Design, synthesis, and biological evaluation of novel piperidine-4-carboxamide derivatives as potent CCR5 inhibitors

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · NOVEMBER 2013

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2013.11.013 · Source: PubMed

9 AUTHORS, INCLUDING:

Xin Xie
Chinese Academy of Sciences
80 PUBLICATIONS **1,371** CITATIONS

SEE PROFILE

European Journal of Medicinal Chemistry 71 (2014) 259–266

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis, and biological evaluation of novel piperidine-4-carboxamide derivatives as potent CCR5 inhibitors

Suwen Hu [a], Quan Gu [a], Zhilong Wang [b], Zhiyong Weng [a,1], Yunrui Cai [a], Xiaowu Dong [a], Yongzhou Hu [a], Tao Liu [a,*], Xin Xie [b,**]

[a] ZJU-ENS Joint Laboratory of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China
[b] State Key Laboratory of Drug Research, National Center for Drug Screening, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 5 May 2013
Received in revised form
28 October 2013
Accepted 9 November 2013
Available online 19 November 2013

Keywords:
CCR5 inhibitor
Anti-HIV-1 agent
Piperidine-4-carboxamide derivatives

ABSTRACT

Based on a putative 'Y shape' pharmacophore model of CCR5 inhibitors, a series of novel piperidine-4-carboxamide derivatives were designed and synthesized using a group-reverse strategy. Among synthesized target compounds, **16g** (IC_{50} = 25.73 nM) and **16i** (IC_{50} = 25.53 nM) showed equivalent inhibitory activity against CCR5 to that of the positive control maraviroc (IC_{50} = 25.43 nM) in calcium mobilization assay. Selected compounds were further tested for their antiviral activity in HIV-1 single cycle assay. Two compounds, **16g** and **16i**, displayed antiviral activity with IC_{50} values of 73.01 nM and 94.10 nM, respectively. Additionally, the pharmacokinetic properties and inhibitory potency against hERG of **16g** were evaluated, providing a foundation for ongoing optimization.

1. Introduction

The unfolding of the close connection between chemokine receptor 5 (CCR5) and HIV-1 provides a better understanding about how HIV-1 enters human cells [1]. CCR5, one of the seven-transmembrane G-protein coupled receptors, plays an intimate role in the R5-tropic HIV-1 entry process by serving as a critical co-receptor for the viral envelope protein glycoprotein-120 (gp120) [2,3]. Homozygous individuals with the CCR5 delta 32 allele deletion do not express the functional receptor and are ultimately resistant to R5-tropic HIV-1 infection, while heterozygotes are prone to be infected but with a slowed progress toward HIV-1 [4]. These facts have stimulated a great amount of efforts in identifying new CCR5 inhibitors, among which include several clinic candidates (TAK-220 in Phase I, **1**, TBR-652 in Phase II, **2**, Fig. 1) and one FDA approved-CCR5 inhibitor, maraviroc (UK-427,857, **3**, Fig. 1) [5–7]. Recently, Dong and co-workers synthesized a novel series of 1,4-disubstituted piperidine/piperazine derivatives, among which compound **B07** (**4**, Fig. 1) exhibited anti-HIV activity with an IC_{50} value of 6.17 nM [8]. Although with the emergence of these encouraging results, various challenges associated with CCR5 inhibitors such as viral tropism, drug resistance and possible long term adverse events still exist. Therefore, current studies have been focused on the development of second generation CCR5 inhibitors with improved properties.

In our previous work, we have designed and synthesized a series of novel piperazine derivatives based on a 'Y shape' pharmacophore model, which contains one basic center, three hydrophobic domains, and an amide linker. The results obtained from CCR5 inhibitory assay were not very satisfactory with inhibitory activities (IC_{50} value) for most compounds varied from several hundred nanomolar to low micromolar [9]. During our continuing searching for more potent compound, an interesting structure **5** disclosed by Long et al. [10], which showed potent inhibitory effect on RANTES-stimulated [^{35}S]-GTPγS binding to CCR5-expressing CHO cell membranes (IC_{50} = 7.00 nM), attracted our attention. A novel series of piperidine-4-carboxamide derivatives were then designed through the utilization of a group-reverse strategy by replacing the piperidin-4-amine moiety of **5** with a piperidine-4-carboxamide scaffold. Evaluation of CCR5 inhibitory activity revealed that the obtained piperidine-4-carboxamide derivative **6** (IC_{50} = 525.00 nM) and lead compound **5** (IC_{50} = 251.67 nM) shared equivalent potency against CCR5 in a calcium mobilization assay (Fig. 2).

* Corresponding author. Tel.: +86 571 88208458; fax: +86 571 88981051.
** Corresponding author. Tel./fax: +86 21 50801313.
E-mail addresses: Lt601@zju.edu.cn, lt601@163.com (T. Liu), xxie@mail.shcnc.ac.cn (X. Xie).
[1] Current address: Hangzhou No. 2 High School of Zhejiang Province, Hangzhou, Zhejiang, China.

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.11.013

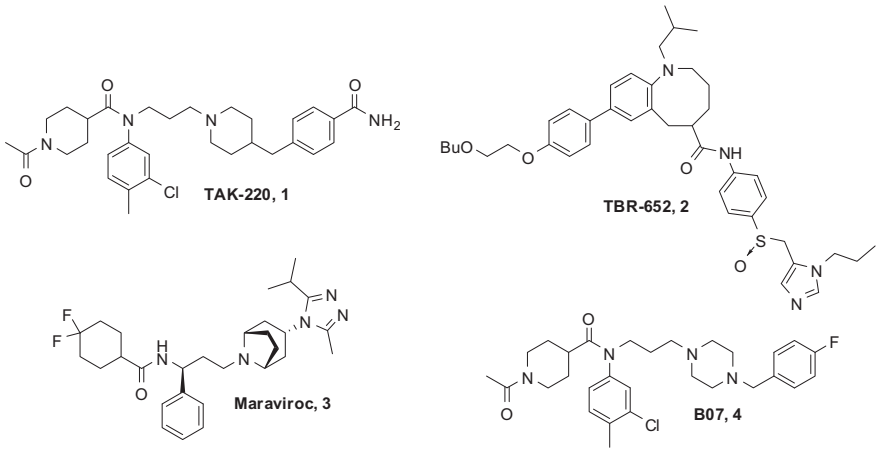


Fig. 1. Structures of TAK-220, TBR-652, maraviroc, and **B07**.

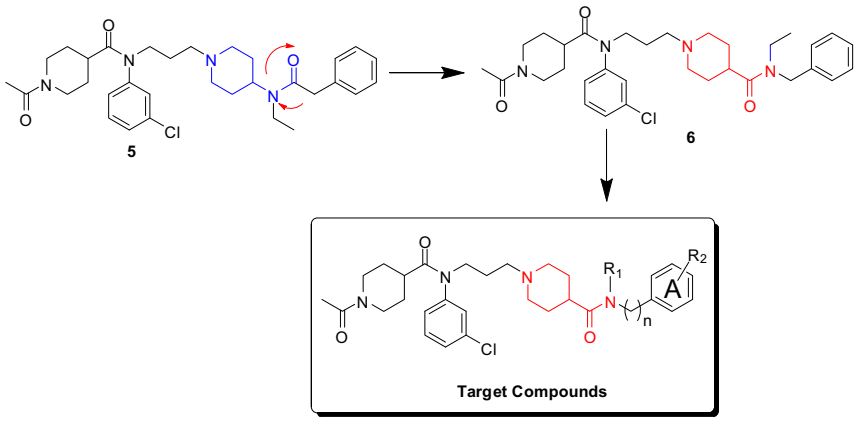


Fig. 2. Design of the target compounds.

In order to explore the structure–activity relationship of these novel piperidine-4-carboxamide derivatives, we firstly modified the phenyl ring **A** of the target compounds with a variety of other functionalities. Then the nitrogen atom of the amide was substituted with different alkyl groups (**16b**–**j**). Thirdly, the length of the linker between the phenyl ring and nitrogen atom of the amide was changed from null to two-methylene units (**16k**–**r**) to determine an optimal linker. Herein, we report the design, synthesis, and biological evaluation of a series of novel piperidine-4-carboxamide derivatives, as well as the antiviral activity of representative compounds, to develop novel compounds as potent CCR5 inhibitors for HIV.

2. Results and discussion

2.1. Chemistry

Target compounds were synthesized as outlined in Scheme 1. The readily available material piperidine-4-carboxylic acid **7** was firstly converted to 1-acetylpiperidine-4-carboxylic acid **8** according to a reported method with minor modification [11]. Then reaction of **8** with thionyl chloride gave chloride **9** [12]. Subsequent *N*-alkylation of 3-chloroaniline **10** with 1-bromo-3-chloropropane in the presence of potassium iodide under microwave conditions afforded substituted aniline **11**. Acylation of **11** with chloride **9** gave amide **12** [13].

Besides, *N*-Boc-4-piperidinecarboxylic acid was added to a solution of the different substituted amines **13a**–**r** at room temperature to afford amides **14a**–**r**, which were de-protected with 6 N HCl to yield the corresponding intermediates **15a**–**r**. **15a**–**r** were then reacted with amide **12** in the presence of potassium iodide and potassium carbonate to afford target compounds **6** and **16b**–**r**.

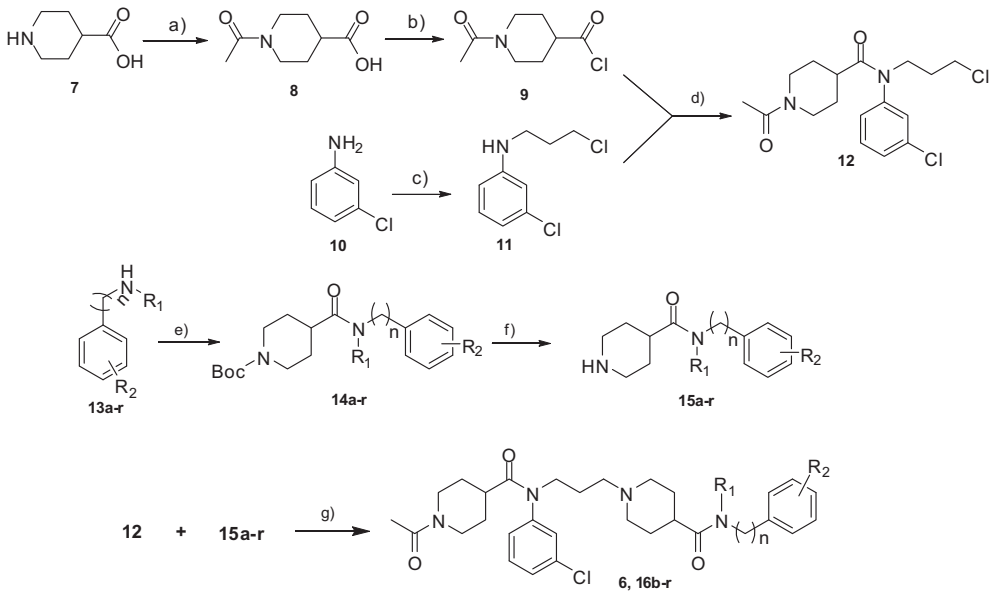
2.2. Cell cytotoxicity assays

A total of 18 novel piperidine-4-carboxamide derivatives (**6** and **16b**–**r**) were tested in CCK-8 assay, showing no significant cytotoxicity against HEK293 cells at a concentration of 50 μM. The purity of all compounds used for biological test was more than 95%.

2.3. Calcium mobilization assay

All compounds have been evaluated by calcium mobilization assay for their CCR5 inhibitory activity with maraviroc employed as the positive control, the results of which were summarized in Table 1.

Compound **6**, with the IC_{50} value of 525.00 nM, was obtained via inverting the amide functionality that tethered to the terminal benzene ring of **5**. Provided its moderate CCR5 inhibitory activity, **6** was employed as a starting point for further optimization to pursue compounds with more attractive potency and probe SARs associated with its derivatives. As an initial step for our structural



Scheme 1. Synthesis of target compounds **6** and **16b**–**r**. Reagents and conditions: a) Ac_2O, DCM, rt, 12 h; b) $SOCl_2$, rt, 30 min; c) 1-bromo-3-chloropropane, KI, MeCN, microwave reactor, 110 °C, 15 min; d) Et_3N, DCM, rt, 5 h; e) *N*-Boc-4-piperidinecarboxylic acid, EDC·HCl, DCM; f) 6 N HCl, EtOAc, rt, 2 h; g) K_2CO_3, KI, MeCN, reflux, 48 h.

derivatization, a variety of substituents were introduced to the 4-position of the phenyl ring **A** while maintaining the ethyl substitution on the nitrogen of the inversed amide functionality (**16b**–**e**). As displayed in Table 1, replacement of the 4-position with methyl group (**16b**, IC_{50} = 202.43 nM) only resulted in a slight improvement in activity compared to **6**. Subsequently, halogen atoms, including fluorine and chloride, were employed as the C-4 substituents of the terminal benzene ring **A**. Between the attained compounds, known as **16c** (IC_{50} = 320.33 nM) and **16d** (IC_{50} = 93.53 nM), the latter one exhibited a 6-fold enhancement in activity by contrast to **6**, indicating that the chloro substitution was more beneficial for CCR5 inhibitory activity. Afterward, structural alterations were examined at the nitrogen of the inversed amide functionality to generate the optimum **R1** replacement. Upon changing the ethyl substitution of **6** into methyl substitution, a 9-fold improvement in activity was obtained by compound **16f** (IC_{50} = 64.07 nM). Encouraged by this observation, we prepared compound **16g** via removal the R1 replacement, which exhibited comparable activity (IC_{50} = 25.73 nM) to the positive control maraviroc. On the insight that the amide functionality without substitution on the nitrogen atom was preferable for potency, we subsequently investigated compounds containing distinct replacement at the 4-position of the phenyl ring **A** on the basis of **16g**. As a consequence, most of the target compounds were evaluated to be more favorable in activity than **6**. Particularly compounds **16g** (IC_{50} = 25.73 nM) and **16i** (IC_{50} = 25.53 nM) which showed equivalent CCR5 inhibitory activity to maraviroc. To this point, it became evident that the inversed amide group without substitution on the nitrogen was optimum for activity.

Further modification was focused on exploring the length of the alkyl linker in order to optimize the spacing between the nitrogen atom of the amide group and the phenyl ring **A** of the target compounds. As shown in Table 1, compounds with a CH_2 (n = 1) linker (**16g** and **16i**) demonstrated better activity while shortening the linker by a carbon (n = 0) caused a loss in potency (**16k**, IC_{50} > 1000.00 nM; **16l**, IC_{50} > 1000.00 nM). Similarly, lengthening the linker by a carbon atom (n = 2) also resulted in a loss of potency, as testified by the comparison of **16g** with **16m**. Moreover, substitution of the 4-position of the phenyl ring **A** with halogen atom (F, Cl) afforded compound **16n** (IC_{50} = 196.90 nM) and **16o** (IC_{50} = 86.70 nM), whose activities were at least 2-fold less potent than compounds **16g** and **16i**, respectively. These experimental results indicated that the longer linker (n = 2) was not preferable compared to the linker with one methylene group (n = 1). In addition, similar results, decrease of activity comparable to compounds **16g** and **16i**, were observed when ethyl group was introduced to the nitrogen atom of compounds **16m**–**o** to afford compound **16p** (IC_{50} = 26.37 nM), **16q** (IC_{50} = 151.80 nM), and **16r** (IC_{50} = 125.81 nM), respectively. To this point, it was clear that compounds with the linker of one methylene displayed better activities than that of compounds with linker of none or two methylenes.

2.4. Viral infectivity assays

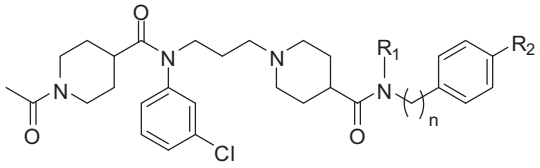
In order to assess the activities of target compounds for inhibition of virus infectivity, the most potent compounds (**16g** and **16i**) were selected for further anti-HIV-1 single cycle antiviral assay. The results are summarized in Table 2. The tested compounds showed nanomolar inhibitory activities (**16g**, IC_{50} = 73.01 nM and **16i**, IC_{50} = 94.10 nM, respectively).

2.5. Evaluation of pharmacokinetic property and cardiovascular safety (hERG) of *16g*

Given that compound **16g** was easily synthesized and exhibited good anti-HIV-1 activity and no cytotoxicity at the concentration of 50 μM, it was selected for further evaluation. The pharmacokinetic properties of **16g** in rat are shown in Table 3. This compound displayed a large volume of distribution after oral administration ($Vdss_{iv}$ = 1.23 L kg^{-1} and $Vdss_{po}$ = 8.99 L kg^{-1}), moderately clearance (Clp_{iv} = 1.25 L h^{-1} kg^{-1} and Clp_{po} = 8.86 L h^{-1} kg^{-1}), a low blood level (AUC_{0-24iv} = 3.25 mg L^{-1} h and AUC_{0-24PO} = 3.13 mg L^{-1} h) and a short half-life ($t_{iv1/2}$ = 0.74 h and $t_{po1/2}$ = 0.71 h), and the oral bioavailability in rats was 15.00%.

Meanwhile a patch clamp assay for **16g** was also conducted. Disappointingly, it exhibited 15.20% inhibition of the hERG potassium current at a concentration of 3 μM.

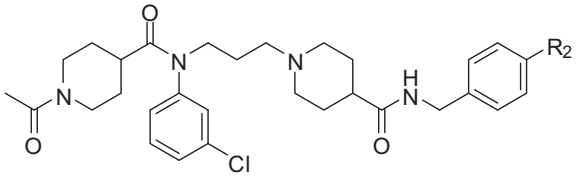
Table 1
CCR5 inhibitory activity of compounds **6** and **16b**–**r** by calcium mobilization assay.



Compd.	R_1	R_2	*n*	IC_{50} (nM)[a]
6	CH_3CH_2	H	1	525.00 ± 191.85
16b	CH_3CH_2	CH_3	1	202.43 ± 111.63
16c	CH_3CH_2	F	1	320.33 ± 169.36
16d	CH_3CH_2	Cl	1	93.53 ± 30.98
16e	CH_3CH_2	CN	1	473.33 ± 155.73
16f	CH_3	H	1	64.07 ± 14.73
16g	H	H	1	25.73 ± 3.85
16h	H	F	1	35.47 ± 5.67
16i	H	Cl	1	25.53 ± 5.84
16j	H	CN	1	75.30 ± 23.86
16k	H	H	0	>1000
16l	CH_3CH_2	H	0	>1000
16m	H	H	2	76.03 ± 12.60
16n	H	F	2	196.90 ± 32.85
16o	H	Cl	2	86.70 ± 6.95
16p	CH_3CH_2	H	2	26.37 ± 8.43
16q	CH_3CH_2	F	2	151.80 ± 25.72
16r	CH_3CH_2	Cl	2	125.81 ± 21.87
Positive control	Maraviroc			25.43 ± 1.35

[a] Values are means of three experiments.

Table 2
Antiviral activity of two promising compounds.



Compd.	R_2	IC_{50}, nM
16g	H	73.01
16i	Cl	94.10
Maraviroc		1.10

3. Conclusions

In conclusion, a series of novel piperidine-4-carboxyamide derivatives were designed and synthesized by using a group-reverse strategy. The CCR5 inhibitory activities of target compounds were evaluated based on calcium mobilization assay. Several compounds showed promising inhibitory activity, with IC_{50} values ranging from 25.53 to 76.03 nM. Among them, two compounds (**16g**, IC_{50} = 25.73 nM and **16i**, IC_{50} = 25.53 nM) showed equivalent similar activity in comparison with that of the positive control, maraviroc (IC_{50} = 25.43 nM). Potent compounds identified by calcium mobilization assay were selected for further antiviral evaluation. As a result, two compounds showed a nanomolar activity (**16g**, IC_{50} = 73.01 nM and **16i**, IC_{50} = 94.10 nM, respectively). Moreover, compound **16g** was chosen for further evaluation of pharmacokinetic property, and it exhibited oral bioavailability of 15.00% in rats. Meanwhile, a patch clamp assay revealed that compound **16g** exhibited 15.20% inhibition of the hERG potassium current at a concentration of 3 μM. Although we were able to identify compound **16g** with acceptable oral bioavailability, it had hERG inhibition in rats, suggesting an ion channel effect on cardiovascular safety. As a consequence, we will describe our efforts in redesigning this chemotype in due course.

Table 3
Pharmacokinetic property of **16g** in rat.[a]

Parameter	IV (4 mg kg^{-1})	PO (25 mg kg^{-1})
Vdss (L kg^{-1})	1.23	8.99
Clp (L h^{-1} kg^{-1})	1.25	8.86
AUC_{0-24} (mg L^{-1} h)	3.25	3.13
$t_{1/2}$ (h)	0.74	0.71
F%[b]	15.00%	

[a] Drug was administered orally (PO) or intravenously (IV) to fasted SD rats ($n = 3$).
[b] $F = (AUCpo/25)/(AUCiv/4) \times 100\%$.

4. Experimental protocols

4.1. Chemistry

4.1.1. General procedures

Melting points were obtained on a B-540 Buchi melting point apparatus which were uncorrected. ^1H NMR spectra was recorded on a Bruker Avance III instrument at 500 MHz (chemical shifts were expressed as δ values relative to TMS as internal standard). Mass spectra (MS) and ESI (positive) were recorded on an Esquire-LC-00075 spectrometer. High-resolution mass (HRMS) were measured by Agilent 6224 LC/MS.

4.1.2. Synthesis of 1-acetylpiperidine-4-carboxylic acid (*8*) and 1-acetylpiperidine-4-carbonyl chloride (*9*)

Compound **8** and compound **9** were obtained according to the known approach in Refs. [11,12].

4.1.3. Synthesis of 3-chloro-N-(3-chloropropyl)aniline (*11*)

A mixture of 3-chloroaniline **10** (1.14 g, 9.00 mmol), 1-bromo-3-chloropropane (0.49 g, 3.00 mmol), and KI (0.05 g, 0.3 mmol) in MeCN (5 ml) was reacted in microwave reactor at 110 °C for 15 min. The solvent was evaporated *in vacuo*, diluted with water (30 ml), and extracted with EtOAc (30 ml × 3). The combined organic layer was dried ($MgSO_4$) and filtered, then the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/PE 1/12) to yield yellow oil (0.34 g, 60%). ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.34–7.20 (m, 2H, Ar–H), 7.18–7.00 (m, 2H, Ar–H), 3.67–3.64 (m, 2H, CH_2), 3.57–3.53 (m, 2H, CH_2), 3.05–2.98 (m, 2H, CH_2). ESI-MS *m/z*: 204 $[M + H]^+$.

4.1.4. Synthesis of 1-acetyl-N-(3-chlorophenyl)-N-(3-chloropropyl) piperidine-4-carboxamide (*12*)

To an ice-cooled stirred suspension of 3-chloro-*N*-(3-chloropropyl)aniline **11** (0.21 g, 1.00 mmol) in DCM (3.00 ml) was added Et_3N (0.40 ml, 3.00 mmol) followed by 1-acetylpiperidine-4-carbonyl chloride **9** (0.23 g, 1.20 mmol), and the mixture was stirred at room temperature for 5 h. Then it was poured to a saturated solution of sodium bicarbonate (10.00 ml). The residue was extracted by EtOAc (20 ml × 3), the organic layer was combined, then washed with water (15 ml × 3) and brine (10.00 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/PE 1/3) to afford the title compound (0.30 g, 85%) as a brown solid; mp: 115–117 °C (dec). ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.31–6.98 (m, 4H, Ar–H), 4.55–4.50 (m, 1H, piperidine–H), 3.83–3.80 (m, 1H, piperidine–H), 3.76 (t, J = 7.0 Hz, 2H, CH_2), 3.54 (t, J = 7.0 Hz, 2H, CH_2), 2.94–2.81 (m, 1H, piperidine–H), 2.44–2.34 (m, 2H, piperidine–H), 2.06 (s, 3H, CH_3),

2.10–2.06 (m, 2H, CH_2), 1.87–1.62 (m, 4H, piperidine–H). ESI-MS *m/z*: 357 $[M + H]^+$.

4.1.5. General procedure for the synthesis of **14a–r**

To a solution of 1-(*tert*-butoxycarbonyl) piperidine-4-carboxylic acid (0.23 g, 1.00 mmol) in DCM (10.00 ml), EDC·HCl (1.00 mmol), **13a–r** (1.00 mmol) and triethylamine (15.00 μL, 0.10 mmol) were added successively at room temperature, and the resulting reaction mixture was stirred for 18 h at the same temperature. Then the reaction mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/PE 1/1) to furnish the title compound.

4.1.6. tert-Butyl-4-(benzyl(ethyl)carbamoyl)piperidine-1-carboxylate (**14a**)

Yellow oil (65%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.55–7.45 (m, 5H, Ar–H), 4.35–4.20 (m, 2H, CH_2), 4.19–4.15 (m, 2H, piperidine–H), 2.79–2.74 (m, 2H, piperidine–H), 2.49–2.43 (m, 2H, CH_2), 2.35–2.29 (m, 1H, piperidine–H), 1.92–1.71 (m, 4H, piperidine–H), 1.43 (s, 9H, Boc-CH_3), 1.03–0.96 (m, 3H, CH_3). ESI-MS *m/z*: 347 $[M + H]^+$.

4.1.7. tert-Butyl 4-(ethyl(4-methylbenzyl)carbamoyl)piperidine-1-carboxylate (**14b**)

Yellow oil (72%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.17–7.04 (m, 4H, Ar–H), 4.54–4.51 (m, 2H, CH_2), 4.26–4.00 (m, 2H, piperidine–H), 2.87–2.76 (m, 2H, piperidine–H), 2.66–2.44 (m, 2H, CH_2), 2.34–2.31 (m, 1H, piperidine–H), 2.21 (s, 3H, Ar–CH_3), 1.95–1.72 (m, 4H, piperidine–H), 1.45 (s, 9H, Boc-CH_3), 1.03–0.97 (m, 3H, CH_3). ESI-MS *m/z*: 361 $[M + H]^+$.

4.1.8. tert-Butyl-4-(ethyl(4-fluorobenzyl)carbamoyl)piperidine-1-carboxylate (**14c**)

Yellow oil (43%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.38–7.19 (m, 4H, Ar–H), 4.52–4.48 (m, 2H, CH_2), 4.17–4.10 (m, 2H, piperidine–H), 3.40–3.30 (m, 2H, CH_2), 2.75–2.68 (m, 2H, piperidine–H), 2.37–2.26 (m, 1H, piperidine–H), 1.89–1.72 (m, 4H, piperidine–H), 1.45 (s, 9H, Boc-CH_3), 1.10–0.95 (m, 3H, CH_3). ESI-MS *m/z*: 365 $[M + H]^+$.

4.1.9. tert-Butyl-4-((4-chlorobenzyl)(ethyl)carbamoyl)piperidine-1-carboxylate (**14d**)

Yellow oil (74%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.40–7.29 (m, 4H, Ar–H), 4.45–4.19 (m, 2H, CH_2), 4.13–4.13 (m, 2H, piperidine–H), 2.78–2.73 (m, 2H, piperidine–H), 2.59–2.52 (m, 2H, CH_2), 2.39–2.30 (m, 1H, piperidine–H), 1.93–1.70 (m, 4H, piperidine–H), 1.44 (s, 9H, Boc-CH_3), 1.01–0.94 (m, 3H, CH_3). ESI-MS *m/z*: 381 $[M + H]^+$.

4.1.10. tert-Butyl-4-((4-cyanobenzyl)(ethyl)carbamoyl)piperidine-1-carboxylate (**14e**)

Yellow oil (73%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.59–7.57 (d, 2H, Ar–H), 7.59–7.57 (d, 2H, Ar–H) 4.16–4.09 (m, 2H, piperidine–H), 3.66 (s, 2H, CH_2), 2.66–2.62 (m, 2H, piperidine–H), 2.56–2.51 (m, 2H, CH_2), 2.34–2.28 (m, 1H, piperidine–H) 1.73–1.64 (m, 4H, piperidine–H), 1.44 (s, 9H, Boc-CH_3), 1.02–0.95 (m, 3H, CH_3). ESI-MS *m/z*: 372 $[M + H]^+$.

4.1.11. tert-Butyl-4-(benzyl(methyl)carbamoyl)piperidine-1-carboxylate (**14f**)

White solid (67%). Mp: 57–62 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.45–7.21 (m, 5H, Ar–H), 4.53–4.49 (m, 2H, CH_2), 4.16–4.11 (m, 2H, piperidine–H), 3.27 (s, 3H, CH_3), 2.78–2.71 (m, 2H, piperidine–H), 2.22–2.19 (m, 1H, piperidine–H), 1.88–1.71 (m, 4H, piperidine–H), 1.41 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 333 $[M + H]^+$.

4.1.12. tert-Butyl-4-(benzylcarbamoyl)piperidine-1-carboxylate (**14g**)

White solid (62%). Mp: 88–92 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.33–7.10 (m, 5H, Ar–H), 6.40 (s, 1H, NH), 4.52–4.48 (m, 2H, CH_2), 4.16–4.10 (m, 2H, piperidine–H), 2.74–2.69 (m, 2H, piperidine–H), 2.22–2.18 (m, 1H, piperidine–H), 1.86–1.70 (m, 4H, piperidine–H), 1.40 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 319 $[M + H]^+$.

4.1.13. tert-Butyl-4-(4-fluorobenzylcarbamoyl)piperidine-1-carboxylate (**14h**)

White solid (59%). Mp: 94–97 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.39–7.22 (m, 4H, Ar–H), 6.45 (s, 1H, NH), 4.53–4.49 (m, 2H, CH_2), 4.17–4.10 (m, 2H, piperidine–H), 2.70–2.65 (m, 2H, piperidine–H), 2.32–2.23 (m, 1H, piperidine–H), 1.89–1.71 (m, 4H, piperidine–H), 1.41 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 337 $[M + H]^+$.

4.1.14. tert-Butyl-4-(4-chlorobenzylcarbamoyl)piperidine-1-carboxylate (**14i**)

White solid (72%). Mp: 121–123 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.41–7.21 (m, 4H, Ar–H), 6.50 (s, 1H, NH), 4.55–4.51 (m, 2H, CH_2), 4.20–4.11 (m, 2H, piperidine–H), 2.70–2.62 (m, 2H, piperidine–H), 2.34–2.28 (m, 1H, piperidine–H), 1.90–1.71 (m, 4H, piperidine–H), 1.43 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 353 $[M + H]^+$.

4.1.15. tert-Butyl-4-(4-cyanobenzylcarbamoyl)piperidine-1-carboxylate (**14j**)

Yellow solid (67%). Mp: 89–94 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.57–7.45 (m, 4H, Ar–H), 6.43 (s, 1H, NH), 4.35–4.17 (m, 2H, CH_2), 4.19–4.13 (m, 2H, piperidine–H), 2.78–2.73 (m, 2H, piperidine–H), 2.33–2.23 (m, 1H, piperidine–H), 1.90–1.71 (m, 4H, piperidine–H), 1.42 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 344 $[M + H]^+$.

4.1.16. tert-Butyl-4-(phenylcarbamoyl)piperidine-1-carboxylate (**14k**)

Yellow solid (65%). Mp: 155–158 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.35–7.06 (m, 5H, Ar–H), 6.63 (s, 1H, NH), 4.18–4.13 (m, 2H, piperidine–H), 2.79–2.74 (m, 2H, piperidine–H), 2.36–2.33 (m, 1H, piperidine–H), 1.91–1.65 (m, 4H, piperidine–H), 1.43 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 305 $[M + H]^+$.

4.1.17. tert-Butyl-4-(ethyl(phenyl)carbamoyl)piperidine-1-carboxylate (**14l**)

White solid (54%). Mp: 77–79 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.39–7.16 (m, 5H, Ar–H), 4.29–4.20 (m, 2H, piperidine–H), 3.77–3.75 (m, 2H, CH_2), 2.79–2.71 (m, 2H, piperidine–H), 2.45–2.42 (m, 1H, piperidine–H), 1.98–1.72 (m, 4H, piperidine–H), 1.45 (s, 9H, Boc-CH_3), 1.04 (t, 3H, $J = 7$ Hz, CH_3). ESI-MS *m/z*: 333 $[M + H]^+$.

4.1.18. tert-Butyl-4-(phenethylcarbamoyl)piperidine-1-carboxylate (**14m**)

White solid (73%). Mp: 139–143 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.38–7.19 (m, 5H, Ar–H), 6.90 (s, 1H, NH), 4.24–4.18 (m, 2H, piperidine–H), 3.41–3.35 (m, 2H, CH_2), 2.84–2.79 (m, 2H, piperidine), 2.75–2.70 (m, 2H, CH_2), 2.32–2.27 (m, 1H, piperidine–H), 1.87–1.70 (m, 4H, piperidine–H), 1.39 (s, 9H, Boc-CH_3). ESI-MS *m/z*: 333 $[M + H]^+$.

4.1.19. tert-Butyl 4-(4-fluorophenethylcarbamoyl)piperidine-1-carboxylate (**14n**)

White solid (78%). Mp: 153–155 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.14–7.11 (m, 2H, Ar–H), 7.01–6.97 (m, 2H, Ar–H), 5.43 (brs, 1H), 4.12–4.10 (m, 2H), 3.51–3.47 (m, 2H), 2.80–2.67 (m, 4H), 2.18–2.11 (m, 1H), 1.74–1.72 (m, 2H), 1.61–1.53 (m, 2H), 1.44 (s, 9H, 3× CH_3). ESI-MS *m/z*: 351 $[M + H]^+$.

*4.1.20. tert-Butyl 4-(4-chlorophenethylcarbamoyl)piperidine-1-carboxylate (**14o**)*

Yellow solid (75%). Mp: 143–145 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.27–7.25 (m, 2H, Ar–H), 7.10–7.08 (m, 2H, Ar–H), 5.53 (brs, 1H), 4.11–4.09 (m, 2H), 3.49–3.45 (m, 2H), 2.79–2.69 (m, 4H), 2.16–2.11 (m, 1H), 1.73–1.70 (m, 2H), 1.60–1.52 (m, 2H), 1.44 (s, 9H, 3× CH_3). ESI-MS *m/z*: 367.8 $[M + H]^+$.

*4.1.21. tert-Butyl-4-(ethyl(phenethyl)carbamoyl)piperidine-1-carboxylate (**14p**)*

Yellow oil (65%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.39–7.20 (m, 5H, Ar–H), 4.25–4.18 (m, 2H, piperidine–H), 3.43–3.36 (m, 2H, CH_2), 2.85–2.79 (m, 2H, piperidine–H), 2.75–2.52 (m, 4H, CH_2), 2.32–2.27 (m, 1H, piperidine–H), 1.89–1.70 (m, 4H, piperidine–H), 1.37 (s, 9H, Boc-CH_3), 1.03–0.98 (m, 3H, CH_3). ESI-MS *m/z*: 361 $[M + H]^+$.

*4.1.22. tert-Butyl 4-(ethyl(4-fluorophenethyl)carbamoyl) piperidine-1-carboxylate (**14q**)*

White solid (78%). Mp: 93–95 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.12–7.06 (m, 2H, Ar–H), 6.98–6.90 (m, 2H, Ar–H), 4.12–4.05 (m, 2H), 3.46–3.29 (m, 3H), 3.17–3.13 (m, 1H), 2.80–2.71 (m, 3H), 2.53–2.47 (m, 1H), 2.16–2.00 (m, 1H), 1.75–1.66 (m, 1H), 1.61–1.53 (m, 2H), 1.42 (s, 9H, 3× CH_3), 1.36–1.24 (m, 1H), 1.11–1.06 (m, 3H, CH_3). ESI-MS *m/z*: 379 $[M + H]^+$.

*4.1.23. tert-Butyl 4-((4-chlorophenethyl)(ethyl)carbamoyl) piperidine-1-carboxylate (**14r**)*

Yellow solid (71%). Mp: 87–89 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.28–7.22 (m, 2H, Ar–H), 7.11–7.06 (m, 2H, Ar–H), 4.15–4.05 (m, 2H), 3.48–3.36 (m, 3H), 3.18–3.14 (m, 1H), 2.81–2.70 (m, 3H), 2.56–2.49 (m, 1H), 2.14–2.10 (m, 1H), 1.77–1.69 (m, 1H), 1.61–1.58 (m, 2H), 1.44 (s, 9H, 3× CH_3), 1.35–1.29 (m, 1H), 1.13–1.08 (m, 3H, CH_3). ESI-MS *m/z*: 396 $[M + H]^+$.

*4.1.24. General procedure for the synthesis of **6** and **16b–r***

To a solution of compound **14a–r** (1.00 mmol) in EtOAc (1.00 ml) was added 6 N HCl (4.00 ml). The mixture was stirred at room temperature for 2 h. The solution was concentrated at reduced pressure, and the residue was poured into water and basified with 10% NaOH aqueous solution (pH = 10). The mixture was extracted with EtOAc (15 ml × 3). The organic phase was dried with Na_2SO_4 and concentrated. The obtained products **15a–r** were used for next step without further purification.

A mixture of 1-acetyl-*N*-(3-chlorophenyl)-*N*-(3-chloropropyl) piperidine-4-carboxamide **11** (0.10 mmol), compound **15a–r** (0.10 mmol), KI (0.10 mmol) and K_2CO_3 (0.15 mmol) in MeCN (8 ml) was stirred at reflux for 48 h. The mixture was concentrated *in vacuo*, diluted with water (10.00 ml) and extracted with EtOAc (15 ml × 3). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/MeOH 1/0 to 9/1) to afford product.

*4.1.25. 1-Acetyl-N-(3-(4-(benzyl(ethyl)carbamoyl)piperidin-1-yl) propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**6**)*

Yellow oil (35%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.46–7.28 (m, 7H, Ar–H), 7.18–7.13 (m, 2H, Ar–H), 4.57–4.53 (m, 4H), 4.07–4.01 (m, 2H), 3.82–3.66 (m, 5H), 3.52–3.43 (m, 4H), 3.32–3.27 (m, 2H), 2.86–2.81 (m, 2H), 2.37–2.29 (m, 4H), 2.05 (s, 3H, CH_3), 1.98–1.75 (m, 5H), 1.09–0.99 (m, 3H, CH_3). HRMS (ESI^+) *m/z* calculated for $C_{32}H_{44}ClN_4O_3$ $[M + H]^+$, 567.3102; found, 567.3080.

*4.1.26. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(ethyl(4-methylbenzyl)carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16b**)*

Yellow solid (44%); mp: 50–56 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ:7.45–7.41 (m, 2H, Ar–H), 7.25–7.23 (m, 1H, Ar–H), 7.17–7.02 (m, 5H, Ar–H), 4.56–4.48 (m, 4H), 4.24–4.11 (m, 2H), 3.78–3.67 (m, 4H), 3.43–3.39 (m, 1H), 3.30–3.16 (m, 3H), 2.86–2.50 (m, 6H), 2.37–2.34 (m, 3H), 2.32 (s, 3H, Ar–CH_3), 2.05 (s, 3H, CH_3), 1.99–1.70 (m, 5H), 1.13–1.02 (m, 3H, CH_3). HRMS (ESI^+) *m/z* calculated for $C_{33}H_{46}ClN_4O_3$ $[M + H]^+$, 581.3258; found, 581.3271.

*4.1.27. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(ethyl(4-fluorobenzyl) carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16c**)*

Brown solid (41%); mp: 48–52 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.47–7.42 (m, 2H, Ar–H), 7.18–7.13 (m, 3H, Ar–H), 7.08–6.99 (m, 3H, Ar–H), 4.58–4.48 (m, 4H), 4.04–3.82 (m, 2H), 3.78–3.64 (m, 4H), 3.59–3.50 (m, 1H), 3.43–3.18 (m, 6H), 3.10–2.75 (m, 6H), 2.38–2.17 (m, 5H), 2.06 (s, 3H, CH_3), 1.10–0.96 (m, 3H, CH_3). HRMS (ESI^+) *m/z* calculated for $C_{32}H_{43}ClFN_4O_3$ $[M + H]^+$, 585.3008; found, 585.3015.

*4.1.28. 1-Acetyl-N-(3-(4-((4-chlorobenzyl)(ethyl)carbamoyl) piperidin-1-yl)propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16d**)*

Brown solid (39%); mp: 43–47 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.45–7.28 (m, 6H, Ar–H), 7.14–7.09 (m, 2H, Ar–H), 4.56–4.49 (m, 4H), 4.14–4.08 (m, 2H), 3.79–3.67 (m, 4H), 3.44–3.27 (m, 3H), 2.98–2.79 (m, 6H), 2.39–2.29 (m, 3H), 2.06 (s, 3H, CH_3), 1.98–1.65 (m, 6H), 1.15–1.04 (m, 3H, CH_3). HRMS (ESI^+) *m/z* calculated for $C_{32}H_{43}Cl_2N_4O_3$ $[M + H]^+$, 601.2712; found, 601.2721.

*4.1.29. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-((4-cyanobenzyl)(ethyl)carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16e**)*

Yellow solid (38%); mp: 42–46 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.68–7.28 (m, 6H, Ar–H), 7.22–7.12 (m, 2H, Ar–H), 4.61–4.52 (m, 3H), 4.23–4.12 (m, 2H), 3.81–3.64 (m, 3H), 3.42–3.29 (m, 2H), 3.08–2.97 (m, 2H), 2.87–2.81 (m, 1H), 2.60–2.30 (m, 5H), 2.06 (s, 3H, CH_3), 1.99–1.65 (m, 10H), 1.19–1.10 (m, 3H, CH_3). HRMS (ESI^+) *m/z* calculated for $C_{33}H_{43}ClN_5O_3$ $[M + H]^+$, 592.3054; found, 592.3059.

*4.1.30. 1-Acetyl-N-(3-(4-(benzyl(methyl)carbamoyl)piperidin-1-yl) propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16f**)*

Yellow solid (46%); mp: 50–54 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.47–7.29 (m, 6H, Ar–H), 7.23–7.12 (m, 3H, Ar–H), 4.59–4.51 (m, 4H), 4.17–4.03 (m, 2H), 3.82–3.63 (m, 5H), 3.47–3.32 (m, 4H), 3.15 (s, 3H, CH_3), 2.62 (s, 2H, CH_2), 2.36–2.29 (m, 4H), 2.05 (s, 3H, CH_3), 1.95–1.73 (m, 5H). HRMS (ESI^+) *m/z* calculated for $C_{31}H_{42}ClN_4O_3$ $[M + H]^+$, 553.2945; found, 553.2953.

*4.1.31. 1-Acetyl-N-(3-(4-(benzylcarbamoyl)piperidin-1-yl)propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16g**)*

Brown solid (25%); mp: 89–92 °C. ^{1}H NMR (500 MHz, CDCl3) δ: 7.37–7.36 (m, 2H, Ar–H), 7.31–7.28 (m, 2H, Ar–H), 7.24–7.18 (m, 4H, Ar–H), 7.07–7.05 (m, 1H, Ar–H), 5.92 (brs, 1H), 4.49 (d, J = 13.5 Hz, 1H), 4.41–4.39 (m, 2H), 3.75 (d, J = 13.5 Hz, 1H), 3.66–3.63 (m, 2H), 2.90–2.78 (m, 3H), 2.34–2.29 (m, 4H), 2.14–2.08 (m, 1H), 2.01 (s, 3H, CH_3), 1.94–1.93 (m, 2H), 1.85–1.59 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.57, 173.72, 168.68, 143.50, 138.30, 135.31, 130.83, 128.63, 128.51, 128.25, 127.63, 127.41, 126.39, 55.66, 53.02, 47.96, 45.44, 43.35, 40.59, 39.40, 28.76, 28.24, 25.10, 21.29. HRMS (ESI^+) *m/z* calculated for $C_{30}H_{40}ClN_4O_3$ $[M + H]^+$, 539.2789; found, 539.2765.

*4.1.32. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(4-fluorobenzylcarbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16h**)*

Yellow solid (43%); mp: 75–78 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.44–7.41 (m, 2H, Ar–H), 7.25–7.19 (m, 4H, Ar–H), 7.03–6.99 (m, 2H, Ar–H), 4.51 (d, J = 13.5 Hz, 1H), 4.38 (d, J = 6.0 Hz, 2H), 3.78–3.66 (m, 4H), 3.22–3.10 (m, 2H), 2.86–2.81 (m, 1H), 2.67–2.63 (m, 1H), 2.62 (s, 2H, CH_2), 2.45–2.30 (m, 5H), 2.18–2.10 (m, 2H), 2.05 (s, 3H, CH_3), 1.92–1.60 (m, 6H). HRMS (ESI^+) m/z calculated for $C_{30}H_{39}ClFN_4O_3$ $[M + H]^+$, 557.2695; found, 557.2705.

*4.1.33. 1-Acetyl-N-(3-(4-(4-chlorobenzylcarbamoyl)piperidin-1-yl) propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16i**)*

Brown solid (45%); mp: 46–49 °C. ^{1}H NMR (500 MHz, CDCl3) δ: 7.38–7.37 (m, 2H, Ar–H), 7.27–7.26 (m, 2H, Ar–H), 7.18–7.15 (m, 3H, Ar–H), 7.07–7.06 (m, 1H, Ar–H), 5.99 (brs, 1H), 4.99 (d, J = 13.5 Hz, 1H), 4.37 (d, J = 6 Hz, 2H), 3.75 (d, J = 13.5 Hz, 1H), 3.67 (t, J = 7.5 Hz, 2H), 2.91–2.79 (m, 3H), 2.32 (brs, 4H), 2.14–2.09 (m, 1H), 2.01 (s, 3H, CH_3), 1.95–1.94 (m, 2H), 1.85–1.83 (m, 2H), 1.76–1.55 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.71, 173.82, 168.75, 143.55, 137.00, 135.40, 133.24, 130.91, 129.03, 128.81, 128.60, 128.32, 126.45, 55.70, 53.04, 48.02, 45.51, 42.69, 40.66, 39.46, 28.83, 28.32, 25.15, 21.36. HRMS (ESI^+) m/z calculated for $C_{30}H_{39}Cl_2N_4O_3$ $[M + H]^+$, 573.2399; found, 573.2406.

*4.1.34. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(4-cyanobenzylcarbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16j**)*

Yellow oil (35%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.63–7.30 (m, 8H, Ar–H), 4.53–4.45 (m, 3H), 3.80–3.48 (m, 5H), 3.33–3.29 (m, 1H), 3.12–3.08 (m, 1H), 2.97–2.82 (m, 2H), 2.40–2.07 (m, 7H), 2.05 (s, 3H, CH_3), 1.98–1.63 (m, 7H). HRMS (ESI^+) m/z calculated for $C_{31}H_{39}ClN_5O_3$ $[M + H]^+$, 564.2741; found, 564.2752.

*4.1.35. 1-Aacetyl-N-(3-chlorophenyl)-N-(3-(4-(phenylcarbamoyl) piperidin-1-yl)propyl)piperidine-4-carboxamide (**16k**)*

Yellow solid (34%); mp: 92–95 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.54–7.40 (m, 5H, Ar–H), 7.34–7.31 (m, 2H, Ar–H), 7.12–7.09 (m, 2H, Ar–H), 4.52 (d, J = 13.5 Hz, 1H), 3.76 (d, J = 13.5 Hz, 1H), 3.71–3.68 (m, 2H), 3.04 (d, J = 10.5 Hz, 2H), 2.87–2.82 (m, 1H), 2.45–2.10 (m, 13H), 2.06 (s, 3H, CH_3), 2.00–1.94 (m, 4H). HRMS (ESI^+) m/z calculated for $C_{29}H_{38}ClN_4O_3$ $[M + H]^+$525.2632; found, 525.2637.

*4.1.36. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(ethyl(phenyl) carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16l**)*

Yellow solid (33%); mp: 70–72 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.40–7.26 (m, 5H, Ar–H), 7.11–7.00 (m, 4H, Ar–H), 4.43 (d, J = 12.5 Hz, 1H), 4.16–4.03 (m, 1H), 3.70–3.50 (m, 5H), 2.95–2.66 (m, 4H), 2.50–2.05 (m, 10H), 1.97 (s, 3H, CH_3), 1.92–1.73 (m, 5H), 1.00 (t, J = 7.0 Hz, 3H, CH_3). HRMS (ESI^+) m/z calculated for $C_{31}H_{42}ClN_4O_3$ $[M + H]^+$553.2945; found, 553.2952.

*4.1.37. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(phenethylcarbamoyl) piperidin-1-yl)propyl)piperidine-4-carboxamide (**16m**)*

Yellow solid (46%); mp: 82–85 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.44–7.29 (m, 4H, Ar–H), 7.25–7.14 (m, 5H, Ar–H), 4.51 (d, J = 13.0 Hz, 1H), 3.83–3.65 (m, 5H), 3.56–3.47 (m, 2H), 3.19–3.05 (m, 2H), 2.90–2.77 (m, 4H), 2.72–2.60 (m, 2H), 2.40–2.28 (m, 4H), 2.05 (s, 3H, CH_3), 1.95–1.65 (m, 8H). HRMS (ESI^+) m/z calculated for $C_{31}H_{42}ClN_4O_3$ $[M + H]^+$553.2945; found, 553.2950.

*4.1.38. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(4-fluorophenethylcarbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16n**)*

White solid (44%); mp: 66–68 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.36–7.35 (m, 2H, Ar–H), 7.16 (s, 1H, Ar–H), 7.10–7.03 (m, 3H, Ar–H), 6.96–6.92 (m, 2H, Ar–H), 5.65 (brs, 1H), 4.48 (d, J = 13.5 Hz, 1H), 3.74–3.71 (d, J = 13.5 Hz, 1H), 3.64–3.61 (m, 2H), 3.45–3.41 (m, 2H), 2.83–2.72 (m, 5H), 2.31–2.24 (m, 4H), 2.00 (s, 3H, CH_3), 1.98–1.94 (m, 1H), 1.87–1.82 (m, 2H), 1.76–1.56 (m, 10H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.77, 173.65, 168.64, 162.47, 160.53, 143.47, 135.25, 134.48, 130.78, 130.07, 128.46, 128.21, 126.33, 55.67, 53.08, 47.97, 45.40, 43.14, 40.55, 39.34, 34.75, 28.75, 28.20, 25.14, 21.25. HRMS (ESI^+) m/z calculated for $C_{31}H_{40}ClFN_4O_3$ $[M + H]^+$571.2851; found, 571.2860.

*4.1.39. 1-Acetyl-N-(3-(4-(4-chlorophenethylcarbamoyl)piperidin-1-yl)propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16o**)*

Yellow solid (49%); mp: 58–60 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.36–7.35 (m, 2H, Ar–H), 7.23–7.21 (d, J = 8 Hz, 2H, Ar–H), 7.16 (s, 1H, Ar–H), 7.07–7.03 (m, 3H, Ar–H), 5.71 (brs, 1H), 4.45 (d, J = 10 Hz, 1H), 3.74–3.61 (m, 3H), 3.45–3.41 (m, 2H), 2.84–2.72 (m, 5H), 2.33–2.24 (m, 4H), 2.00 (s, 3H, CH_3), 1.98–1.94 (m, 1H), 1.87–1.83 (m, 2H), 1.72–1.54 (m, 10H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.83, 173.66, 168.68, 143.43, 137.30, 135.26, 132.24, 130.79, 130.00, 128.57, 128.48, 128.20, 126.32, 55.65, 53.06, 47.97, 45.41, 43.13, 40.56, 39.34, 34.91, 28.71, 28.20, 25.11, 21.25. HRMS (ESI^+) m/z calculated for $C_{31}H_{40}Cl_2N_4O_3$ $[M + H]^+$587.2555; found, 587.2561.

*4.1.40. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(ethyl(phenethyl) carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16p**)*

Yellow oil (32%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.44–7.28 (m, 4H, Ar–H), 7.25–7.14 (m, 5H, Ar–H), 4.56–4.53 (m, 1H), 3.80–3.62 (m, 4H), 3.56–3.41 (m, 3H), 3.20–3.11 (m, 3H), 2.86–2.80 (m, 3H), 2.78–2.61 (m, 2H), 2.40–2.25 (m, 4H), 2.20–2.06 (m, 4H), 2.07 (s, 3H, CH_3), 1.96–1.62 (m, 6H), 1.16–1.08 (m, 3H, CH_3). HRMS (ESI^+) m/z calculated for $C_{33}H_{46}ClN_4O_3$ $[M + H]^+$581.3258; found, 581.3266.

*4.1.41. 1-Acetyl-N-(3-chlorophenyl)-N-(3-(4-(ethyl(4-fluorophenethyl)carbamoyl)piperidin-1-yl)propyl)piperidine-4-carboxamide (**16q**)*

White oil (42%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.34–7.33 (m, 2H, Ar–H), 7.16–7.15 (m, 1H, Ar–H), 7.11–7.04 (m, 3H, Ar–H), 6.96–6.88 (m, 2H, Ar–H), 4.47 (d, J = 12 Hz, 1H), 3.72–3.70 (d, J = 12 Hz, 1H), 3.63–3.62 (m, 2H), 3.43–3.40 (m, 2H), 3.34–3.30 (m, 1H), 3.14–3.11 (m, 1H), 2.86–2.74 (m, 5H), 2.29–2.21 (m, 5H), 1.99 (s, 3H, CH_3), 1.89–1.85 (m, 1H), 1.81–1.55 (m, 10H), 1.34–1.32 (m, 1H), 1.08–1.04 (m, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.62, 173.72, 168.69, 143.57, 135.28, 134.96, 130.82, 130.27, 130.16, 128.48, 128.26, 127.77, 126.40, 55.82, 53.21, 48.08, 45.46, 42.75, 40.61, 39.41, 34.96, 33.27, 28.78, 28.26, 25.17, 21.30, 14.73, 12.87. HRMS (ESI^+) m/z calculated for $C_{33}H_{44}ClFN_4O_3$ $[M + H]^+$599.3164; found, 599.3156.

*4.1.42. 1-Acetyl-N-(3-(4-((4-chlorophenethyl)(ethyl)carbamoyl) piperidin-1-yl)propyl)-N-(3-chlorophenyl)piperidine-4-carboxamide (**16r**)*

Yellow oil (48%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.36 (brs, 2H, Ar–H), 7.26–7.25 (m, 1H, Ar–H), 7.22–7.17 (m, 2H, Ar–H), 7.11–7.05 (m, 3H, Ar–H), 4.50 (d, J = 13.5 Hz, 1H), 3.75–3.73 (d, J = 13.5 Hz, 1H), 3.67–3.62 (m, 2H), 3.46–3.41 (m, 2H), 3.36–3.32 (m, 1H), 3.16–3.11 (m, 1H), 2.89–2.76 (m, 5H), 2.35–2.24 (m, 5H), 2.02 (s, 3H, CH_3), 1.92–1.88 (m, 1H), 1.84–1.58 (m, 10H), 1.37–1.35 (m, 1H), 1.10–1.06 (m, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.61, 173.67, 168.64, 143.43, 137.74, 135.24, 131.91, 130.78, 130.14, 128.78, 128.39, 128.21, 126.36, 55.77, 53.14, 48.03, 45.42, 42.75, 40.57, 39.36, 35.12, 33.41, 28.74,

28.21, 25.13, 21.26, 14.70, 12.83. HRMS (ESI^+) m/z calculated for $C_{33}H_{44}ClFN_4O_3$ $[M + H]^+$615.2868; found, 615.2870.

4.2. Biological assay methods

4.2.1. Cell cytotoxicity assays

Cytotoxicities of target compounds were tested using CCK-8 (Sigma–Aldrich) assay. Briefly, 100 μL of HEK293 cell suspension (5000 cells/well) were dispensed in a 96-well plate, and then preincubated in the plate for 24 h at 37 °C in 5% CO_2. 10 μL of various concentrations of substances to be tested were added to the plate. After 7 h incubation, 10 μL of CCK-8 solution were added to each well of the plate. The plate was incubated at 37 °C for another 2 h in the incubator and the optical absorbance was measured at 430 nm using a microplate reader.

4.2.2. Calcium mobilization assay

CHO cells stably expressing CCR5 and $G\alpha_{16}$ were loaded with 2.00 μmol/L Fluo-4 AM in Hanks balanced salt solution (HBSS, containing KCl 5.40 mmol/L, Na_2HPO_4 0.30 mmol/L, KH_2PO_4 0.4 mmol/L, $NaHCO_3$ 4.20 mmol/L, $CaCl_2$ 1.30 mmol/L, $MgCl_2$ 0.50 mmol/L, Mg_2SO_4 0.60 mmol/L, NaCl 137.00 mmol/L, BSA 5.00 g/L, glucose 5.60 mmol/L, sulfinpyrazone 250 μmol/L, pH 7.4) at 37 °C for 45 min. After the cells being rinsed with the reaction buffer, 50 μL HBSS containing known antagonists (positive control), compounds of interest or DMSO (negative control, final concentration 1%) were added. After incubation at room temperature for 10 min, 25 μL RANTES (final concentration 3 nmol/L) was dispensed into the well using a FlexStation II microplate reader (Molecular Devices, Sunnyvale, CA, USA) and intracellular calcium change was recorded with an excitation wavelength of 485 nm and emission wavelength of 525 nm. The half maximal inhibitory concentrations (IC_{50}) of compounds were determined with GraphPad Prism software by constructing their dose–response curves.

4.2.3. Viral infectivity assays

Plasmids: HIV-1 proviral indicator construct pNL-Luc-E- contains a full-length HIV-1 genome, in which env was replaced by firefly Luciferase coding sequence. pENV-Ad8 expresses R5-tropic envelope (AD8).

Viral infectivity assays: Single-cycle HIV-1 replication assays were performed as described previously. In brief, 4×10^5 293T cells were co-transfected with 0.40 μg of pNL-Luc-E- and 0.4 μg of pENV-R5. After 48 h, the supernatant containing pseudovirion was harvested by filtration through a 0.45 μm filter and the amount of viral capsid protein was measured by p24 antigen capture ELISA (Biomerieux). The resultant supernatant (10.00 μL) was used to infect SupT1 cells (1×105) in 96-well plates in the presence of testing compound at the concentration indicated. The SupT1 cells were lysed 48 h post-infection and firefly luciferase activities were determined using a firefly Luciferase Assay System (Promega). Values were normalized to the control group treated with DMSO and represented relative infectivity of each sample testing.

Acknowledgments

The authors are grateful to the support of the National Natural Science Foundation of China (81072515 and 81202341), the Ministry of Science and Technology of China (2012ZX09301001-005), the Ministry of Health-medical Science Critical Technological Program of Zhejiang Province (WKJ20122024)and the Shanghai Commission of Science and Technology (12XD1402100). The authors also thank Dr. Shan Cen (Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing, China) for evaluation of antiviral activity of representative compounds and Jianyang Pan (Institute of Pharmaceutical Informatics, Zhejiang University, China) for performing NMR spectrometry and Mass spectrometry for structure elucidation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.11.013.

References

[1] E.A. Berger, P.M. Murphy, J.M. Farber, Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease, Annu. Rev. Immunol. 17 (1999) 657–700.
[2] L. Wu, N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A.A. Cardoso, E. Desjardin, W. Newman, C. Gerard, J. Sodroski, CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR5, Nature 384 (1996) 179–183.
[3] P.D. Kwong, R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski, W.A. Hendricks, Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody, Nature 393 (1998) 648–659.
[4] R. Liu, W.A. Paxton, S. Choe, D. Ceradini, S.R. Martin, R. Horuk, M.E. MacDonald, H. Stuhlmann, R.A. Koup, N.R. Landau, Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection, Cell 86 (1996) 367–377.
[5] M. Nishikawa, K. Takashima, T. Nishi, R.A. Furuta, N. Kanzaki, Y. Yamamoto, J. Fujisawa, Analysis of binding sites for the new small-molecule CCR5 antagonist TAK-220 on human CCR5, Antimicrob. Agents Chemother. 49 (2005) 4708–4715.
[6] O.M. Klibanov, S.H. Williams, C.A. Iler, Cenicriviroc, an orally active CCR5 antagonist for the potential treatment of HIV infection, Curr. Opin. Investig. Drugs 11 (2010) 940–950.
[7] T. Liu, Z. Weng, X. Dong, Y. Hu, Recent advances in the development of small-molecule CCR5 inhibitors for HIV, Mini-Rev. Med. Chem. 10 (2010) 1277–1292.
[8] M.X. Dong, L. Lu, X. Wang, H. Lu, S. Jiang, Q.Y. Dai, Design, synthesis, and biological activity of novel 1,4-disubstituted piperidine/piperazine derivatives as CCR5 antagonist-based HIV-1 entry inhibitors, Bioorg. Med. Chem. Lett. 22 (2012) 3284–3286.
[9] T. Liu, Z. Weng, X. Dong, L. Chen, L. Ma, S. Cen, N. Zhou, Y. Hu, Design, synthesis and biological evaluation of novel piperazine derivatives as potential CCR5 antagonists, PLoS ONE 8 (2013) e53636.
[10] Y.Q. Long, D.Z. Feng, L. Chen, R.H. Chen, 1-(3-Amino-propyl)-piperidin-4-yl-amides, pharmaceutical compositions, processes for their preparation and uses, PCT Int. Appl., 30 April, 2009, WO 2009052708 A1, 27.
[11] J. Klaveness, B. Brudeli, F.O. Levy, 5-HTX modulators, PCT Int. Appl., 18 Jan, 2007, WO 2007007072 A1, 17.
[12] R.L. Duncan Jr., G.C. Helsley, W.J. Welstead Jr., J.P. DaVanzo, W.H. Funderburk, C.D. Lunsford, Aroylpiperidines and pyrrolidines. A new class of potent central nervous system depressants, J. Med. Chem. 13 (1970) 1–6.
[13] S. Imamura, T. Ichikawa, Y. Nishikawa, N. Kanzaki, K. Takashima, S. Niwa, Y. Iizawa, M. Baba, Y. Sugihara, Discovery of a piperidine-4-carboxamide CCR5 antagonist (TAK-220) with highly potent anti-HIV-1 activity, J. Med. Chem. 49 (2006) 2784–2793.