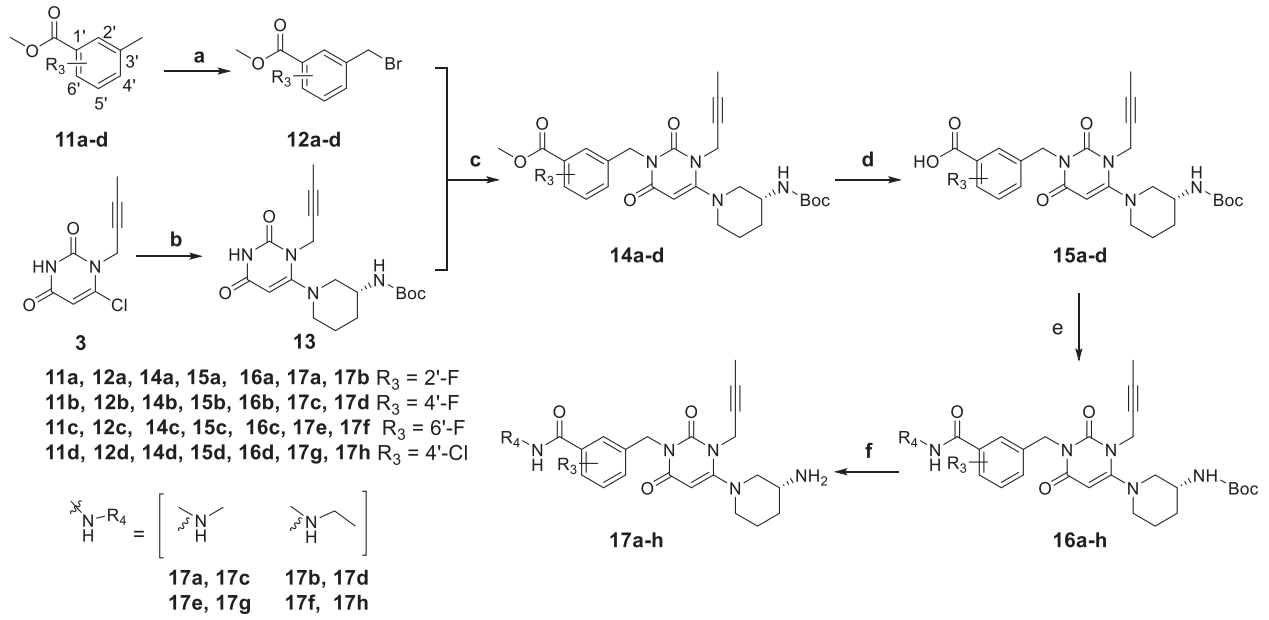
The target compounds **17a–h** were prepared as shown in Scheme 2. The commercially available substituted methyl 3-methylbenzoates **11a–d** were converted to 3-bromomethylbenzoates **12a–d** by bromination of 3-methyl with *N*-bromosuccinimide (NBS). Treatment of **3** with (*R*)-3-*N*-Boc-aminopiperidin gave compound **13.** Compound **13** was subsequently alkylated with compounds **12a–d**, then hydrolyzed with LiOH to afford compounds **15a–d.** Compounds **15a–d** were condensed with amines via the recently reported procedure [28] to give compounds **16a–h**, which underwent deprotection of Boc group to afford the target compounds **17a–h**.

2.2. In vitro DPP-4 inhibition and SAR study

Human recombinant DPP-4 was used to evaluate *in vitro* DPP-4 inhibitory activities of the designed compounds, with the approved drug alogliptin as references. In our previous work, we disclosed potent DPP-4 inhibitor such as **1** in Fig. 1, containing *o*-carboxyl benzene moiety that were expected to form salt bridges with Lys554 and π-stacking with Trp629 in S_2' site. Inspired by this work, we moved the carboxyl group to *meta*-position of benzene ring, as shown in Table 1, the resulting compound **8** preserved potent activity (IC_{50} = 7.5 nM). Interestingly, the methyl ester of compound **6** (IC_{50} = 5.9 nM) showed even more potent activity than their corresponding carboxyl-based compound **8**, suggesting that an ionized carboxylic acid is not essential for potent binding to DPP-4. These results demonstrated that the new series of analogues with *m*-carboxylic acid exhibit a considerably different SAR compared with what we obtained from the previous *o*-carboxyl-derived compounds [27], that the carboxylic acid has a key role in the potent DPP-4 inhibitory activity [27]. The structure of compound **1** contains a carboxyl group and the amino group, leading to low clogP value and poor oral bioavailability. Encouraged by the result that the ionized carboxylic acid is not essential for potent binding to DPP-4 in this series, we explored amides as replacements for the carboxylic acid to compromise lipophilicity, and thus improve oral activity. As shown in Table 1, it was reassuring to observe that primary amide **10a** (IC_{50} = 8.9 nM) retained potent DPP-4 inhibitory activity. we, therefore, introduced a great variety of amides to the *meta*-position of benzene ring. The methyl amide **10b**, an isostere of methyl ester, exhibited approximately 2-fold improvement in DPP-4 activity (IC_{50} = 3.6 nM) compared to ester **8**. The ethyl amide **10d** (IC_{50} = 4.6 nM) also exhibited slightly more potent activity than ester **8**. However, extending the carbon chain (**10f**, **10g**) led to decrease in activity. The N, *N*-



Scheme 1. Synthesis of target compounds **6**, **8**, **10a–i**. Reagents and conditions: (a) 1-bromo-but-2-yne, DIPEA, DMF, r.t., 12 h, 86%; (b) methyl 3-(bromomethyl) benzoate, K_2CO_3, DMF, r.t. 12 h, 88%; (c) (*R*)-3-*N*-Boc-aminopiperidine, K_2CO_3, DMF, 60 °C, 10 h, 87%; (d) HCl gas, EA/ether, 0 °C, 65–88%; (e) LiOH, H_2O/MeOH, 40 °C, 89%; (f) **9a**, NH_3OH, EtOH, seal tube 72%; **9b–i**, NHR_1R_2, EDCI, HOBt, DIPEA, CH_3CN, r.t. 12 h, 76–92%.



Scheme 2. Synthesis of target compounds **17a–h**. Reagents and conditions: (a) NBS, $(PhCO_2)_2$, $ClCH_2CH_2Cl$, 80 °C, 12 h, 55–82%; (b) (*R*)-3-*N*-Boc-aminopiperidine, K_2CO_3, DMF, 60 °C, 10 h, 91%; (c) K_2CO_3, DMF, r.t. 12 h; (d) LiOH, H_2O/MeOH, r.t., 60–79%; (e) NH_2R_4, N,N,N′,N′-tetramethylchloroformamidinium hexafluorophosphate (TFCH), *N*-methylimidazole (NMI), r.t., 1 h; (f) HCl gas, EA/ether, 0 °C, two steps yeilds:41–52%.

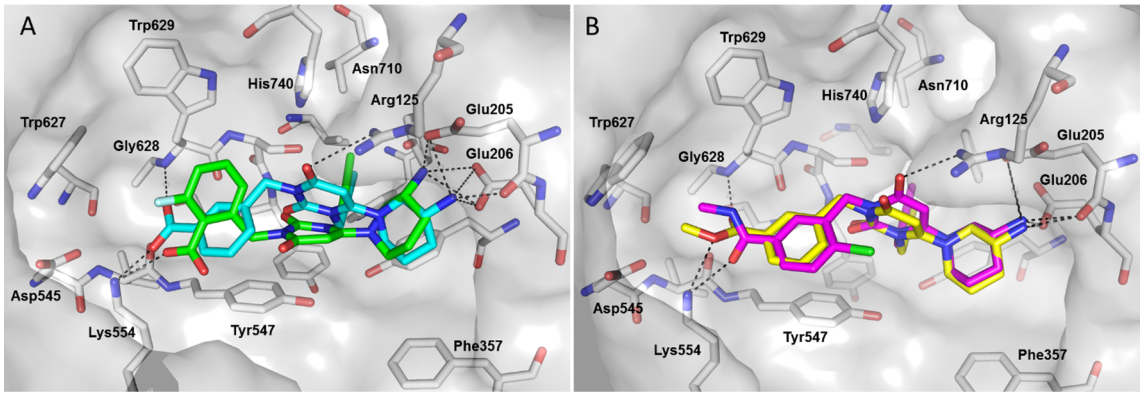


Fig. 2. The docking binding modes of compounds **17g** and its structural superposition in DPP-4 active site. Hydrogen bonds are depicted as dashed black lines. The active site of DPP-4 is represented in white surface. Compounds and residues are shown in sticks. Fig. 2A, the overlay structure of **8** (cyan) with compound **1** (green). Fig. 2B, the superposition of **17g** (maganta) and **6** (yellow).

dimethyl **10c** and diethyl **10e** provided less potency. Increasing the bulk, such as *tert*-butyl **10h**, resulted in pronounced loss in potency, and less bulky cyclopropyl **10i** (IC_{50} = 8.2 nM) had potency similar to that of ester **8.** These results revealed that there may be a small pocket that would accommodate small methyl chain in S_2' site, but do not tolerate bulky groups.

Based on the obtained SAR above, we selected methyl amide **10b** and ethyl amide **10d** as lead for further modification by the introduction of a variety of halogen to the benzene ring (Table 2). As fluorine has been extensively applied as a bioisosteric replacement for the hydrogen atom due to its small size and the high C—F bond strength [29], a fluorine was introduced to 2′, 4′ and 6′-position of the benzene ring, respectively. When the fluorine was introduced at the 4′-position, the methyl amide **17c** (IC_{50} = 2.2 nM) showed an improvement in potency compared with that of **10b,** the activity of compounds **17c** was slightly more potent than that of alogliptin (IC_{50} = 2.6 nM). But the ethyl amide **17d** (IC_{50} = 6.1 nM) displayed decreased activity relative to **10d**. The substitution of the fluorine at 2′-position (**17a**, IC_{50} = 16.6 nM, **17b**, IC_{50} = 24.0 nM) and 6′-position (**17e**, IC_{50} = 5.7 nM, **17f**, IC_{50} = 29.0 nM) of benzene ring resulted in a pronounced loss in DPP-4 inhibitory activity. With 4′-fluorine compound **17d** as a potent DPP-4 inhibitor, we decided to introduce the chlorine to 4′-position of the benzene ring, the 4′-chlorine substituted amide **17g** (IC_{50} = 1.6 nM) and **17h** (IC_{50} = 3.2 nM) turned out to be more potent than 4′-fluorine amide **17c** and **17d**, in which compound **17g** was one of the most potent DPP-4 inhibitor, which was approximately 2-fold more potent than alogliptin. The comparison between halogen-substituted methyl amide series and ethyl series revealed that methyl amide series were more potent activity than ethyl series, which was in accordance with the order of hydrogen-substituted compounds **10b** and **10d**. Furthermore, the calculated clogP values indicated that cLogP values of **10a-i** and **17a-h** were ranging from 0.51 to 2.35, indicating amide compounds might have optimum lipophilicity for good oral bioavailability.

2.3. Molecular docking study

To illustrate the probable molecular interaction between designed compounds and DPP-4 receptor, molecular docking was carried out by using program GLIDE 5.9. Compounds **6**, **8** and **17g** were docked into a DPP-4 crystal structure (PDB ID: 2RGU) with compound **1** as reference. Amino acids in the active site of DPP-4 and the interaction of the best docking pose of compounds **6**, **8** and **17g** are shown in Fig. 2. The

superposition of compound **6** with compound **1** reveals that the compound **6** shares two key binding interactions with compound **1**, the 2-butynyl group inserts into the S_1 pocket to form hydrophobic interaction, and the primary amine of (3*R*)-aminopiperidine interact with Glu205 and Glu206 via a salt bridge interaction. However, the uracil ring and the phenyl ring of **6** have different binding modes compared with the compound **1**: the phenyl ring of **1** makes an upwards bend to interact with Trp629 via π-stacking, while the phenyl ring of **6** twists in the opposite direction to form a π-stacking with Tyr547. On the other hand, the uracil ring of **1** forms a π-stacking with Tyr547, but that of **6** loses this π-stacking, while the carbonyl of uracil of **6** gains an additional hydrogen bonding with Arg125. Furthermore, the carboxyl group of **6** extends to spaces between Lys554 and Trp629 to form a salt bridge with the amine of Lys554 and a hydrogen bonding with amine of Trp629 in S_2' subsite, while the carboxyl group of compound **1** have only a salt bridge with Lys554. As shown in Fig. 2B, compounds **8** and **17g** interact with the active site of DPP-4 in a similar way with **6**, the uracil ring, 2-butynyl group and (*3R*)-aminopiperidine group almost completely superimposed on that of compound **6**. The binding modes of compounds **8** and **17g** were different with the compound **1,** resulting in retaining the DPP-4 activity when replacing the carboxylic acid by esters (**8**) or amides (**17g**), whose carbonyl groups preserve the key hydrogen binding with Lys554. The enhancement of DPP-4 activity of compound **17g** compared with **8** may contribute to the electron migration induced by the chlorine in **17g**, leading to stronger π-stacking with Tyr547. In addition, the methyl of ester and amide perfectly inserts into a small hydrophobic pocket surrounding by Trp629, Gly628, Trp627, Val546 and Tyr447 in S_2' subsite to accommodate the methyl group. This may be the reason for decreasing in DPP-4 activity when increasing the bulk, as illustrated by the compounds in Table 1. All the evidences supported the exploration of S_2' subsite can be a promising strategy to design novel DPP-4 inhibitors.

2.4. DPP-8 and DPP-9 selectivity study

Compounds **17a-h** were selected for serine dipeptidyl peptidase DPP-8 and DPP-9 selectivity study, due to their inhibitions are likely associated with severe toxicities in preclinical species [24]. As shown in Table 2, compounds **17a-h** exhibited the IC_{50} values > 100 μM for both DPP-8 and DPP-9, while reference drug vildagliptin displayed the IC_{50} of 2.3 μM and 0.14 μM for DPP-8 and DPP-9, respectively. The results revealed that the designed compounds have excellent selectivity against DPP-8 and DPP-9.

2.5. Effects of compounds *17d, 17g* and *17h* on cell viability

Based on the potent DPP-4 activity and selectivity, compounds **17d, 17g** and **17h** were selected for toxicity evaluation on normal hepatic LO2 cell using the MTT assay. Anticancer drug Doxorubicin (DOX) was used as controls. As shown in Fig. 3, the % viability of compounds **17d, 17g** and **17h** were approximately 100% up to 100 μM, indicating those compounds exhibited low cytotoxicity against normal hepatic L-02 cell line. In contrast, the positive control DOX was found to exhibit higher toxicity (% viability at 10 μM was 50%) in the cell line.

2.6. Metabolic stability study of *17d, 17g* and *17h*

As majority of drug metabolism occurs through liver, metabolic stability was assessed in ICR mice liver microsomes and rat plasma applying LC-MS/MS for analysis and processing. **17c, 17g** and **17h** were incubated separately for 60 min in liver microsomal systems (ICR mice) with the cofactors NADPH and rat plasma. As shown in Table 3, all tested analogues exhibited excellent stability in liver microsomes as the percentage remaining > 92% after 60 min incubation. Furthermore, the **17c, 17g** and **17h** are also stable in rat plasma, suggesting that the molecule has adequate metabolic stability in both liver microsomes and plasma to be a drug.

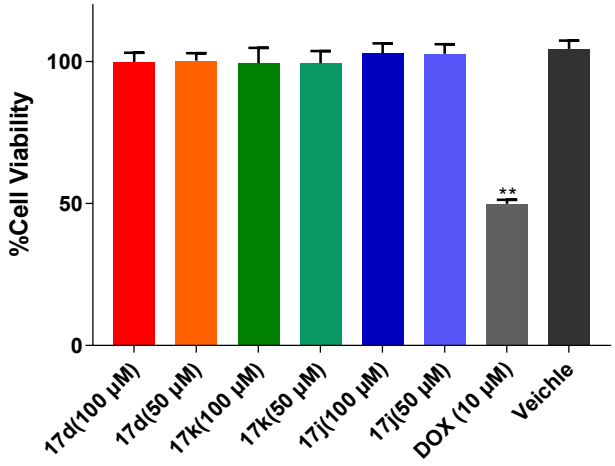


Fig. 3. Cytotoxicity of compounds **17d, 17g** and **17h** against normal hepatic LO2 cell. Values are mean ± SD (n = 5). **P ≤ 0.01 compared to vehicle-treated group by using a one-way ANOVA with Dunnett's multiple comparisons test.

Table 3
Compounds metabolic properties in the rat liver microsome and plasma.

Compounds[a]	Remaining ratio (%) SD rat- plasma	ICR-liver microsome
17d	95.22 ± 2.32	92.37 ± 3.37
17g	99.38 ± 1.18	101.83 ± 1.69
17h	94.28 ± 2.64	95.68 ± 1.51

[a] Values are the means of at least two independent determinations.

2.7. Hypoglycemic effects of *17d, 17g* and *17h* in normal mice

On the basis of potent DPP-4 activity and low toxicity, compounds **17d, 17g** and **17h** further underwent *in vivo* glucose-lowering effect evaluation by the oral glucose tolerance test (OGTT). **17d, 17g** and **17h** (10 mg kg^{-1}) were orally administered to Kunming mice. The time-dependent changes in plasma glucose levels and the corresponding area under the curve ($AUC_{0-120\ min}$) are shown in Fig. 4. Among all compounds, compound **17g** exerts strongest hypoglycemic effect with the reduced the $AUC_{0-120\ min}$ value of 16.4%, while compound **17d** and **17h** reduced $AUC_{0-120\ min}$ value to 12.90% and 6.58%, respectively. The hypoglycemic activity of the compound **17g** was slightly more potent than that of referenced drug alogliptin, which produced a decrease in glucose level with the decreased $(AUC)_{0-120\ min}$ of 13.78% at a dose of 10 mg/kg.

2.8. Pharmacokinetic evaluation of compounds *17g*

Compounds **17g** were further selected for *in vivo* PK study. Compounds **17g** was administrated orally and intravenously at the dose of 10 mg/kg and 3 mg/kg, respectively. The key parameters are shown in Table 4. The compound **17g** displayed a low clearance (0.35 L/h/kg), long half-life (11.1 h) and good bioavailability (27.3%), indicating compound **17g** had reasonable PK properties.

3. Conclusion

Inspired by our recent identification of *o*-benzoic acid-based uracil **1** as a potent DPP-4 inhibitor, further optimization was carried out by designing benzamide fragment targeting the S_2' site. Our design thought originated from the finding that moving the benzoic acid from

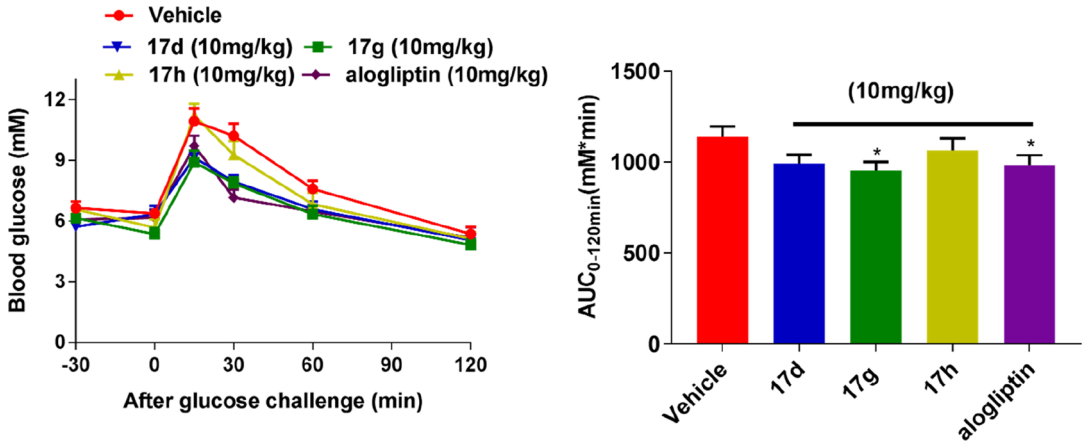


Fig. 4. Effects of compounds **17d, 17g** and **17h** on blood glucose levels during an OGTT in male Kunming mice (A) Time-dependent changes of plasma glucose levels of compound **17d, 17g** and **17h** at the dose of 10 mg/kg. (B) $AUC_{0-120\ min}$ of blood glucose levels of compounds **17d, 17g** and **17h** at the dose of 10 mg/kg. Values are mean ± SEM (n = 8). *P ≤ 0.05 compared to vehicle-treated group by using a one-way ANOVA with Dunnett's multiple comparisons test.

ortho-position to *meta*-position retained potent DPP-4 inhibitory activity, while replacement of acid with ester improved the activity. After exploring the SAR by introducing a variety of amide and halogen on benzene ring, the 4′-chlorine substituted methyl amide **17g** was identified as a potent DPP-4 inhibitor with the IC_{50} value of 1.6 nM superior to reference alogliptin. Furthermore, **17g** was found to exhibit good selectivity over other related enzymes including DPP-8, and DPP-9. Subsequent docking study ideally verified the remarkable DPP-4 activities of designed compounds. Importantly, compound **17g** displayed low toxicity toward the LO2 cell line up to 100 μM, and reduced blood glucose excursion in normal mice. Notably, **17g** exhibited reasonable AUC, C_{max} value and moderate oral bioavailability for oral delivery. These evidences supported compound **17g** promises for further investigation.

4. Experimental section

4.1. Chemistry

All starting materials, reagents and solvents were obtained from commercial sources and used without further purification. Column chromatography was carried on silica gel (200–300 mesh). NMR spectra were recorded on a Bruker instrument in the indicated solvents (DMSO-d_6, $CDCl_3$, TMS as internal standard), chemical shifts are expressed in δ values (ppm) and the coupling constants (*J*) in Hz. MS spectra were determined on ThermoFisher TSQ Series Mass spectrometer system (ESI, TQU04153). Melting points were tested on capillary tube and were uncorrected. %Purity of the target compounds were determined by HPLC analysis (UV detector, wavelength: 254 nm, mobile phase composed of mathanol (0.1% HCOOH) and H_2O.

4.1.1. 1-(but-2-ynyl)-6-chlorouracil (3)

DIPEA (9.3 g, 72.1 mmol) and 1-bromo-2-butyne (9.5 g, 71.7 mmol) were added successively to the solution of 6-chlorouracil (10 g, 68.2 mmol) in DMF (60 mL), then the mixture was stirred at r.t. for 12 h. Water (150 mL) was added, the precipitate was collected by filtration, washed with water and EtOH, and dried to give compound **3** as light yellow solid (11.7 g, yield: 86%). mp: 216–217 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.71 (s, 1H), 5.99 (s, 1H), 4.65 (s, 2H), 1.80 (s, 3H); MS (ESI) *m/z*: 199.02 $[M+H]^+$.

4.1.2. Methyl 3-((3-(but-2-yn-1-yl)-4-chloro-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl) benzoate (4)

To a mixture of compound **3** (12.7 g, 63.7 mmol) and K_2CO_3 (17.6 g, 127.5 mmol) in DMF (50 mL) was added methyl 3-(bromomethyl) benzoate (14.6 g, 63.7 mmol). After stirring at r.t. for 12 h, the mixture was poured into water. The precipitate was separated by filtration, washed with water, and dried to give compound **4** as a brown solid (19.4 g, yield: 88%), mp: 121–123 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.91 (d, *J* = 1.8 Hz, 1H), 7.90 – 7.84 (m, 1H), 7.60 – 7.57 (m, 1H), 7.49 (t, *J* = 7.7 Hz, 1H), 6.24 (s, 1H), 5.02 (s, 2H), 4.73 (d, *J* = 2.5 Hz, 2H), 3.85 (s, 3H), 1.80 (t, *J* = 2.4 Hz, 3H). MS (ESI) *m/z*: 369.43 $[M+Na]^+$.

4.1.3. Methyl (R)-3-((3-(but-2-yn-1-yl)-4-(3-((tert-butoxycarbonyl) amino)piperidin-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl) benzoate (5)

A mixture of compound **4** (5.0 g, 14.4 mmol), (*R*)-3-(*N*-Boc-amino) piperidine (3.5 g, 17.3 mmol) and K_2CO_3 (6.0 g, 43.3 mmol) in DMF (30 mL) was stirred at 60 °C for 10 h. After cooling to r.t., water was added, and extracted with dichloromethane (3 × 20 mL). The resulting organic layer was washed thrice with saturated brine, dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to give the crude product, which was purified by chromatography on silica gel (dichloromethane/MeOH 100:1–60:1) to give product **5** as light yellow solid (6.4 g, yield: 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (d, *J* = 1.8 Hz, 1H), 7.99 – 7.92 (m, 1H), 7.74 – 7.64 (m, 1H), 7.39 (t, *J* = 7.7 Hz, 1H), 5.28 (s, 1H), 5.20 – 5.11 (m, 2H), 4.69 – 4.61 (m, 1H), 4.53 – 4.44 (m, 1H), 3.91 (s, 3H), 3.89 – 3.82 (m, 1H), 3.39 – 3.22 (m, 1H), 3.17 – 3.02 (m, 1H), 3.00 – 2.79 (m, 1H), 2.75 – 2.61 (m, 1H), 1.97 – 1.84 (m, 2H), 1.83 (t, *J* = 2.3 Hz, 3H), 1.79 – 1.69 (m, 1H), 1.61 – 1.51 (m, 1H), 1.46 (s, 9H). MS (ESI) *m/z*: 533.64 $[M+Na]^+$.

Table 4
Pharmacokinetic Parameters of **17g** after po and i.v. administration.

Parameters[a]	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/L)	$AUC_{0\text{-}inf}$ (μg/L*h)	Cl (L/h/kg)	F%
iv (3 mg/kg)	6.34 ± 2.58	–	4607.04 ± 46.59	8596.84 ± 157.74	0.35 ± 0.01	–
po (10 mg/kg)	11.10 ± 3.68	0.5	639.29 ± 188.06	7823.32 ± 1771.84	0.13 ± 0.03	27.3%

[a] Compounds were dosed to equal number of male Sprague-Dawley rats in po and iv administration (n = 3).

*4.1.4. Methyl (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)benzoate hydrochloride (**6**)*

To a solution of compound **5** (300 mg, 0.6 mmol) in EtOAc/ether (v/v, 1/3, 30 mL) was bubbled with freshly prepared HCl gas at 0 °C. After the completed consumption of starting materials, the precipitate was separated by filtration, dried in vacuum to afford compound **6** as white solid (210 mg, yield: 87%); HPLC purity: 95.4%; mp: 176.5–178.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.28 (s, 3H), 7.90 (s, 1H), 7.86 (d, J = 7.8 Hz, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.48 (t, J = 7.8 Hz, 1H), 5.30 (s, 1H), 5.06 – 4.93 (m, 2H), 4.63 (d, J = 17.5 Hz, 1H), 4.46 (d, J = 19.5 Hz, 1H), 3.85 (s, 3H), 3.44 – 3.39 (m, 1H), 3.34 – 3.26 (m, 1H), 3.18 – 2.94 (m, 2H), 2.92 – 2.83 (m, 1H), 2.02 – 1.84 (m, 2H), 1.78 (t, J = 2.1 Hz, 3H), 1.71 – 1.53 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.52, 162.22, 159.53, 152.04, 138.46, 133.25, 130.15, 129.32, 129.01, 128.52, 88.83, 80.25, 74.94, 52.66, 52.60, 51.80, 46.46, 43.75, 36.10, 27.44, 21.77, 3.60.; MS (ESI) *m/z*: 411.68 [M + H]$^+$.

*4.1.5. (R)-3-((3-(but-2-yn-1-yl)-4-(3-((tert-butoxycarbonyl)amino) piperidin-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)benzoic acid (**7**)*

To a solution of compound **5** (6 g, 9.4 mmol) in methanol (30 mL) was added LiOH (1 M, 23.5 mL), and then stirred at r.t. for 24 h. After the completed consumption of starting materials, the mixture was concentrated, the remainder was dissolved in water (130 mL) and acidified with HCl to adjust the pH value to 3. The precipitate was collected by filtration and washed with water. After drying at 50 °C for 10 h, compound **7** (5.2 g, 89%) was obtained as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.94 (s, 1H), 7.91 – 7.84 (m, 1H), 7.86 – 7.80 (m, 1H), 7.57 – 7.51 (m, 1H), 7.48 – 7.41 (m, 1H), 5.25 (s, 1H), 4.99 (s, 2H), 4.58 – 4.39 (m, 2H), 3.62 – 3.47 (m, 1H), 3.29 – 3.23 (m, 1H), 3.21 – 3.13 (m, 1H), 2.80 – 2.69 (m, 1H), 2.57 – 2.52 (m, 1H), 1.84 – 1.77 (m, 2H), 1.76 (t, J = 2.3 Hz, 3H), 1.65 – 1.55 (m, 1H), 1.39 (s, 9H), 1.37 – 1.32 (m, 1H). MS (ESI) *m/z*: 519.50 [M + Na]$^+$.

*4.1.6. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)benzoic acid hydrochloride (**8**)*

Using the procedure described for compound **6, 8** was prepared from compound **7**. White solid (109 mg, yield 85%). HPLC purity: 98.9%; mp: 196.8–198.2 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.97 (s, 1H), 8.42 (s, 3H), 7.86 (s, 1H), 7.83 (d, J = 7.7, 1.5 Hz, 1H), 7.54 (d, J = 7.7, 1.5 Hz, 1H), 7.45 (t, J = 7.7 Hz, 1H), 5.29 (s, 1H), 5.00 (s, 2H), 4.65 (d, J = 17.3 Hz, 1H), 4.52 – 4.42 (m, 1H), 3.40 – 3.35 (m, 2H), 3.09 (s, 1H), 2.99 (s, 1H), 2.95 – 2.87 (m, 1H), 2.01 – 1.94 (m, 1H), 1.92 – 1.87 (m, 1H), 1.78 (t, J = 2.4 Hz, 3H), 1.72 – 1.57 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.59, 162.24, 159.53, 152.05, 138.24, 132.79, 131.33, 129.10, 129.01, 128.66, 88.84, 80.27, 74.94, 52.61, 51.81, 46.46, 43.79, 36.09, 27.44, 21.84, 3.61. MS (ESI) *m/z*: 397.39 [M + H]$^+$.

*4.1.7. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl) benzamide hydrochloride (**10a**)*

To a solution of compound **5** (1.0 g, 2.0 mmol) in ethanol (10 mL) in a sealed tube was added ammonia (10 mL). The resulting solution was stirred at 80 °C for 12 h. After cooling to r.t., the reaction mixture was acidified with HCl to adjust the pH value to 5, the precipitate was separated by filtration to give the crude product, which was purified by chromatography (dichloromethane/MeOH 100:1–10:1) on silica gel to afford intermediate **9a**. The Boc group of **9a** was removed using a similar procedure for the preparation of compound **6** to give compound **10a** as white solid (500 mg, yield 65%). mp: 239.0–240.0 °C; HPLC purity: 96.0%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (s, 3H), 7.98 (s, 2H) 7.77 – 7.66 (m, 2H), 7.40 – 7.32 (m, 2H), 5.27 (s, 1H), 5.02 – 4.88 (m, 2H), 4.61 (d, J = 17.4 Hz, 1H), 4.51 – 4.37 (m, 1H), 3.41 – 3.32 (m, 1H), 3.31 – 3.21 (m, 1H), 3.13 – 2.92 (m, 2H), 2.91 – 2.77 (m, 1H), 2.01 – 1.85 (m, 2H), 1.75 (s, 3H), 1.69 – 1.50 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.46, 168.26, 162.24, 159.50, 152.08, 137.89, 134.85, 130.78, 128.63, 127.26, 88.83, 80.25, 75.00, 52.57, 51.84, 46.46, 43.93, 36.10, 27.42, 21.74, 3.62; MS (ESI) *m/z*: 418.19 [M + Na]$^+$.

*4.1.8. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-methylbenzamide (**10b**)*

Compound **7** (2 g, 4.0 mmol) and methylamine hydrochloride (271.9 mg, 4.0 mmol) were dissolved in acetonitrile. EDCI (1.16 g, 6.0 mmol), HOBt (816.4 mg, 6.0 mmol) and DIPEA (1.67 mL, 12.1 mmol) were added, the resulting mixture was stirred at r.t. until the completed consumption of starting materials. Acetonitrile was evaporated, the residue was dissolved in EtOAc (80 mL), and washed successively with 0.5 M of NaOH, 0.5 M of HCl and brine, dried over anhydrous Na_2SO_4. After removal of the solvent, the remainder was purified by chromatography (dichloromethane/methanol, 200:1–20:1) on silica gel to afford amide **9b**. Boc group of the **9b** was removed by using a similar procedure for the preparation of compound **6**, After the removal of the Boc group, the product was dissolved in water (20 mL). Then the mixture was neutralized with $NaHCO_3$, and extracted with dichloromethane (30 mL). The solvent was removed to to afford compound **10b** as white solid (1.4 g, 68%); HPLC purity: 96.7%; mp: 112.1–114.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (s, 1H), 7.73 (s, 1H), 7.69 (s, 1H), 7.39 (s, 2H), 5.21 (s, 1H), 4.98 (s, 2H), 4.49 (d, J = 5.8 Hz, 2H), 3.29 – 3.21 (m, 1H), 3.20 – 3.12 (m, 1H), 2.92 – 2.81 (m, 1H), 2.80–2.70 (m, 3H), 2.51 (s, 1H), 2.49 – 2.39 (m, 1H), 1.90 – 1.80 (m, 1H), 1.78 (s, 3H), 1.64 – 1.50 (m, 1H), 1.29 – 1.14 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.04, 162.28, 159.90, 152.24, 138.08, 135.17, 130.64, 128.69, 127.05, 126.09, 88.01, 80.15, 75.02, 58.93, 51.26, 47.67, 43.82, 36.06, 33.08, 26.70, 21.24, 3.55. MS (ESI) *m/z*: 410.55 [M + H]$^+$.

*4.1.9. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N,N-dimethylbenzamide (**10c**)*

Using the procedure described for compound **10b**, **10c** was prepared from compound **7** and dimethylamine hydrochloride. Light yellow solid (132 mg, yield 84%); HPLC purity: 95.8%; mp: 102.7–103.9 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.42 – 7.32 (m, 2H), 7.32 – 7.23 (m, 2H), 5.20 (s, 1H), 4.97 (s, 2H), 4.48 (s, 2H), 3.26 – 3.09 (m, 2H), 2.97 (s, 3H), 2.88 (s, 3H), 2.85 – 2.77 (m, 1H), 2.76 – 2.63 (m, 1H), 2.46 – 2.31 (m, 1H), 1.88 – 1.80 (m, 1H), 1.77 (s, 3H), 1.77 – 1.69 (m, 1H), 1.66 – 1.48 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.58, 162.23, 159.51, 152.04, 138.23, 132.78, 131.31, 129.10, 129.00, 128.65, 88.83, 80.27, 74.94, 52.59, 51.81, 46.45, 43.79, 36.08, 31.31, 27.43, 26.66, 21.76, 3.61; MS (ESI) *m/z*: 424.54 [M + H]$^+$.

*4.1.10. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-ethylbenzamide (**10d**)*

Following a similar procedure for the preparation of **10b**. **10d** was prepared starting from compound **7** and ethylamine. Light yellow solid (152 mg, yield 88%). HPLC purity: 97.6%; mp: 113.6–114.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.48 (t, J = 5.5 Hz, 1H), 7.74 (s, 1H), 7.71 – 7.66 (m, 1H), 7.47 – 7.32 (m, 2H), 5.20 (s, 1H), 4.98 (s, 2H), 4.57 – 4.38 (m, 2H), 3.30 – 3.25 (m, 2H), 3.25 – 3.13 (m, 2H), 2.87 – 2.76 (m, 1H), 2.76 – 2.61 (m, 1H), 2.48 – 2.32 (m, 1H), 1.92 – 1.80 (m, 2H), 1.78 (s, 3H), 1.75 – 1.71 (m, 1H), 1.65 – 1.48 (m, 1H), 1.11 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.31, 162.27, 159.91, 152.24, 138.04, 135.29, 130.57, 128.63, 127.16, 126.13, 87.95, 80.13, 75.02, 58.24, 51.21, 47.69, 43.81, 36.04, 34.49, 33.33, 23.39, 15.26, 3.54; MS (ESI) *m/z*: 424.55 [M + H]$^+$.

*4.1.11. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N,N-diethylbenzamide hydrochloride (**10e**)*

Following a similar procedure for the preparation of **10b**. **10e** was prepared starting from compound **7** and diethylamine hydrochloride. The Boc group was removed, the product was precipitated, and

separated by filtration to give compound **10e** as a white solid (112 mg, yield 84%). HPLC purity: 95.9%; mp: 178.0–180.2 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (s, 3H), 7.40 – 7.33 (m, 2H), 7.24 – 7.20 (m, 2H), 5.28 (s, 1H), 4.98 (s, 2H), 4.65 (d, J = 17.3 Hz, 1H), 4.51 – 4.42 (m, 1H), 3.44 – 3.32 (m, 5H), 3.18 – 3.07 (m, 2H), 3.03 – 2.95 (m, 1H), 2.94 – 2.86 (m, 1H), 2.02 – 1.94 (m, 1H), 1.94 – 1.87 (m, 1H), 1.78 (t, J = 2.3 Hz, 3H), 1.64 (t, J = 8.3 Hz, 2H), 1.19 – 1.09 (m, 3H), 1.04 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.20, 162.21, 159.50, 152.03, 137.89, 137.67, 128.94, 128.90, 125.62, 125.48, 88.87, 80.20, 74.96, 52.60, 51.81, 46.46, 43.80, 43.23, 39.14, 36.05, 27.43, 21.77, 14.44, 13.26, 3.60; MS (ESI) m/z: 452.53[M + H]$^+$.

4.1.12. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-propylbenzamide hydrochloride (10f)

Following a similar procedure for the preparation of **10e**. **10f** was prepared starting from compound **7** and propan-1-amine. White solid (126 mg, yield 88%). HPLC purity: 98.6%; mp: 169.8–171.8 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.49 (t, J = 5.6 Hz, 1H), 8.24 (s, 3H), 7.77 – 7.66 (m, 2H), 7.42 – 7.35 (m, 2H), 5.30 (s, 1H), 5.08 – 4.91 (m, 2H), 4.64 (d, J = 17.2 Hz, 1H), 4.56 – 4.36 (m, 1H), 3.34 – 3.25 (m, 2H), 3.25 – 3.16 (m, 2H), 3.14 – 2.95 (m, 2H), 2.95 – 2.80 (m, 1H), 2.03 – 1.84 (m, 2H), 1.78 (t, J = 2.4 Hz, 3H), 1.70 – 1.57 (m, 2H), 1.58 – 1.46 (m, 2H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.49, 162.23, 159.49, 152.06, 137.90, 135.33, 130.56, 128.65, 127.15, 126.20, 88.84, 80.25, 74.98, 52.58, 51.83, 46.45, 43.92, 41.43, 36.09, 27.43, 22.85, 21.55, 11.94, 3.61; MS (ESI) m/z: 438.39 [M + H]$^+$.

4.1.13. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-butylbenzamide hydrochloride (10g)

Following a similar procedure for the preparation of **10e**. **10g** was prepared starting from compound **7** and butan-1-amine. White solid (74 mg, yield 72%). HPLC purity: 96.5%; mp: 169.0–171.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.48 (m, 1H), 8.46 – 8.37 (m, 3H), 7.79 – 7.68 (m, 2H), 7.43 – 7.33 (m, 2H), 5.28 (s, 1H), 4.99 (s, 2H), 4.65 (d, J = 17.7 Hz, 1H), 4.46 (m, 1H), 3.43 – 3.35 (m, 2H), 3.33 – 3.22 (m, 2H), 3.12 – 3.05 (m, 2H), 2.95 – 2.86 (m, 1H), 2.01 – 1.87 (m, 2H), 1.78 (t, J = 2.3 Hz, 3H), 1.68 – 1.55 (m, 2H), 1.54 – 1.44 (m, 2H), 1.39 – 1.30 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.43, 162.21, 159.48, 152.05, 137.89, 135.32, 130.54, 128.62, 127.16, 126.19, 88.83, 80.24, 74.90, 52.59, 51.84, 46.46, 44.88, 43.92, 36.08, 27.42, 24.22, 20.13, 16.92, 14.20, 3.61; MS (ESI) m/z:452.42 [M + H]$^+$.

4.1.14. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-(tert-butyl)benzamide hydrochloride (10h)

Following a similar procedure for the preparation of **10e**. **10h** was prepared starting from compound **7** and *tert*-butylamine. White solid (86 mg, yield 79%). HPLC purity: 99.0%; mp: 192.0 °C − 194.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.25 (s, 3H), 7.77 (s, 1H), 7.71 – 7.65 (m, 2H), 7.39 – 7.29 (m, 2H), 5.30 (s, 1H), 5.04 – 4.93 (m, 2H), 4.64 (d, J = 17.6 Hz, 1H), 4.46 (d, J = 17.8 Hz, 1H), 3.41 – 3.35 (m, 1H), 3.34 – 3.28 (m, 1H), 3.12 – 2.98 (m, 2H), 2.95 – 2.81 (m, 1H), 2.04 – 1.84 (m, 2H), 1.79 (s, 3H), 1.72 – 1.54 (m, 2H), 1.37 (s, 9H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.69, 162.23, 159.48, 152.05, 137.69, 136.42, 130.28, 128.42, 127.29, 126.50, 88.85, 80.25, 74.98, 52.59, 51.82, 51.23, 46.45, 43.90, 36.07, 29.04, 27.41, 21.55, 3.62; MS (ESI) m/z: 452.61 [M + H]$^+$.

4.1.15. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-cyclopropylbenzamide hydrochloride (10i)

Following a similar procedure for the preparation of **10e**. **10i** was prepared starting from compound **7** and cyclopropanamine.White solid (84 mg, yield 77%). HPLC purity: 97.3%; mp:209.2 °C − 211.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.47 (d, J = 4.2 Hz, 1H), δ 8.40 (s, 3H), 7.72 (s, 1H), 7.71 – 7.67 (m, 1H), 7.40 – 7.35 (m, 2H), 5.28 (s, 1H), 4.98 (s, 2H), 4.65 (d, J = 17.1 Hz, 1H), 4.50 – 4.43 (m, 1H), 3.45 – 3.36 (m, 2H), 3.35 – 3.33 (m, 1H), 3.09 (s, 1H), 3.01 (s, 1H), 2.96 – 2.87 (m, 1H), 2.01 – 1.94 (m, 1H), 1.94 – 1.88 (m, 1H), 1.79 (d, J = 2.3 Hz, 3H), 1.65 (t, J = 8.1 Hz, 2H), 0.71 – 0.66 (m, 2H), 0.59 – 0.55 (m, 2H).^{13}C NMR (126 MHz, DMSO-d_6) δ 167.88, 162.23, 159.50, 152.07, 137.89, 135.04, 130.63, 128.62, 127.15, 126.28, 88.85, 80.26, 74.98, 52.60, 51.83, 46.46, 43.90, 36.09, 27.43, 23.52, 21.74, 6.18, 3.62; MS (ESI) m/z: 436.44 [M + H]$^+$.

4.1.16. Methyl 3-(bromomethyl)-2-fluorobenzoate (12a)

A mixture of methyl 2-fluoro-3-methylbenzoate (1 g, 6.0 mmol), NBS (1.3 g, 7.1 mmol) and di-benzoyl peroxide (BPO) (144.0 mg, 84.4 mmol) in 1,2-dichloroethane (5 mL) was heated to 80 °C, and stirred for 12 h. After cooling to r.t., the precipitated solid was separated by filtration. The filtrate was evaporated in vacuo and the residue was dissolved in ethers (15 mL) and washed with $NaHCO_3$ (2 N, 15 mL). The organic layer dried over Na_2SO_4, filtered and concentrated to give a crude product (1.2 g, 82%), which was used in the next step reaction without further purification.

4.1.17. tert-butyl (R)-(1-(3-(but-2-yn-1-yl)-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)piperidin-3-yl) carbamate (13)

Compound **3** (5 g, 25.2 mmol) was dissolved in DMF, then (*R*)-3-*N*-Boc-aminopiperidine (6.2 g, 30.1 mmol) and K_2CO_3 (10.4 g, 75.6 mmol) were added. The mixture was stirred at 60 °C for 10 h. Water was added, the precipitated solid was separated by filtration to give compound **13** as light yellow solid (8.3 g, 91%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (s, 1H), 5.20 (s, 1H), 4.71 – 4.59 (m, 1H), 4.52 – 4.40 (m, 1H), 3.94 – 3.83 (m, 1H), 3.38 – 3.24 (m, 1H), 3.17 – 3.04 (m, 1H), 3.01 – 2.82 (m, 1H), 2.79 – 2.62 (m, 1H), 1.97 – 1.86 (m, 2H), 1.84 (t, J = 2.3 Hz, 3H), 1.81 – 1.67 (m, 2H), 1.47 (s, 9H); MS (ESI) m/z: 385.43 [M + Na]$^+$.

4.1.18. Methyl (R)-3-((3-(but-2-yn-1-yl)-4-(3-((tert-butoxycarbonyl) amino)piperidin-1-yl)-2,6-dioxo- 3,6-dihydropyrimidin-1(2H)-yl)methyl)-4-fluorobenzoate (14a)

Following a similar procedure for the preparation of **4**. **14a** was prepared starting from compound **13** and **12a**. Compound **14a**, colorless oil (230 mg, 76%), which was used in the next step reaction without further purification.

4.1.19. (R)-3-((3-(but-2-yn-1-yl)-4-(3-((tert-butoxycarbonyl)amino) piperidin-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-2-fluorobenzoic acid (15a)

Following a similar procedure for the preparation of **7**. Compound **14a** was converted to compound **15a** as a white solid (2.4 g, yield 86%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 – 7.86 (m, 1H), 7.56 – 7.45 (m, 1H), 7.15 (t, J = 7.7 Hz, 1H), 5.32 (s, 1H), 5.25 (s, 2H), 4.74 – 4.61 (m, 1H), 4.56 – 4.47 (m, 1H), 3.92 – 3.80 (m, 1H), 3.46 – 3.28 (m, 1H), 3.21 – 3.06 (m, 1H), 2.95 – 2.83 (m, 1H), 2.78 – 2.60 (m, 1H), 2.02 – 1.86 (m, 2H), 1.82 (t, J = 2.6 Hz, 3H), 1.78 – 1.71 (m, 1H), 1.62 – 1.52 (m, 1H), 1.47 (s, 9H).

4.1.20. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)- 2-fluoro-N-methylbenzamide hydrochloride (17a)

Compound **15a** (1 g, 1.9 mmol) was dissloved in acetonitrile, methylamine hydrochloride (157.5 mg, 2.3 mmol), TCFH (817.9 mg, 6.0 mmol), NMI (816.4 mg, 6.0 mmol) were added, then the reaction mixture was stirred at r.t. After completed consumption of starting materials, the solvent was evaporated, and the residue was dissolved in EtOAc (80 mL) and washed successively with 0.5 M of NaOH, 0.5 M of HCl and brine, dried over anhydrous Na_2SO_4, and evaporated to give

crude product. The crude product was purified by chromatography (dichloromethane/methanol, 100:1 ~ 20:1) to afford amide **16a**. The Boc group of **16a** was removed by using a similar procedure for the preparation of compound **6** to afford compound **17a** as white solid (336 mg, yield 49%). HPLC purity: 95.6%; mp: 186.0–188.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.40 (s, 3H), 8.32 – 8.27 (m, 1H), 7.51 – 7.42 (m, 1H), 7.24 – 7.11 (m, 2H), 5.29 (s, 1H), 5.02 (s, 2H), 4.71 – 4.59 (m, 1H), 4.55 – 4.38 (m, 1H), 3.40 – 3.37 (m, 1H), 3.35 – 3.32 (m, 1H), 3.18 – 3.08 (m, 1H), 3.05 – 2.97 (m, 1H), 2.95 – 2.87 (m, 1H), 2.77 (d, J = 4.6 Hz, 3H), 2.03 – 1.87 (m, 2H), 1.79 (t, J = 2.3 Hz, 3H), 1.70 – 1.60 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.56, 162.09, 157.16 (d, J = 249.9 Hz), 156.18, 152.04, 130.45 (d, J = 4.4 Hz), 129.04 (d, J = 3.0 Hz), 125.13 (d, J = 15.4 Hz), 124.84 (d, J = 15.2 Hz), 124.56 (d, J = 3.8 Hz), 88.70, 80.29, 74.98, 52.59, 51.82, 46.46, 38.14, 36.23, 27.48, 26.69, 21.80, 3.64. MS (ESI) m/z: 428.32 $[M+H]^+$.

*4.1.21. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-ethyl-2-fluorobenzamide hydrochloride (**17b**)*

Following a similar procedure for the preparation of **17a**. **17b** was prepared starting from compound **15a** and ethylamine. White solid (136 mg, yield 47%). HPLC purity: 96.8%; mp: 169.5 °C − 171.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (s, 3H), 8.37 (t, J = 5.8 Hz, 1H), 7.52 – 7.37 (m, 1H), 7.23 – 7.13 (m, 2H), 5.29 (s, 1H), 5.11 – 4.92 (m, 2H), 4.66 (d, J = 17.4 Hz, 1H), 4.51 – 4.39 (m, 1H), 3.39 – 3.32 (m, 2H), 3.31 – 3.22 (m, 2H), 3.17 – 3.05 (m, 1H), 3.04 – 2.95 (m, 1H), 2.94 – 2.84 (m, 1H), 2.04 – 1.87 (m, 2H), 1.79 (t, J = 2.3 Hz, 3H), 1.70 – 1.58 (m, 2H), 1.11 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.90, 162.08, 159.62, 157.11 (d, J = 249.7 Hz), 152.03, 130.29 (d, J = 4.1 Hz), 128.95 (d, J = 3.2 Hz), 125.18 (d, J = 7.7 Hz), 125.05 (d, J = 7.8 Hz), 124.51 (d, J = 3.9 Hz), 88.70, 80.27, 74.97, 52.58, 51.82, 46.46, 38.13, 36.23, 34.49, 27.48, 21.80, 15.08, 3.64; MS (ESI) m/z: 442.48 $[M+H]^+$.

*4.1.22. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-4-fluoro-N-methylbenzamide hydrochloride (**17c**)*

Following a similar procedure for the preparation of **17a**. Compound **17c** was prepared starting from compound **15b** and methylamine hydrochloride**,** white solid (350 mg, 42%); HPLC purity: 97.1%; mp: 185.7–187.7 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.67 – 8.48 (m, 4H), 7.85 – 7.76 (m, 1H), 7.59 (dd, J = 7.4, 2.2 Hz, 1H), 7.27 (t, J = 9.2 Hz, 1H), 5.28 (s, 1H), 5.07 – 4.93 (m, 2H), 4.73 – 4.63 (m, 1H), 4.50 – 4.41 (m, 1H), 3.42 – 3.32 (m, 2H), 3.20 – 3.00 (m, 2H), 2.99 – 2.86 (m, 1H), 2.73 (d, J = 3.6 Hz, 3H), 2.04 – 1.84 (m, 2H), 1.77 (t, J = 2.3 Hz, 3H), 1.71 – 1.58 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.97, 162.15, 161.88 (d, J = 249.7 Hz), 152.04, 131.28 (d, J = 3.1 Hz), 128.41, 128.33, 128.29, 124.61 (d, J = 14.7 Hz), 115.57 (d, J = 22.1 Hz), 88.69, 80.30, 74.92, 52.53, 51.84, 46.48, 38.03, 36.23, 27.38, 26.66, 21.78, 3.60; MS (ESI) m/z: 428.53 $[M+H]^+$.

*4.1.23. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-ethyl-4-fluorobenzamide hydrochloride (**17d**)*

Following a similar procedure for the preparation of **17a**. **17d** was prepared starting from compound **15b** and ethanamine. White solid (139 mg, yield 45%). HPLC purity: 95.8%; mp: 183.2–185.2 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.55 (t, J = 5.5 Hz, 1H), 8.38 (s, 3H), 7.84 – 7.76 (m, 1H), 7.60 (dd, J = 7.4, 2.2 Hz, 1H), 7.28 (dd, J = 9.9, 8.6 Hz, 1H), 5.31 (s, 1H), 5.08 – 4.93 (m, 2H), 4.66 (d, J = 17.3 Hz, 1H), 4.51 – 4.38 (m, 1H), 3.38 – 3.31 (m, 2H), 3.29 – 3.20 (m, 2H), 3.16 – 3.00 (m, 2H), 2.97 – 2.88 (m, 1H), 2.01 – 1.85 (m, 2H), 1.78 (t, J = 2.3 Hz, 3H), 1.71 – 1.59 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.28, 162.13, 161.92 (d, J = 249.8 Hz), 159.62, 152.00, 131.44 (d, J = 3.0 Hz), 128.64 (d, J = 4.7 Hz), 128.25 (d, J = 9.1 Hz), 124.62 (d, J = 14.7 Hz), 115.51 (d, J = 22.3 Hz), 88.75, 80.34, 74.89, 52.58, 51.85, 46.44, 38.02, 36.18, 34.51, 27.40, 21.65, 15.24, 3.59; MS (ESI) m/z: 442.39 $[M+H]^+$.

*4.1.24. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-6-fluoro-N-methylbenzamide (**17e**)*

Following a similar procedure for the preparation of **17a**. **17e** was prepared starting from compound **15c** and methylamine hydrochloride. The Boc group was removed by using the procedure described for compound **10b.** Compound **17e**, white solid (350 mg, 42%); HPLC purity: 99.2%; mp: 185.7–187.7 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.23 (s, 1H), 7.52 (dd, J = 6.9, 2.3 Hz, 1H), 7.45 – 7.39 (m, 1H), 7.23 (dd, J = 10.4, 8.5 Hz, 1H), 5.19 (s, 1H), 4.93 (s, 2H), 4.51 – 4.43 (m, 2H), 3.25 – 3.20 (m, 1H), 3.19 – 3.10 (m, 2H), 2.86 – 2.78 (m, 1H), 2.76 (d, J = 4.5 Hz, 3H), 2.72 – 2.62 (m, 1H), 2.46 – 2.38 (m, 1H), 1.86 – 1.79 (m, 1H), 1.78 (t, J = 2.3 Hz, 3H), 1.76 – 1.68 (m, 1H), 1.61 – 1.52 (m, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.35, 162.19, 159.52, 158.65 (d, J = 248.0 Hz), 152.03, 134.00, 132.32 (d, J = 8.6 Hz), 129.87, 124.31 (d, J = 15.4 Hz), 116.45 (d, J = 23.0 Hz), 88.85, 80.28, 74.95, 52.59, 51.80, 46.45, 43.20, 36.09, 27.43, 26.71, 21.74, 3.60; MS (ESI) m/z: 428.17 $[M+H]^+$.

*4.1.25. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-N-ethyl-6-fluorobenzamide hydrochloride (**17f**)*

Following a similar procedure for the preparation of **17a**. **17f** was prepared starting from compound **15c** and ethanamine. White solid (154 mg, yield 49%). HPLC purity: 96.0%; mp: 180.3–182.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.39 (s, 3H), 8.31 (d, J = 2.5 Hz, 1H), 7.51 (dd, J = 6.9, 2.4 Hz, 1H), 7.46 – 7.39 (m, 1H), 7.22 (dd, J = 10.3, 8.5 Hz, 1H), 5.27 (s, 1H), 5.00 – 4.88 (m, 2H), 4.64 (d, J = 17.5 Hz, 1H), 4.51 – 4.38 (m, 1H), 3.41 – 3.30 (m, 2H), 3.28 – 3.22 (m, 2H), 3.12 – 2.96 (m, 2H), 2.93 – 2.78 (m, 1H), 2.00 – 1.85 (m, 2H), 1.78 (s, 3H), 1.71 – 1.58 (m, 2H), 1.10 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.69, 162.17, 159.50, 159.44 (d, J = 257.6 Hz), 152.01, 133.96 (d, J = 3.2 Hz), 132.17 (d, J = 8.6 Hz), 129.84 (d, J = 3.4 Hz), 124.63 (d, J = 15.5 Hz), 116.41 (d, J = 23.1 Hz), 88.85, 80.26, 74.94, 52.59, 51.80, 46.45, 43.19, 36.08, 34.53, 27.42, 21.72, 15.06, 3.60; MS (ESI) m/z: 442.34 $[M+H]^+$.

*4.1.26. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-4-chloro-N-methylbenzamide hydrochloride (**17g**)*

Following a similar procedure for the preparation of **17a**. **17g** was prepared starting from compound **15d** and methanamine hydrochloride. White solid (139 mg, yield 51%). HPLC purity: 97.9%; mp: 210.8 – 212.9 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.67 (d, J = 4.5 Hz, 1H), 8.63 – 8.49 (m, 3H), 7.77 (dd, J = 8.3, 2.1 Hz, 1H), 7.56 (d, J = 8.2 Hz, 1H), 7.42 (d, J = 1.8 Hz, 1H), 5.32 (s, 1H), 5.12 – 4.94 (m, 2H), 4.70 (d, J = 17.3 Hz, 1H), 4.54 – 4.40 (m, 1H), 3.45 – 3.34 (m, 2H), 3.21 – 3.00 (m, 2H), 3.01 – 2.90 (m, 1H), 2.73 (d, J = 4.2 Hz, 3H), 2.03 – 1.88 (m, 2H), 1.77 (s, 3H), 1.73 – 1.61 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.90, 162.20, 159.76, 152.10, 134.79, 134.78, 133.86, 129.64, 127.43, 126.13, 88.67, 80.34, 74.89, 52.56, 51.87, 46.48, 42.18, 36.35, 27.41, 26.69, 21.79, 3.63; MS (ESI) m/z: 444.31 $[M+H]^+$.

*4.1.27. (R)-3-((4-(3-aminopiperidin-1-yl)-3-(but-2-yn-1-yl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl)methyl)-4-chloro-N-ethylbenzamide hydrochloride (**17h**)*

Following a similar procedure for the preparation of **17a**. **17h** was prepared starting from compound **15d** and ethanamine. White solid (39 mg, yield 41%). HPLC purity: 96.8%; mp: 189.0 – 191.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.61 (t, J = 5.5 Hz, 1H), 8.30 (s, 3H), 7.76 (dd, J = 8.3, 2.2 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.41 (d, J = 2.0 Hz, 1H), 5.36 (s, 1H), 5.08 – 4.97 (m, 2H), 4.74 – 4.61 (m, 1H), 4.54 – 4.43 (m,

1H), 3.47 – 3.40 (m, 2H), 3.28 – 3.19 (m, 2H), 3.17 – 3.01 (m, 2H), 2.99 – 2.87 (m, 1H), 2.00 – 1.90 (m, 2H), 1.78 (t, J = 2.3 Hz, 3H), 1.71 – 1.61 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.24, 162.18, 159.72, 152.04, 134.85, 134.80, 134.00, 129.59, 127.20, 126.44, 88.73, 80.41, 74.83, 52.59, 51.88, 46.44, 42.11, 36.27, 34.54, 27.40, 21.54, 15.18, 3.59; MS (ESI) m/z: 458.35 $[M+H]^+$.

4.2. In vitro assay for inhibition of DPP-4, DPP-8, and DPP-9

According to the literature [30,31], human recombinant DPP-4, DPP-8 and DPP-9 enzymes (Sigma) were used for the assay. The fluorescent aminomethylcoumarin (AMC), released from a substrate Gly-Pro-AMC (Sigma) by DPP-4 hydrolyzation, was continuously monitored using an excitation wavelength of 360 nm and an emission wavelength of 460 nm every 1 min for 15 min using a BioTek microplate reader. The reaction contained different concentrations of the test compounds, 17.3 μU/μL enzyme, 10 μM Gly-Pro-AMC, and assay buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.12 mg/mL BSA) in a total reaction volume of 100 μL. And the IC_{50} data were calculated using the software GraphPad Prism 7. The inhibitory effect of selected compounds on DPP-8 and DPP-9 were determined by the same fluorometric method as the DPP4 assay system. The pH of the assay buffer for DPP-8 and DPP-9 was 8.0. The selective dose response of inhibition was tested at least in duplicate.

4.3. Molecular modeling

Docking studies were performed using Glide 5.9 in Schrödinger 2013 suite. The DPP-4 protein was extracted from RCSB Protein Data Bank (PDB ID: 2RGU). Ligands were built using Maestro build panel and prepared by the LigPrep application using default parameters. Protein structures were prepared using Maestro protein preparation wizard applying the default parameters. A docking grid was constructed by using the centroid of the bound ligand. Molecular docking of all molecules into the generated grid was performed by using the extra precision (XP) docking mode.

4.4. Cell viability assay

Cell viability was tested by MTT method. Hepatic LO2 cells were maintained in DMEM medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cell lines were grown at 37 °C in a 5% CO_2 atmosphere. Hepatic LO2 cells during logarithmic growth phase were seeded in 96-well microtiter plates at 1×10^4 cells per well. Cells were incubated in the presence of compounds **17d**, **17g**, and **17h** for 48 h. MTT dye (20 μL of 5 mg/mL in PBS) was added to each well 4 h prior to experiment termination. The supernatant was discarded without disturbing the formazan crystals and cells in the wells, while the MTT formazan crystals were dissolved in 150 μL of DMSO and the plates agitated on a plate shaker for 10 min. The optical density (OD) was read on a microplate reader (Molecular Devices, USA) with a wavelength of 490 nm.

4.5. Typical microsomal and plasma incubation procedures

Microsomal incubations were conducted as previously reported [32]. Testostrone was used as a positive control. Typically, 1 μM of test compounds were mixed with 1 mg/mL mouse liver microsomes (Meilunbio, Dalian, China) in 100 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$. After a 5 min preincubation at 37 °C, and the reactions (final volumes 600 μL) were started by the addition of NADPH (Sigma–Aldrich, St. Louis, MO, USA) at a final reaction concentration of 1.0 mM. Incubation mixtures without NADPH served as controls (with deactivated microsomes). The incubations were carried out for 0–60 min at 37 °C and were quenched by the addition of 300 μL of a ice-cold methanol with 0.5% of formic acid. Reaction mixtures were centrifuged (10 000 g, 10 min) to sediment the precipitated proteins and aliquots of the supernatants were injected onto LC-MS/MS system. The values for % remaining were analyzed in presence of NADPH. Control experiments were carried out under identical conditions but without protein.

The stability of tested compounds was evaluated in rat plasma, as previously reported [33]. Rat plasma was collected from adult male Sprague–Dawley rats and was stored at −20 °C. The tested compounds at concentration of 1 μM were incubated with rat plasma at 37 °C. A 100 μL of plasma was aliquoted from the incubation solution at 0, 5, 15, 30, 60, 120 min time points, then it was quenched by the addition of 300 μL of a ice-cold methanol with 0.5% of formic acid. Reaction mixtures were centrifuged (14000 RMP, 10 min) to sediment the precipitated proteins and aliquots of the supernatants were injected onto LC/MS/MS system.

4.6. In vivo oral glucose tolerance test (OGTT) in Kunming(KM) mice

All the animal studies were strictly performed according to the protocols issued by the Animal Research Committee and the Institutional Animal Care and Use Committee of Guangxi Medical University. Male KM mice (20 ± 2 g) were purchased from the Laboratory Animal Center of Guangxi Medical University (Nanning, China). The mice were housed at a temperature and humidity controlled environment with free access to food and water and a 12 h light–dark cycle. The mice were fasted overnight (12 h), weighed, bled via tail tip, and randomized into groups (n = 8). Mice were administrated orally with a single dose of vehicle (water solution), alogliptin (dissolved in the vehicle; 10 mg/kg) or tested compounds (dissolved in the vehicle; 10 mg/kg), subsequently dosed orally with glucose aqueous solution (3 g/kg) after 30 min. Blood samples were collected immediately before drug administration (−30 min), before glucose challenge (0 min), and at 15, 30, 60 and 120 min postdose. The blood glucose was measured by blood glucose test strips (SanNuo Changsha, China). Data and statistical analyses were performed using GraphPad version 7.00 (GraphPad Software, San Diego, CA, USA). General effects were analyzed using a one-way ANOVA with Dunnett's multiple comparisons test.

4.7. Pharmacokinetics assay in Sprague–Dawley rats

The pharmacokinetic parameters of tested compounds were evaluated in male SD rats (n = 3, 230 ± 20 g). After an overnight fasting, each animal received tested compounds by intravenous injection (5 mg/kg, dissolved in saline solution) or orally gavage (10 mg/kg, dissolved in purified water). Serial blood samples were collected into tubes containing EDTA at predose, 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h postdose. Approximately 300 μL of blood was collected and centrifuged at 10 000 rpm for 2 min. The obtained aliquots of plasma samples were stored at −20 °C until analysis. Plasma proteins was precipitated with two volumes of 0.5% formic acid/acetonitrile (v/v). After centrifugation (14,000 rpm, 10 min), the supernatants were analyzed via LC-MS/MS. PK parameters were estimated by DAS 2.0 software (China).

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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