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Structural optimization of pyrazolo[1,5-*a*]pyrimidine derivatives as potent and highly selective DPP-4 inhibitors

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ABSTRACT

Our previous discovery of pyrazolo [1,5-*a*]pyrimidin-7*(4H)*-one scaffold-based DPP-4 inhibitors yielded two potent compounds **b2** (IC_{50} = 79 nM) and **d1** (IC_{50} = 49 nM) but characterized by cytotoxicity. Herein, with scaffold hopping and fragment-based drug design strategies, highly potent and selective pyrazolo [1,5-*a*]pyrimidine DPP-4 inhibitors were found featured by reduced or diminished cytotoxicity. Specifically, **c24** (IC_{50} = 2 nM) exhibits a 25 to 40-fold increase of inhibitory activity respect to those of **b2** and **d1**, respectively, 2-fold from Alogliptin (IC_{50} = 4 nM), and remarkable selectivity over DPP-8 and DPP-9 (>2000 fold). Further docking studies confirmed that the pyrazolo [1,5-*a*]pyrimidine core interacts with the S1 pocket whereas its substituted aromatic ring interacts with the sub-S1 pocket. The interactive mode in this case resembles that of Alogliptin and Trelagliptin. Further *in vivo* IPGTT assays in diabetic mice demonstrated that **c24** effectively reduces glucose excursion by 48% at the dose of 10 mg/kg, suggesting that **c24** is worthy of further development as a potent anti-diabetes agent.

1. Introduction

Diabetes is a fast-growing chronic metabolic disorder. To date, 415 million people worldwide live with diabetes, and the number is expected to be 592 million by 2035. Type 2 diabetes mellitus (T2DM) accounts for more than 90% of all diabetic patients [1]. The complications of diabetes, such as coronary artery disease, hypertension, retinopathy and stroke, severely affect patients' quality of life and increase the risk of death.

Traditionally, there are five categories of oral antidiabetic drugs, biguanides, thiazolidinediones, sulfonylureas, meglitinides, and α-glucosidase inhibitors. These drugs were reported to have adverse events such as hypoglycemia, weight gain, gastrointestinal stress and cancer risk [2]. Recently, three new categories, DPP-4 inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonist and sodium-glucose co-transporter 2 (SGLT2) inhibitors were developed [3–6]. Nevertheless, despite their excellent regulation of blood glucose level, respective inconvenient administration and side effects restrict their compliance in patients. For example, GLP-1 receptor agonists need by injection and induce gastrointestinal stress [7], and SGLT2 inhibitors lower blood pressure and increase genital infection [8].

Among all categories of antidiabetic drugs, DPP-4 inhibitors have demonstrated their advantages in terms of mode-of-action, good patient compliance and tolerance [4]. While DPP-4 inhibitors have drawn great attention, some side effects, such as the elevated risk of cardiovascular death, heart failure, pancreatitis and pancreatic cancer were reported [9,10]. New design and development strategies were employed, resulting in Omarigliptin [11], Trelagliptin [12] and compound **1** [4] with low cytotoxicity and long-acting effect (Fig. 1).

Our research on DPP-4 inhibitors began with full structural analyses of DPP family binding sites and the preparation of two tool compounds **b2** and **d1** (Fig. 2) [13], that are structurally analogs of Alogliptin. While potent activities were achieved with **b2** (IC_{50} = 79 nM) [13] and **d1** (IC_{50} = 49 nM), *in vitro* cytotoxicity was introduced as well.

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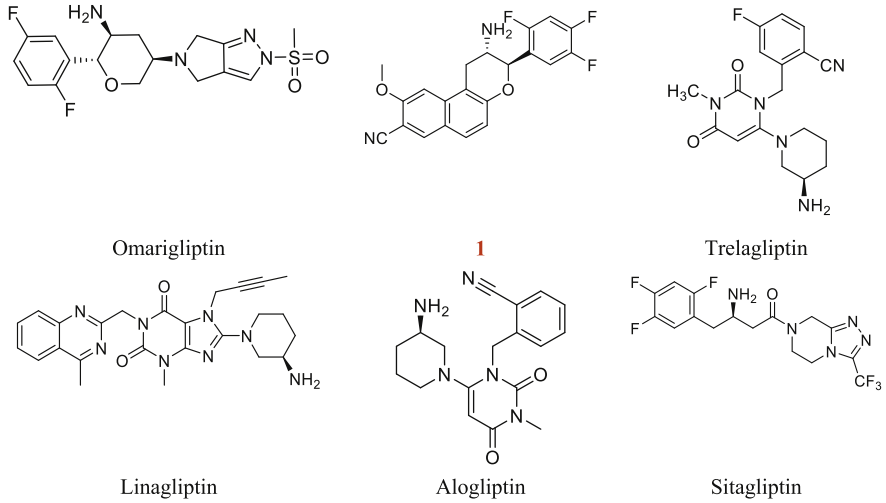


Fig. 1. Representative Potent DPP-4 inhibitors.

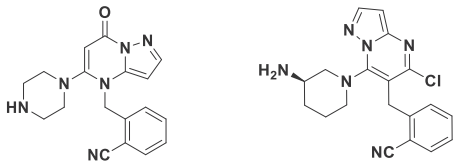


Fig. 2. Our Tool Compounds as DPP-4 inhibitors [13].

Further SAR studies on **b2**, **d1**, Trelagliptin and Alogliptin revealed that (R)-piperidin-3-amine and substituted benzyl groups are key components that impact DPP-4 inhibitory activity. Specifically, the substituted benzyl group is supposed to stretch into the S1 pocket, and (R)-piperidin-3-amine can bind with amino acid residues E205 and E206 (Fig. 3) [5]. Our preliminary results from these tool compounds also suggested that re-orientation of the core structure stabilizes ligand's conformation and improves the activity [13].

Herein we utilized the pyrazolo [1,5-*a*]pyrimidine core structure, and applied the scaffold hopping and fragment-based drug discovery strategies to design a series of new DPP-4 inhibitors. These ligands' structures featured in an amine stands at 5′ position and a substituted aromatic ring occupies 7' position (Fig. 3). We proposed that these novel chemical entities would remain the typical binding mode to DPP-4 [14–18], and generate inhibitory activity for DPP-4 and better selectivity over other DPP family members such as DPP-8 and DPP-9.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds is depicted in Schemes 1 and 2. Specifically, **1** was obtained by condensation and cyclization of 1*H*-pyrazol-5-amine and diethyl malonate. Chlorination of **1** with $POCl_3$ using *N, N′*-dimethyl-aniline base yielded the key intermediate **2**, which underwent condensation reactions with respective anilines or amines at ambient temperature to offer **3**. Substitution of **3** with (R)-piperidin-3-yl carbamate yielded **4** followed by deprotection giving **c1**-**c14** as the final product. The **c16** – **c25** was synthesized by substitution of **3** with piperazine. The **c27**

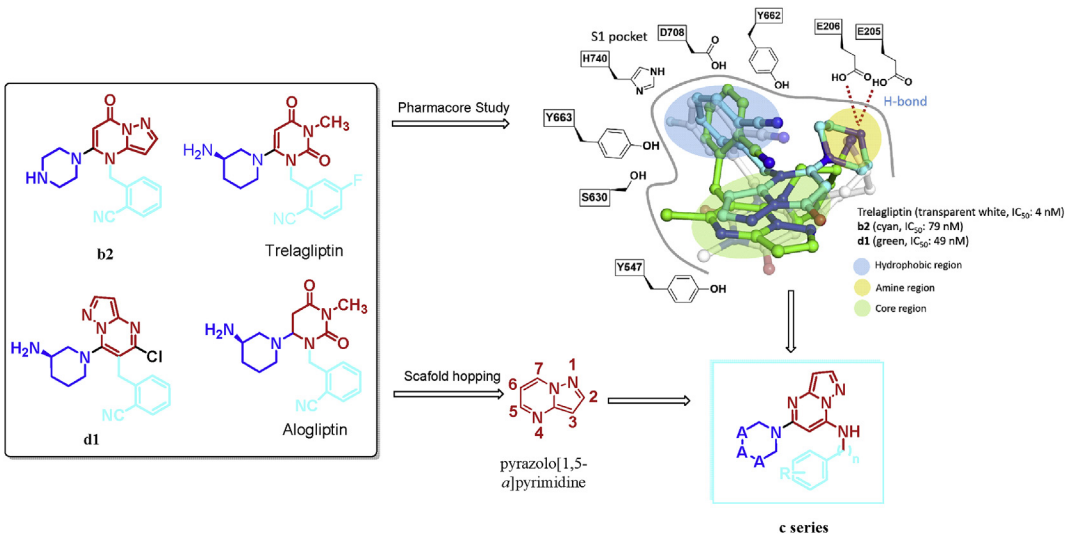
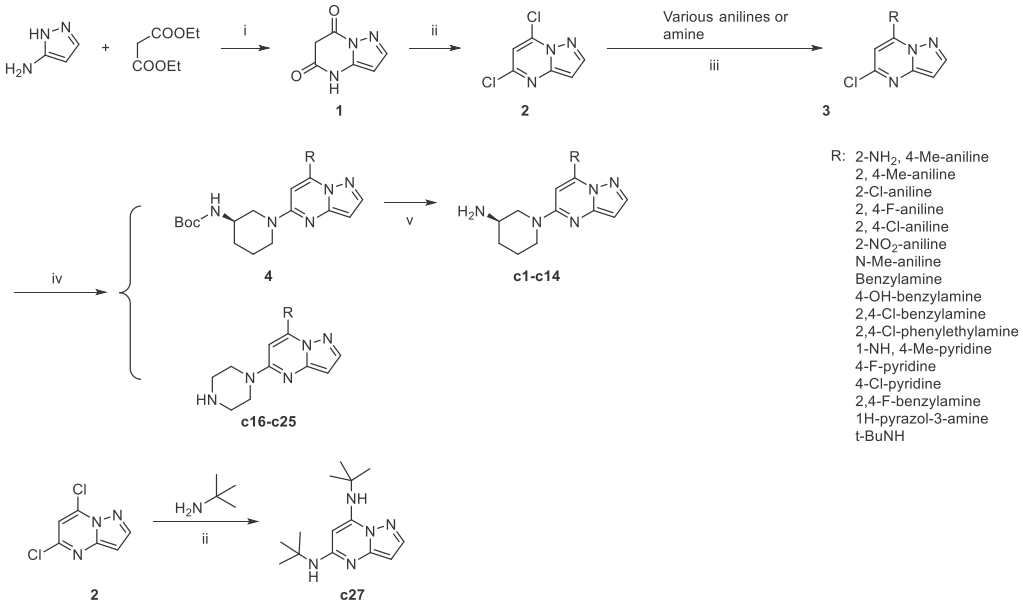
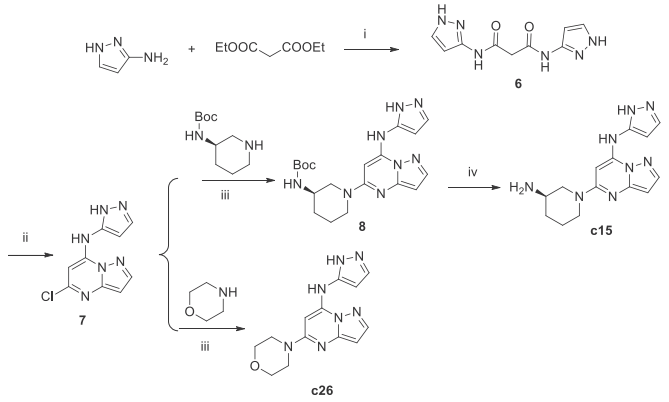


Fig. 3. Design of pyrazolo [1,5-*a*]pyrimidine derivatives as novel DPP-4 inhibitors based on known DPP-4 inhibitors and our previous DPP-4 inhibitors.



Scheme 1. i. *n*-Bu_3N, 130 °C; ii. $POCl_3$, *N,N′*-dimethyl-aniline, 100 °C; iii. TEA, *i*-PrOH or 60% NaH, DMF; iv. 1. *N,N′*-dimethyl-aniline, DMF, 90 °C; v. TFA, DCM.



Scheme 2. i. 130 °C, neat; ii. $POCl_3$, *N,N′*-dimethyl-aniline, 100 °C; iii. *N,N′*-dimethyl-aniline, DMF, 90 °C; iv. TFA, DCM.

was constructed by substitution of **2** with 2 equivalents of t-butylamine.

Compounds **c15** and **c26** were synthesized via a slightly different path, as shown in Scheme 2. 1*H*-pyrazol-5-amine and *N, N′*-dimethylaniline were dissolved in diethyl malonate and heated to 110 °C overnight to yield the diamide **6**. Treating **6** with $POCl_3$ at 90 °C for 3 h led to **7** by chlorination and cyclization process. Subsequently, the substitution of **7** with (R)-piperidin-3-yl carbamate yielded **8.8** was treated with TFA in DCM gave **c15**. **c26** was synthesized by substitution of **7** with morpholine.

2.2. Bioassay and SAR analyses

The DPP-4 inhibitory activity of all compounds was examined on a human DPP-4 enzyme derived from Caco-2 cells according to the previously reported method [13]. The results are shown in Table 1.

To understand the structure-activity relationship (SAR), we focused on 1) the hydrophobic region as aniline, benzylamine, aromatic heterocycles and tert-butylamine, 2) the amine region as (R)-piperidin-3-amine, tert-butylamine, piperazine and morpholine and 3) the linker between the hydrophobic region and core region.

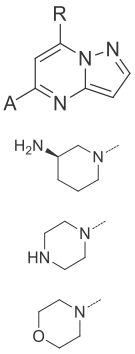
The SAR analysis revealed that in the hydrophobic region, substituted aromatic rings such as aniline and benzylamine are very important for the activity. Ligands with electron donating groups (EDG) exhibit better activities than those with electron withdrawing groups (EWG), such as **c1** (1.2 μM) and **c2** (9.1 μM) vs. **c3** (25.3 μM), **c5** (22.2 μM). The hydrogen on the amino group is pivotal for the activity, because the introduction of methyl group on the amino group of aniline resulted in a dramatic decrease of DPP-4 inhibitory activity, e.g. **c1** (1.2 μM) and c16 (0.8 μM) vs. **c7** (>100 μM) and **c21** (80 μM). We proposed that the conformation restriction of the ligands is the reason why the *N*- methyl group leads to the decrease of the DPP-4 inhibitory activity. Ligands with aromatic heterocycles as substituted aminopyridines and 1H-pyrazol-3-amine exhibited moderate activity, compared with aniline and benzylamine, such as **c13** (14.4 μM), **c15** (31.8 μM) and **c26** (>100 μM).

The amine region is fundamental for the potency. The ligands with piperazine substitution exhibited better activity than those with (R)-piperidin-3-amine substitution, e.g. **c16** (0.8 μM) vs. **c1** (1.2 μM), **c19** (0.4 μM) vs. **c4** (17.7 μM), **c17** (1.6 μM) vs. **c2** (9.1 μM). The piperazine modification effectively boosted ligand activity up to 40 times (**c4**:17.7 μM vs. **c19**:0.4 μM). The amine with a free NH is critical to the activity, because the replacement of piperazine with morpholine or tert-butylamine led to the loss of activity, such as **c26** (>100 μM) and **c27** (>100 μM).

So far, the compounds exhibited excellent activity, but it is still far from our expectations. Subsequently, we compared the structures of **c1** and **d1** and realized that what distinguishes them is the position of substituent on the core structure and the distance between the aromatic group and core structure. We reasonably assumed that prolonging the distance between the aromatic group and the core structure with a linker may gain us more potent inhibitors. We designed and synthesized compounds **c8-c11** bearing a benzylamine group or phenylethylamine to test the hypothesis. Indeed, the length of the linker between the core and the aromatic ring holds a concrete and robust influence on inhibitory activity. For instance, about 4–5 times enhanced activity was observed by adding just one atom to the linker, such as **c5** (22.2 μM) vs. **c10** (5.7 μM) vs. **c11** (1.1 μM).

Thus far, we can draw out that the nature of the amine and the linker are key factors to enhance the inhibitory activity. So, we

Table 1
DPP-4 inhibitory activity (IC_{50}) of the pyrazolo [1,5-*a*]pyrimidine derivatives (c series).



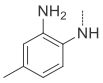
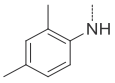
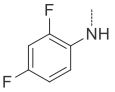
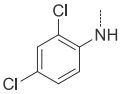
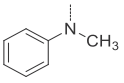
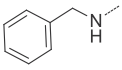
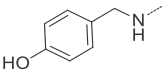
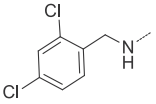
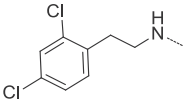
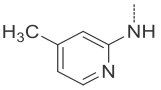
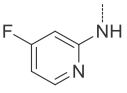
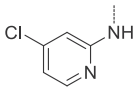
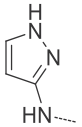
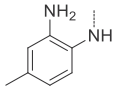
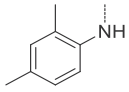
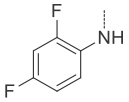
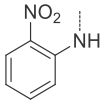
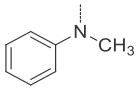
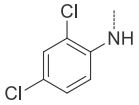
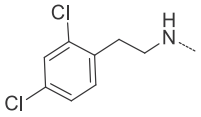
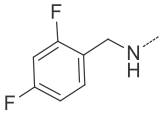
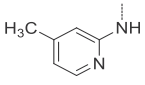
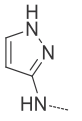
No.	Structures		DPP-4 IC_{50} (μM)
	A	R	
c1	A1		1.2 ± 0.5
c2	A1		9.1 ± 0.5
c3	A1		25.3 ± 3.5
c4	A1		17.7 ± 2.4
c5	A1		22.2 ± 3.1
c6	A1		11.7 ± 3.4
c7	A1		>100
c8	A1		0.3 ± 0.1
c9	A1		7.9 ± 0.3
c10	A1		5.7 ± 1.1
c11	A1		1.1 ± 0.2
c12	A1		8.1 ± 1.1
c13	A1		14.4 ± 3.2

Table 1 (*continued*)

No.	Structures		DPP-4 IC_{50} (μM)
	A	R	
c14	A1		2.4 ± 0.4
c15	A1		31.8 ± 1.3
c16	A2		0.8 ± 0.3
c17	A2		1.6 ± 0.1
c18	A2		23.6 ± 1.3
c19	A2		0.4 ± 0.2
c20	A2		3.5 ± 0.1
c21	A2		80.5 ± 4.4
c22	A2		0.47 ± 0.2
c23	A2		0.09 ± 0.01
c24	A2		0.002 ± 0.001
c25	A2		2.8 ± 0.5
c26	A3		>100
c27	*t*-BuNH		>100
Alogliptin			0.004 ± 0.001

Data are represented as mean ± SD ($n = 3$).

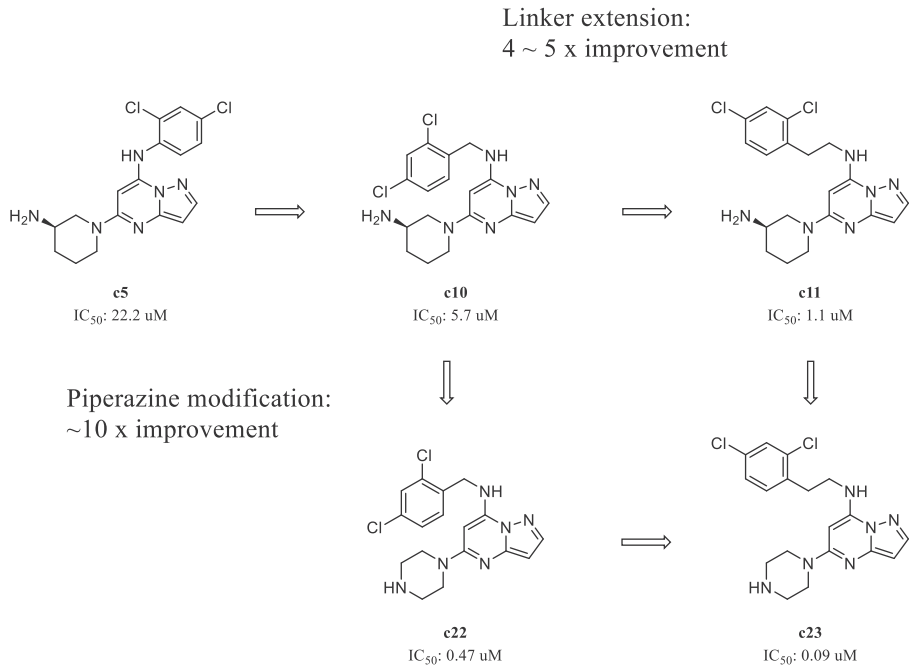


Fig. 4. Linker extension and piperazine modification strategy.

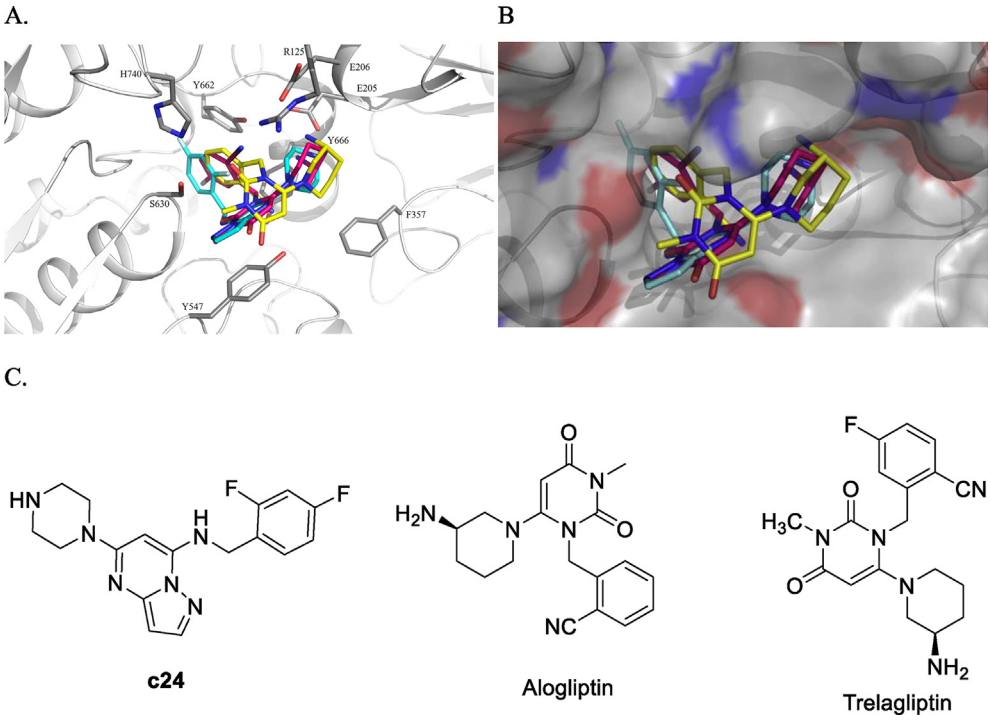


Fig. 5. A. B. Superimpose of Compound **c24** and Alogliptin and Trelagliptin in the active site of DPP-4. Compound **c24** is colored in cyan, Alogliptin is colored in violet and Trelagliptin is colored in yellow; DPP-4 is colored in white and shown as cartoon and surface. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

presume to design new compounds applying this new strategy, as shown in Fig. 4. The compounds with piperazine as amino and phenylethylamine as the aromatic ring should produce efficient inhibitory activity. This guided us to create c23 (0.09 μM), which comprised of piperazine and 2, 4-Cl phenylethylamine, and showed nearly 250-fold higher inhibitory activity than compound **c5** (22.2 μM) (Table 3).

Based on our SAR findings and design strategy which are summarized as piperazine modification and linker extension, redesigning of compound **c4** (17.7 μM) that possess (R)-piperidin-3-

Table 2
Selectivity assays of the selected compounds.

No.	DPP-4 (μM)	DPP-8 (μM)	DPP-8/DPP-4	DPP-9 (μM)	DPP-9/DPP-4
c1	1.2	>100	>100	>100	>100
c2	9.1	>100	>10	>100	>10
c3	25.3	>100	>4	>100	>4
c8	0.3	>100	>1000	>100	>1000
c9	7.9	>50	>6	>50	>6
c10	5.7	>100	>17	>100	>17
c11	1.1	>100	>100	>100	>100
c12	8.1	>100	>12	>100	>12
c14	2.4	>50	>20	>50	>20
c17	1.6	>100	>60	>100	>60
c19	0.4	>100	>200	>100	>200
c20	3.5	>100	>30	>100	>30
c22	0.47	>100	>200	>100	>200
c23	0.089	>100	>1000	>100	>1000
c24	0.002	>50	>2000	>50	>2000
c25	2.8	>100	>35	>100	>35
alogliptin	0.004	>50	>2000	>50	>2000

Table 3
IPGTT assays of compound **c24** in diabetic C57BL/6 mice.

	Blood glucose (mM)					AUC
	0 h	0.5 h	1 h	1.5 h	2 h	
Diabetes control	15.6 ± 2.4	33.6 ± 2.3	25.0 ± 1.2	23.9 ± 2.9	23.9 ± 0.7	51.2 ± 3.9
c24 intraperitoneal						
1 mg/kg	12.7 ± 0.7	25.7 ± 1.9	18.6 ± 2.6	17.9 ± 2.8	14.8 ± 1.0	38.0 ± 4.0
5 mg/kg	13.4 ± 1.1	23.4 ± 4.4	18.5 ± 1.9	17.4 ± 3.7	15.1 ± 1.0	36.7 ± 5.8
10 mg/kg	12.3 ± 0.3	16.2 ± 1.2	14.4 ± 0.1	13.0 ± 1.6	10.9 ± 1.5	27.1 ± 1.9
c24 oral						
10 mg/kg	15.8 ± 4.5	26.7 ± 4.5	19.1 ± 3.3	18.5 ± 3.3	15.6 ± 3.1	40.0 ± 4.1
Alogliptin						
10 mg/kg	15.3 ± 2.3	22.9 ± 3.7	17.4 ± 3.7	16.3 ± 2.1	15.0 ± 5.6	45.9 ± 3.8

Data are represented as mean ± SD ($n = 4$).

amine and 2,4-difluoroaniline as substitutions brought us to compound **c24** (Fig. 4). The later has a piperazine substitution at 5′ position and a 2,4-difluorobenzoylamine at 7' position. It has an explicit superior potency by 14,000-fold increase, presenting IC_{50} of 2 nM, 2-fold higher than Alogliptin.

Subsequently, compounds with IC_{50} value less than 10 μM were selected to perform the selectivity assay over DPP-8 and DPP-9 (Table 2). Data and results showed that the tested compounds showed excellent selectivity over the DPP-4 homologues proteins. Compound **c24** selectivity is heightened by 2000 times compared to Alogliptin over DPP-8 and DPP-9. Therefore, we selected this compound for the extensive *in vivo* bioassays in mice to test its potential to treat type 2 diabetes.

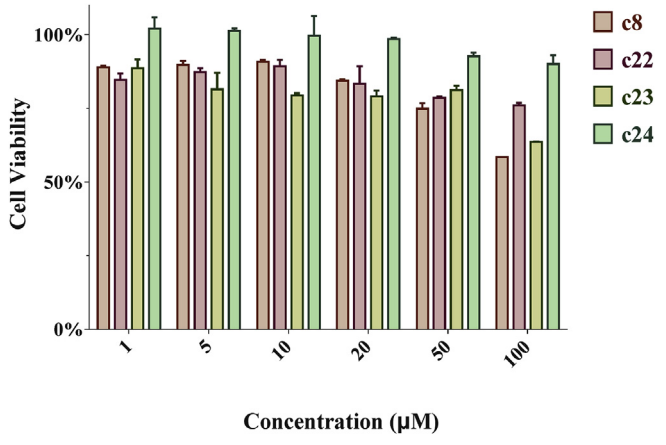


Fig. 6. Cytotoxicity effect of compounds **c8, c22, c23** and **c24** on HepG2 cells after 48 h is evaluated by MTT assay. Each value was presented as mean ± SD, n = 3.

2.3. Molecular docking analysis

The binding of **c24** in the DPP-4 active site generates a similar binding mode to those of Alogliptin and Trelagliptin (Fig. 5). The difluorobenzyl group occupies S1 pocket comprised of S630, Y662 and H740. Furthermore, the piperazine forms H-bond interaction with E205. The pyrazolo [1,5-*a*]pyrimidine core locates at the center of the active site where a π-π interaction with Y547 is formed to stabilize the binding conformation. Such binding conformation sheds light on the molecular mechanism of the potent inhibitory activity of **c24**.

2.4. Cytotoxicity bioassay

Previous studies clarified that pyrazolo [1,5-*a*]pyrimidin analogs could be developed as anti-cancer agents [19,20]. Consequently, we investigated the cytotoxicity of our synthetic ligands in order to recognize the compounds' safety. Cellular growth and viability of compounds **c8**, **c22**, **c23** and **c24** were performed on HepG2 cells using MTT assay. HepG2 cells were incubated with the inhibitors at varying concentrations for 48 h. As illustrated in Fig. 6, **c8**, **c22** and **c23** displayed a dose-dependent manner on cell viability. Interestingly, Compound **c24**, which is the most potent ligand in our study, presents very low cytotoxicity at concentrations ranging from 1 μM to 100 μM. Even when the dose raised to 100 μM, **c24** exhibits more than 90% cell viability, which suggests **c24** is a potent and safe ligand for further *in vivo* anti-hyperglycemic assays.

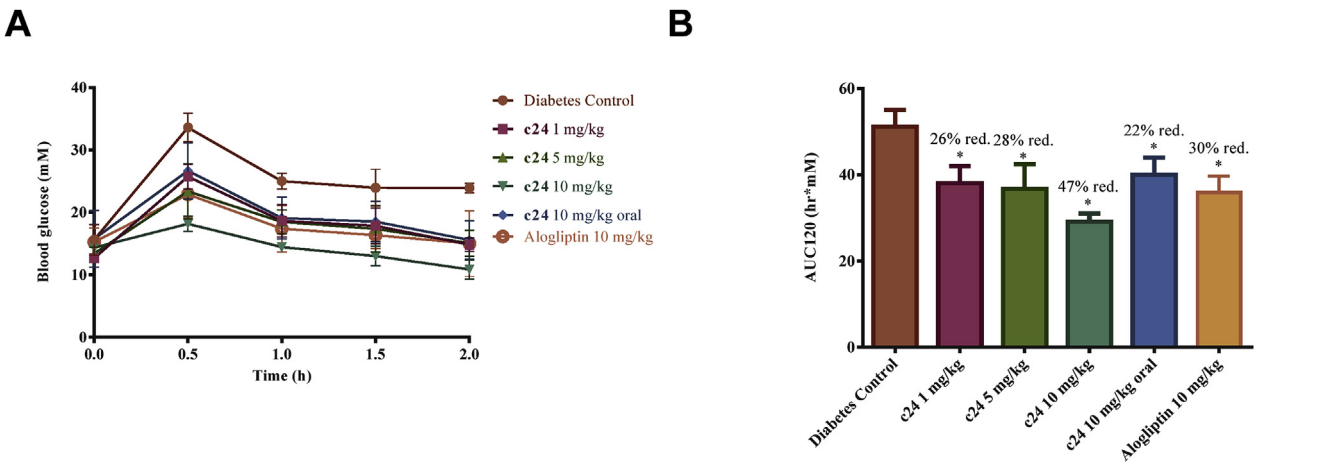


Fig. 7. A. Effects of compound **c24** in various concentrations and 10 mg/kg Alogliptin on glucose levels after IPGTT or OGTT in diabetic male C57BL/6 mice. B. The glucose AUC (diabetes C57BL/6 mice) was determined from 0 to 120 min. Percent reduction values for each treatment were generated from the AUC data normalized to the saline-challenged controls. Data are represented as mean ± SD ($n = 4$), *P < 0.05 versus negative control for 2 h, **P < 0.01 versus negative control for 2 h, one-way ANOVA followed by LSD post-test.

2.5. Glucose tolerance test (IPGTT and OGTT)

The *in vivo* anti-hyperglycemia effects of compound **c24** were evaluated by performing intraperitoneal glucose tolerance test (IPGTT) in high-fat diet-fed/streptozotocin (HFD/STZ) induced T2DM male C57BL/6 mice following our previously reported method [13]. A single dose of compound **c24** administered to male C57BL/6 mice 30 min prior to an IPGTT reduced plasma glucose excursion in a dose-dependent manner from 1 mg/kg to 10 mg/kg. Alogliptin was used as a positive control. The results are shown in Table 3 and Fig. 7. Compound **c24** reduced the area under the curve from 0 to 120 min $(AUC)_{0\text{-}120\ min}$ to 43% in diabetic C57BL/6 mice, which transcended the effect of Alogliptin (30% reduction, Fig. 6) at the same dose. We also performed OGTT with 10 mg/kg **c24** to evaluate the oral effectiveness of the ligand in reducing blood glucose. Oral administration of **c24** induced an AUC reduction of 22% which indicated a significant blood glucose lowering effect.

2.6. Anti-hyperglycemic bioassays in HFD/STZ induced T2DM mice

Based on the DPP-4 inhibitory activities and selectivity, compound **c24** was selected for the chronic effects experiment. The chronic effects of compound **c24** were investigated in HFD/STZ induced type 2 diabetic male C57BL/6 mice with a single dose (10 mg/kg/day) for 14 days, with Alogliptin as a positive control. The results are depicted in Fig. 8. Compound **c24** significantly decreased the blood glucose levels compared to the diabetic control group on the 14th day of treatment (46% reduction) while Alogliptin managed to lower the blood glucose by 57% compared with the control group. During the starting 5 days, the decrease of blood glucose is not significant in either the **c24** group or Alogliptin, then the gradual decline was observed.

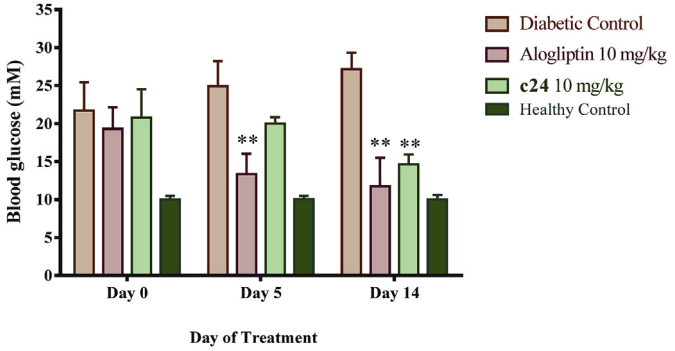


Fig. 8. Effects of compound **c24** (10 mg/kg, single dose) in plasma glucose levels in chronically treating HFD/STZ mice for 14 days. Data are presented as means ± SD of 4 mice. *P < 0.05 versus diabetic control for 14 days, **P < 0.01 versus diabetic control for 14 days, one-way ANOVA followed by LSD post-test.

3. Conclusion

In conclusion, we have designed, synthesized, and evaluated a series of novel pyrazolo [1,5-*a*]pyrimidine derivatives as potent and selective DPP-4 inhibitors which had a similar binding mode as Alogliptin to DPP-4. Compound **c24** demonstrates excellent *in vitro* DPP-4 inhibitory activity with IC_{50} of 2 nM, high selectivity over DPP-8 and DPP-9 and low cytotoxicity profile. Compound **c24** also possessed good *in vivo* efficacy in IPGTTs in healthy and diabetic mice, and demonstrated its capability to be a potential antidiabetic agent.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112850.

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