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Design, synthesis and biological evaluation of 3-substituted 2,5-dimethyl-*N*-(3-(1*H*-tetrazol-5-yl)phenyl)pyrroles as novel potential HIV-1 gp41 inhibitors

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ABSTRACT

Based on the structure of HIV-1 gp41 binding site for small-molecule inhibitors, optimization of lead **2** resulted in the discovery of a new series of 2,5-dimethyl-3-(5-(*N*-phenylrhodaninyl)methylene)-*N*-(3-(1*H*-tetrazol-5-yl)phenyl)pyrrole compounds with improved anti-HIV-1 activity. The most active compounds **13a** and **13j** exhibited significant potency against gp41 6-HB formation with IC_{50} values of 4.4 and 4.6 μM and against HIV-1 replication in the MT-2 cells with EC_{50} values of 3.2 and 2.2 μM, respectively, thus providing a new starting point to develop highly potent small-molecule HIV fusion inhibitors targeting gp41.

1. Introduction

HIV-1 envelope glycoprotein (Env) transmembrane subunit gp41 plays a crucial role in mediating virus fusion and entry. When the HIV virus fuses to the host cell, the N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) of gp41 interact to form a six-helix bundle (6-HB) core structure, bringing the viral and host cell membranes into sufficient proximity to allow fusion.[1] Licensed in 2003 by the US FDA, enfuvirtide (T-20), a 36-amino acid synthetic peptide, is the first HIV fusion inhibitor drug.[2] It was designed by mimicking the partial peptide structure of the gp41 CHR to inhibit the formation of gp41 6-HB. The effectiveness of T-20 for treatment of HIV-1 infection strongly supported the idea that HIV-1 entry steps and the viral Env could be attractive targets for developing novel anti-HIV drugs.[3,4] However, clinical use of the peptide drug T-20 is limited by its lack of oral availability, metabolic instability in vivo and high production cost.[5,6] Therefore, it is essential to discover and develop orally available, non-peptide, small-molecule fusion inhibitors. Although most small molecule inhibitors designed to inhibit gp41 reported in past years showed potency within the micromole concentration range in both molecular and cellular levels,[7–9,4] more attempts and efforts toward high potent small molecule inhibitors targeting gp41 are still intense.

The binding site of the small-molecule inhibitors on gp41 is believed to be the highly conserved, deep hydrophobic pocket formed by the internal coiled-coil N-trimer,[10,11] which could be inserted by the side chains of three amino acids, including W628, W631, and I635, of the CHR to form the stable 6-HB core. Based on a known active compound denoted **NB-64** (**1**), which targets gp41[12], our previous modification studies resulted in the discovery of a new lead *N*-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl pyrrole (**2**)[13] with an EC_{50} value of 7.7 μM in the MT-2 cells and an IC_{50} value of 25.6 μM against gp41 6-HB formation, which was more potent than known hit **1** (IC_{50} = 58.7 μM) in the same assays. These results indicated that the tetrazole moiety, a carboxylic group isostere, was favorable for enhancing inhibitory potency against gp41 6-HB formation. As shown in Figure 1, our docking studies found that the negatively charged carboxyl group in **1** or tetrazolyl in **2** orientated to the positively charged residue R579 on the surface of the 'pocket' to form a 'salt bridge' through electrostatic force,[14] and both compounds **1** and **2** only partially occupied the gp41 binding pocket, thus leaving more modifiable chemical space for the development of more potent new inhibitors targeting the gp41 binding site.

As a continuation of our studies, further modification focused on expanding the molecular size of lead **2** to provide more

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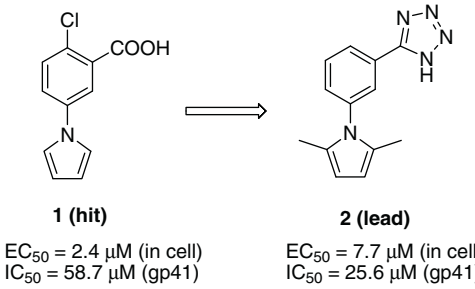


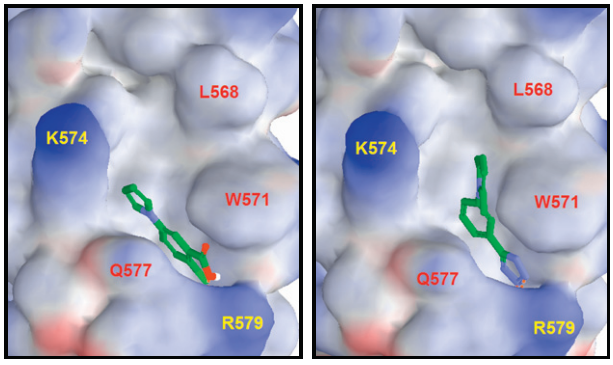


Figure 1. Compounds **1** (left) and **2** (right) and their docking conformation inside the hydrophobic pocket of HIV-1 gp41 (PDB: 1aik). Each compound occupies only part of the binding pocket, and the negatively charged carboxyl in **1** or tetrazolyl in **2** is orientated to positively charged R579.

complementary shape and more interaction points with the binding pocket, thus enhancing inhibitory potency against gp41 6-HB formation. Especially, key amino acid K574 is critical for gp41 6-HB formation; therefore, it is anticipated that the new compound will interact with it on the binding site.[15,16] Consequently, various electronegative groups and fragment moieties were introduced successively at the 3-position on the pyrrole ring of lead **2** to explore more linear potential new inhibitors. Among our several attempts, a substituted *N*-phenyl rhodanine[17,18] was a preferred structural fragment to build a new multiple-ring molecular scaffold because of its convenient synthesis and lead-like structure.[19] In addition, a hydantoin moiety, a bioisostere of rhodanine, was also introduced to explore the effect of the hetero-atom replacement for inhibitory activity against gp41 6-HB formation. Moreover, the effect of a linker with 1 or 2 atoms between the rhodanine and its *N*-phenyl rings was also explored. Herein, we reported the synthesis and anti-HIV activities of a series of 3-substituted *N*-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethylpyrrole derivatives with a conjugated side chain or heterocyclic ring (Fig. 2) as novel small-molecule fusion inhibitors.

2. Chemistry

The syntheses of new target compounds were shown in Schemes 1–3. The Paal–Knorr reaction was performed to synthesize 3-(2,5-dimethyl-1*H*-pyrrol-1-yl) benzonitrile (**4**) by the condensation of 3-aminobenzonitrile (**3**) with 2,5-hexanedione. Then, an aldehyde group was introduced at the 3-position on the pyrrole-ring by using Vilsmeier–Hacck reaction to obtain the desired 3-(3-formyl-2,5-dimethyl-1*H*-pyrrol-1-yl) benzonitrile (**5**). Next, the cyano group in **5** was converted into tetrazolyl by treating with sodium azide and triethylamine hydrochloride in refluxed toluene[20] to afford the key intermediate 2,5-dimethyl-*N*-(3-(1*H*-tetrazol-5-yl)phenyl)pyrrole-3-carbaldehyde (**6**) with a total yield of 73% for the three-step synthesis. Without purification, compound **6** directly reacted with active methylene reagents **7–10**,

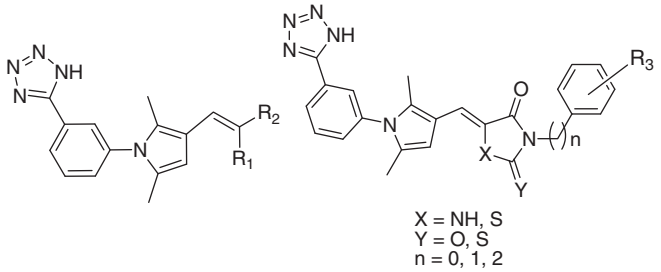


Figure 2. New target compounds.

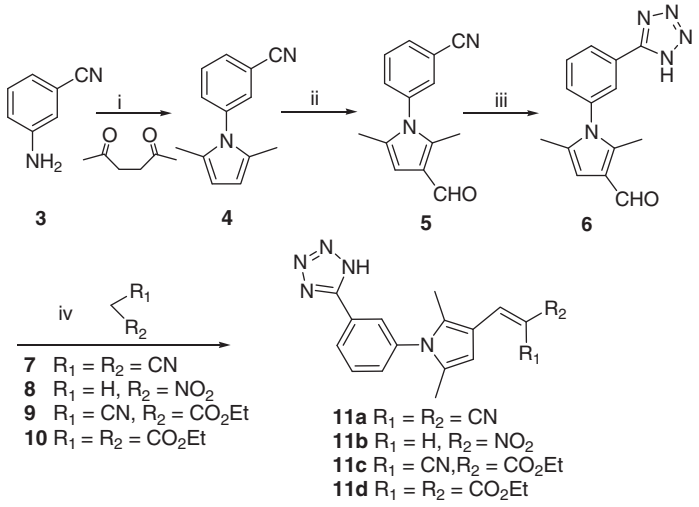
respectively, by Knoevenagel condensation in the presence of piperidine under reflux in ethanol to afford corresponding α, β-unsaturated compounds **11a–d** with a yield range of 33–60%. To expand the structural scaffold in a more linear manner, a series of N-substituted rhodanine derivatives (**12**) were prepared according to methods in the literature,[21] as shown in Scheme 2. Most N-substituted rhodanine building blocks **12a–g** and **12i–j** were synthesized by treating substituted anilines with bis(carboxylmethyl)trithiocarbonate.[13] On the other hand, **12h** was achieved by the condensation of 4-trifluoromethylphenyl isothiocyanate with 2-mercaptoacetic acid, and intermediates **12k** and **12l** were prepared by treating an amine with carbon disulfide and 2-bromoacetic acid successively. Subsequently, rhodanine derivatives **12a–l** reacted with **6** in toluene and methanol (2:1, v/v) refluxed for 3–5 h to afford corresponding new compounds **13a–l** with a yield range of 55–95%. As shown in Scheme 3, N-substituted hydantoin intermediates **15a** and **15b** were prepared from *p*-methoxy or *m*-triflurometh aniline by treating with ethyl glycinate and triphosgene, following a basic hydrolysation of corresponding ureides **14a** and **14b**, and subsequent intramolecular cyclization. Similar to the synthesis of **13**, the condensation between **15a** or **15b** and **6** provided the hydantoin derivatives **16a** and **16b**, respectively, with yields around 80%.

In general, Knoevenagel condensation predominantly provides a thermo-dynamically stable *trans*-isomer (*E*-isomer) product, such as compound **11b** with a 12.8 Hz of *trans*-vinyl coupling constant (*J*) in ^{1}H NMR spectra. However, compared with data of known rhodanine derivatives found in the literatures (7.70–7.75 ppm),[22–24] a series of new 5-arylidene rhodanine compounds **13a–l** were identified as *cis*-isomer (*Z*-isomer) for the exocyclic C=C bond with olefine proton signals at 7.70–7.82 ppm by the deshielding effect of the *cis*-carbonyl (C=O). Similarly to **13**, hydantoin compounds **16a** and **16b** were also identified as *cis*-isomers.[25,26]

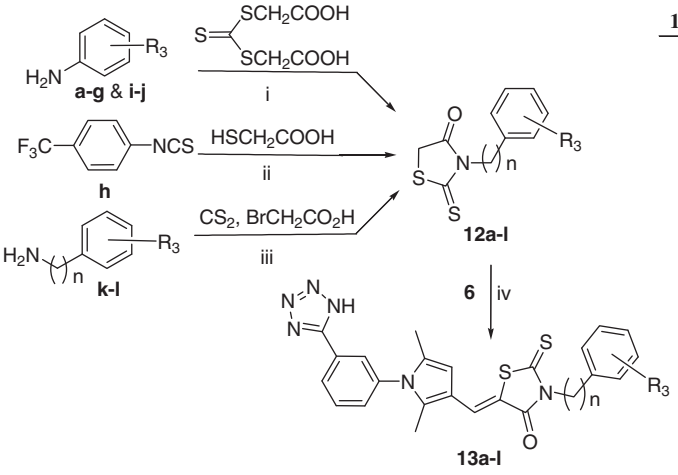
3. Results and discussion

Eighteen newly synthesized 3-substituted *N*-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethylpyrrole derivatives were evaluated against both gp41 six-helix bundle formation (IC_{50}) and HIV replication in MT-2 cell line (EC_{50}), respectively, by enzyme-linked immunosorbent assays (ELISA), as previously described.[12,27] The colorimetric XTT assay[24] was also conducted for the cytotoxicity of these compounds on MT-2 cells (CC_{50}). All assay data were shown in Table 1 and paralleled with **1** (NB-64) and **2** as references.

Among these new compounds, **13a** and **13j** showed significant potency against gp41 6-HB formation with IC_{50} values of 4.4 and 4.6 μM respectively, and against HIV-1 replication in the MT-2 cell line with EC_{50} values of 3.2 and 2.2 μM and selective index (SI) values of 19 and 21, respectively. Both compounds were much more potent than **2** (IC_{50} 25.6 μM and EC_{50} 7.7 μM) in the same assays. In the **13** series, other compounds **13b**, **13d**, **13f**, **13g**, **13h**, and **13k** also exhibited improved potency against gp41 6-HB formation

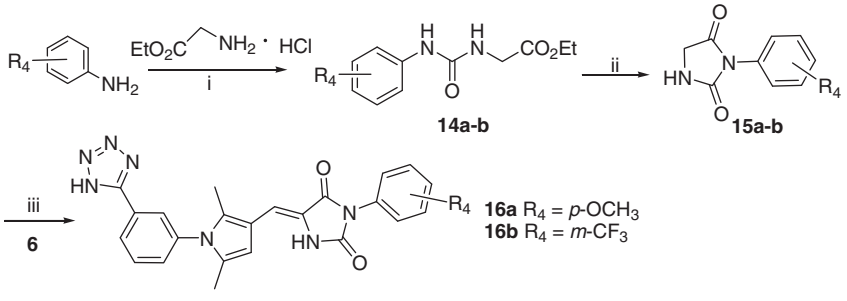


Scheme 1. Synthesis of compounds **11a–d**. Reagents and conditions: (i) AcOH, MW, 160 °C, 30 min; (ii) $POCl_3$, DMF, 0 °C to rt; (iii) NaN_3, Et_3N, HCl, toluene, reflux, 20 h; (iv) piperidine, EtOH, reflux, 5 h.



12, 13	n	R_3
a	0	H
b	0	*m*-OMe
c	0	*m*-CF_3
d	0	*m*-OH
e	0	*m*-Br
f	0	*m*-NO_2
g	0	*p*-OMe
h	0	*p*-CF_3
i	0	*p*-OH
j	0	*p*-Br
k	1	H
l	2	H

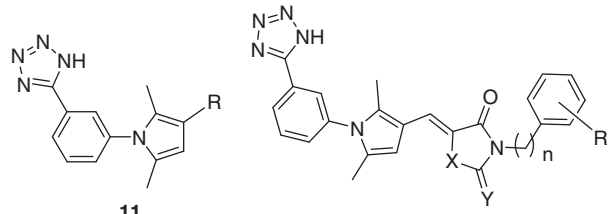
Scheme 2. Synthesis of **12** and **13**. Reagents and conditions: (i) H_2O, 100 °C, 19 h for **12a–g** and **12i–j**; (ii) CH_3OH, reflux, 4 h for **12h**; (iii) (a) CS_2, Et_2O, 0.5 h; (b) $BrCH_2CO_2H$, EtOH, reflux, 1 h for **12k–l**; (iv) NH_4OAc, toluene/CH_3OH, reflux, 3–5 h.



Scheme 3. Synthesis of **16a–b**. Reagents and conditions: (i) (a) triphosgene, Et_3N, CH_2Cl_2, −10 °C, 1 h, (b) corresponding anilines, CH_2Cl_2, 0 °C, 3 h; (ii) (a) NaOH, H_2O, reflux, 1 h, (b) HCl, H_2O, overnight; (iii) piperidine, EtOH, reflux, 8 h.

with an IC_{50} value range of 2.7–12.7 μM, thus indicating a linear multiple aromatic-ring scaffold containing a rhodanine motif greatly favorable to enhance affinity of **13** with the gp41 binding site. The obvious potency of most **13** compounds against HIV-1 replication in the cellular assay provided further supports even though some of them showed inconsistent potency in the cellular assay. Meanwhile, it was found that various substituents at *m*- or *p*-positions on the phenyl ring that connected to rhodanine greatly affected the inhibitory potency in both molecular and cellular assays, although no trend could be observed. Among the *N*-phenylrhodanine derivatives **13b–13f**, the presence of an *m*-substituted methoxy, hydroxyl, or nitro obviously improved the potency against gp41 6-HB formation (see **13b**, **13d** and **13f**) comparing with corresponding *p*-substituted compounds (**13g**, **13i**), but a *m*-trifuloromethyl or *m*-bromo substituent completely abolished the inhibitory activity for gp41 6-HB (see **13c** and **13e**). In contrast,

Table 1
Anti-HIV activity data of target compounds **11**, **13**, and **16**[a]



#	*n*	R	IC_{50}[b] (μM)	EC_{50}[c] (μM)	CC_{50}[d] (μM)	SI[e]
11a	—	CH═$(CN)_2$	NA[f]	NA	>400	—
11b	—	CH═$CHNO_2$	NA	29.3 ± 1.6	78.8 ± 5.2	2.7
11c	—	CH═$C(CN)CO_2Et$	NA	18.0 ± 4.9	76.8 ± 10.5	4.3
11d	—	CH═$C(COOEt)_2$	12.1 ± 2.1	17.2 ± 1.8	77.4 ± 11.7	4.5
13a	0	H	4.4 ± 0.3	3.2 ± 1.1	61.0 ± 7.4	19
13b	0	*m*-OCH_3	3.1 ± 0.1	6.0 ± 0.9	40.6 ± 4.1	6.8
13c	0	*m*-CF_3	NA	6.8 ± 1.1	15.8 ± 0.6	2.3
13d	0	*m*-OH	7.7 ± 0.1	6.3 ± 0.3	35.6 ± 20.2	5.7
13e	0	*m*-Br	NA	9.8 ± 0.1	22.6 ± 0.6	2.3
13f	0	*m*-NO_2	3.2 ± 0.3	23.9 ± 7.2	77.0 ± 1.0	3.2
13g	0	*p*-OCH_3	9.2 ± 1.4	47.9 ± 7.1	87.0 ± 9.1	1.8
13h	0	*p*-CF_3	2.7 ± 0.1	13.1 ± 1.5	27.1 ± 1.9	2.1
13i	0	*p*-OH	20.6 ± 4.9	25.7 ± 4.1	45.6 ± 1.2	1.8
13j	0	*p*-Br	4.6 ± 0.9	2.2 ± 0.4	46.8 ± 7.9	21
13k	1	H	12.7 ± 0.8	12.3 ± 0.5	149.5 ± 8.6	12
13l	2	H	28.1 ± 1.6	4.1 ± 0.4	45.7 ± 16.1	11
16a	0	*p*-OCH_3	22.6 ± 4.7	51.6 ± 2.6	101.4 ± 5.0	2.0
16b	0	*m*-CF_3	41.6 ± 4.6	48.9 ± 2.3	76.5 ± 5.7	1.6
2			25.6	7.7	249.5	32
1			58.7	2.4	335.7	140

[a] Compounds were tested in triplicate and the data was presented as the mean ± SD.
[b] Inhibiting gp41 6-helix bundle formation measured by sandwich ELISA.
[c] Inhibiting p24 production in the MT-2 cells.
[d] Cytotoxicity.
[e] SI: selectivity index (CC_{50}/EC_{50}).
[f] NA: not active.

para-trifuloromethyl and *p*-bromo compounds **13h** and **13j** showed significant high potency against gp41 6-HB (IC_{50} 2.7 and 4.6 μM) and against HIV-1 replication in the cellular assay (EC_{50} 13.1 and 2.2 μM) respectively. In comparison with **13a**, compound **13k** with a one-atom length linker (CH_2) between the rhodanine and its *N*-phenyl rings ($n = 1$) exhibited less potency (IC_{50} 12.7 μM and EC_{50} 12.3 μM) in both assays, but its lower cytotoxicity resulted in a SI value of 12. Another compound **13l** with a two-atom length linker ($n = 2$) was also less potent than **13a** against gp41 6-HB formation even in a similar potent to **13a** in cellular assay. In contract with **13**, the anti-HIV activities of compounds **11a–d** with a conjugated opening side chain were obviously low in cellular assays and did not inhibit gp41 6-HB formation. Furthermore, when the effect of hetero-atom replacement for anti-HIV activity was investigated, results indicated that hydantoin compounds **16a** and **16b** exerted more adverse effect on the anti-HIV-1 activity than the corresponding rhodanine derivatives **13g** and **13c**, implying that the hetero-atom replacement on the rhodanine moiety was not favorable for inhibitory activities of target compounds.

To better understand the nature of the interaction between the new class of small-molecule inhibitors and gp41, docking studies were performed by using the most active compound **13j**, the crystal structure of gp41 (PDB code: 1aik), and Accelrys Discovery Studio 3.0 software. As shown in Figure 3, compound **13j** occupied more space in the gp41 binding pocket and matched well with a complementary shape. Unlike **1** and **2**, a reversal in orientation of molecule **13j** allowed the tetrazolyl group to be in close proximity to the key residue K574, thus forming a 'salt-bridge' (see Fig. 3), as

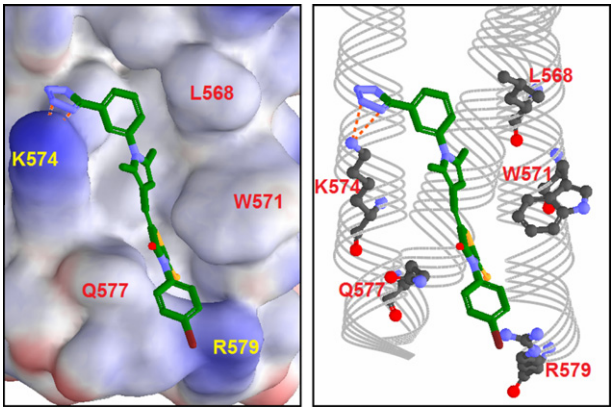


Figure 3. Docking of **13j** in the gp41 hydrophobic cavity (PDB: 1aik). Left: Surface representation of the gp41 core with bound ligand **13j**. Right: The stereoview of **13j** docked, showing possible interactions with the neighboring amino acids and positively charged residue K574 on the surface of binding pocket. The 'salt-bridge' between the negatively charged tetrazolyl and K574 was shown in red dashed line. Compound **13j** and residues K574, Q577, R579, W571 and L568 were shown by stick model with green color and ball–stick model with gray color, respectively.

we expected. Meanwhile, the *p*-bromophenyl moiety of **13j** was pointed toward the positively charged R579, thus providing an expanding linear binding conformation and occupying more space on the binding pocket. Figure 3 also indicated that the up-phenyl and pyrrole rings maintained a T-shaped conformation and that the rhodanine ring inserted into a hydrophobic groove between Q577 and W571. Therefore, the improved inhibitory potency of **13j** might be related to its binding conformation, more interaction points, and the 'salt-bridge' with key amino acid K574.

4. Conclusions

A new series of 5-((1-(3-(1*H*-tereazol-5-yl)phenyl)-2,5-dimenthyl-1*H*-pyrrol-3-yl)methylene)-*N*-phenylrhodanine derivatives has been discovered with obviously improved inhibitory activities against gp41 6-HB formation and HIV-1 replication. The most active compounds, **13a** and **13j**, showed IC_{50} values of 4.4 and 4.6 μM against gp41 6-HB formation, EC_{50} values of 3.2 and 2.2 μM against HIV-1 replication in MT-2 cells, and SI values of 19 and 21, respectively. Structure–activity relationship (SAR) analysis indicated that (1) the new linear molecular scaffold 2, 5-dimenthyl-3-(5-(*N*-phenyl rhodaninyl)methylene)-*N*-(3-(1*H*-tetrazol-5-yl)phenyl)pyrrole is favorable to develop a new class of high potent small-molecule inhibitors targeting gp41, (2) substituent on the phenyl ring connected with the rhodanine ring greatly affect the molecular inhibitory potency in both assays, regardless their substituted positions, (3) rhodanine might be a preferable moiety, rather than opening side chains, such as **11**, and (4) replacement of the rhodanine ring with an isosteric hydantoin ring reduced molecular anti-HIV activity. Docking results provided reasonable support for active compound **13j** with a new multi-aromatic-ring scaffold. Therefore, the most active compounds, **13a** and **13j**, could serve as new starting points for further structure optimization to discover more potent small-molecule fusion inhibitors targeting HIV-1 gp 41.

5. Experimental section

5.1. Chemistry

The proton nuclear magnetic resonance (^1H NMR) spectra were measured on a JNM-ECA-400 (400 MHz) spectrometer using tetramethylsilane (TMS) as internal standard. The solvent used was $CDCl_3$, unless otherwise indicated. Mass spectra (MS) were

measured on API-150 mass spectrometer with the electrospray ionization source from ABI Inc. Melting points were measured with a RY-1 melting apparatus without correction. The microwave reactions were performed on a microwave reactor from Biotage, Inc. All reactions were monitored by thin-layer chromatography (TLC) performed on silica gel GF254 plates. Medium-pressure column chromatography was performed using a CombiFlash Companion purification system. All chemicals were obtained from Beijing Chemical Works or Sigma–Aldrich, Inc. Purities of all target compounds (**11**, **13**, and **16**) were determined by an Agilent 1200 HPLC system with a Eclipse XDB-C18 column, UV detector at 254 nm, mobile MeOH (100%) and flow rate of 1 mL/min.[19]

5.1.1. 3-(2,5-Dimethyl-1*H*-pyrrol-1-yl)benzonitrile (4)

To a suspension of 3-aminobenzonitrile **3** (2.36 g, 20 mmol) in 3 mL of glacial acetic acid was added 2,5-hexanedione (2.5 mL, 21 mmol) and irradiated under microwave at 150 °C with stirring for 30 min. The mixture was dissolved in acetone (ca. 20 mL), poured into ice water, followed by collection of the precipitated solid, which was washed with water to neutral and dried to afford 3.7 g of **4**, gray solid, 94% yield. Mp 92–94 °C; ^{1}H NMR δ ppm 7.72 (1H, d, J = 8.0 Hz, ArH-4), 7.60 (1H, t, J = 8.0 Hz, ArH-5), 7.52 (1H, s, ArH-2), 7.49 (1H, d, J = 8.0 Hz, ArH-6), 5.93 (2H, s, PyH-3, 4), 2.03 (6H, s, $CH_3 \times 2$); MS m/z 197 (M+1, 100).

5.1.2. 3-(2,5-Dimethyl-3-formyl-1*H*-pyrrol-1-yl) benzonitrile (5)

Anhydrous DMF (5 mL) was placed in a dried 50-mL 3-neck flask equipped with a temperature thermometer. Then 1.4 mL of $POCl_3$ (15.5 mmol) was added dropwise into the above flask with stirring and controlled below 10 °C with ice bath. After another 30 min stirring to ensure complete conversion to the Vilsmeier reagent, a solution of **4** (1.96 g, 10 mmol) in 15 mL DMF was added slowly for 20 min keeping the temperature below 10 °C with stirring, and then reacted for another 2 h at room temperature. After quenching with ice, the solution was basified with 5% NaOH aq to pH 9–10 and then rapidly heated to reflux for 30 min. The mixture was slowly poured into ice water with stirring and left to stay overnight. The precipitated solid was filtered out, washed with water to neutral, and dried to afford 1.95 g of **5**, gray solid, 87% yield. Mp 114–116 °C; ^{1}H NMR δ ppm 9.89 (1H, s, CHO), 7.80 (1H, d, J = 8.0 Hz, ArH-4), 7.69 (1H, t, J = 8.0 Hz, ArH-5), 7.55 (1H, s, ArH-2), 7.51 (1H, d, J = 8.0 Hz, ArH-6), 6.42 (1H, s, PyH-4), 2.30 and 2.00 (each 3H, s, $CH_3 \times 2$); MS m/z 225 (M+1, 100).

5.1.3. 2,5-Dimethyl-1*H*-pyrrole-1-(3-(1*H*-tetrazol-5-yl) phenyl)-3-carbaldehyde (6)

The mixture of **5** (4.5 g, 20.0 mmol), sodium azide (1.7 g, 26.0 mmol), and triethylamine hydrochloride (3.6 g, 26.0 mmol) in toluene (40 mL) with water (40:1, v/v) was heated to reflux for 12 h. When the reaction finished, the product was extracted with water and the combined aqueous phase acidified by 36% HCl to pH 2–3. The precipitate was collected, washed with water to neutral, and dried to afford 4.8 g of crude product **6**, gray solid, 90% yield. Mp 180 °C (dec); ^{1}H NMR (acetone-d_6) δ ppm 9.90 (1H, s, CHO), 8.34 (1H, d, J = 8.0 Hz, ArH-4), 8.09 (1H, s, ArH-2), 7.87 (1H, t, J = 8.0 Hz, ArH-5), 7.63 (1H, d, J = 8.0 Hz, ArH-6), 6.35 (1H, s, PyH-4), 2.30 and 2.00 (each 3H, s, $CH_3 \times 2$); MS m/z 266 (M−1, 100).

5.1.4. General procedure for preparation of 11a–d

A mixture of **6** and an active methylene reagent in 10 mL of ethanol in the presence of piperidine (ca. 0.1 mL) was refluxed for 3–5 h and monitored by TLC until reaction was completely finished. The reaction solution was poured into ice water, acidified with 5% HCl to pH 2–3 and extracted with ethyl acetate three times. After removal of solvent under reduced pressure, the residue was purified with a flash silica column [gradual eluant: EtOAc/petroleum ether with AcOH (3%), 0–30%] to afford pure products.

5.1.5. 5-(3-(3-(2,2-Dicyanovinyl)-2,5-dimethyl-1*H*-pyrrol-1-yl)phenyl)-1*H*-tetrazole (11a)

Starting with **6** (110 mg, 0.4 mmol) and malononitrile **7** (60 mg, 0.9 mmol) to afford 42 mg of **11a**, brown powder, 33% yield. Mp 142–144 °C; ^{1}H NMR δ ppm 8.35 (1H, d, J = 8.0 Hz, ArH-4), 8.04 (1H, s, ArH-2), 7.76 (1H, t, J = 8.0 Hz, ArH-5), 7.64 (1H, s, =CH), 7.39 (1H, d, J = 8.0 Hz, ArH-6), 6.95 (1H, s, PyH-4), 2.21 and 2.06 (each 3H, s, $CH_3 \times 2$); MS m/z 314 (M−1, 100); HPLC purity 97.95%.

5.1.6. 5-(3-(3-(2-Nitrovinyl)-2,5-dimethyl-1*H*-pyrrol-1-yl) phenyl)-1*H*-tetrazole (11b)

Starting with **6** (267 mg, 1.0 mmol) and nitromethane **8** (0.2 mL, 1.8 mmol) to afford 193 mg of **11b**, yellow solid, 62% yield. Mp 112–114 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.21 (1H, d, J = 8.0 Hz, ArH-4), 8.10 (1H, d, J = 12.8 Hz, =$CHNO_2$), 7.94 (1H, s, ArH-2), 7.81–7.78 (2H, m, ArH-5 and CH=C-NO_2), 7.62 (1H, d, J = 8.0 Hz, ArH-6), 6.52 (1H, s, PyH-4), 2.19 and 2.01 (each 3H, s, $CH_3 \times 2$); MS m/z 311 (M+1, 100); HPLC purity 95.72%.

5.1.7. 5-(3-(3-(2-Cyano-2-ethoxycarboxylvinyl)-2,5-dimethyl-1*H*-pyrrol-1-yl) phenyl)-1*H*-tetrazole (11c)

Starting with **6** (110 mg, 0.4 mmol) and ethyl 2-cyanoacetate **9** (90 mg, 0.8 mmol) to afford 86 mg of **11c**, yellow solid, 60% yield. Mp 170–172 °C; ^{1}H NMR δ ppm 8.38 (1H, d, J = 8.0 Hz, ArH-4), 8.19 (1H, s, =CH), 8.06 (1H, s, ArH-2), 7.75 (1H, t, J = 8.0 Hz, ArH-5), 7.40 (1H, d, J = 8.0 Hz, ArH-6), 6.99 (1H, s, PyH-4), 4.32 (2H, q, J = 6.8 Hz, CH_2), 2.22 and 2.03 (each 3H, s, $CH_3 \times 2$), 1.37 (3H, t, J = 6.8 Hz, CH_3); MS m/z 361 (M−1, 100); HPLC purity 97.30%.

5.1.8. 5-(3-(3-(2,2-Diethoxycarboxylvinyl)-2,5-dimethyl-1*H*-pyrrol-1-yl) phenyl)-1*H*-tetrazole (11d)

Starting with **6** (133 mg, 0.5 mmol) and diethyl malonate **10** (80 mg, 0.5 mmol) to afford 76 mg of **11d**, yellow solid, 37% yield. Mp 92–94 °C; ^{1}H NMR δ ppm 8.30 (1H, d, J = 8.0 Hz, ArH-4), 7.95 (1H, s, ArH-2), 7.71 (1H, s, =CH), 7.67 (1H, t, J = 8.0 Hz, ArH-5), 7.34 (1H, d, J = 8.0 Hz, ArH-6), 6.04 (1H, s, PyH-4), 4.42 and 4.27 (each 2H, q, J = 7.2 Hz, $CH_2CH_3 \times 2$), 2.10 and 1.94 (each 3H, s, $CH_3 \times 2$), 1.41 and 1.31 (each 3H, t, J = 7.2 Hz, $CH_2CH_3 \times 2$); MS m/z 408 (M−1, 20), 264 (M−145, 100); HPLC purity 99.04%.

5.1.9. General procedure for preparation of 3-aryl-2-thioxothiazolidin-4-one 12a–d and 12f–j

A suspension of aniline analogues (1 equiv) in water (ca. 2 mL/mmol) was heated to 95 °C until the aniline was fully dissolved. Then bis(carboxylmethyl) trithiocarbonate (1.1 equiv) was added and heated at 100 °C for 19 h. After cooling to room temperature, the precipitated solid was filtered out and washed with water. The dried solid was purified by flash silica column [gradual eluent: EtOAc/petroleum ether, 0–50%] to afford the rhodanine derivatives. The preparations of **12c**, **12g**, and **12i** were reported in our previous publication.[13]

5.1.10. 3-Phenyl-2-thioxothiazolidin-4-one (12a)

Starting with aniline (465 mg, 5 mmol) to afford 730 mg of **12a**, pale-yellow solid, 70% yield. Mp 184–186 °C; ^{1}H NMR δ ppm 7.57–7.19 (5H, m, ArH × 5), 4.20 (2H, s, SCH_2); MS m/z 208 (M−1, 100).

5.1.11. 3-(3-Methoxyphenyl)-2-thioxothiazolidin-4-one (12b)

Starting with 3-methoxyaniline (615 mg, 5 mmol) to afford 800 mg of **12b**, pale-yellow solid, 67% yield. Mp 112–114 °C; ^{1}H NMR δ ppm 7.44 (1H, t, J = 8.0 Hz, ArH-5), 7.03 (1H, dd, J = 2.4 and 8.0 Hz, ArH-4), 6.79 (1H, d, J = 8.0 Hz, ArH-6), 6.72 (1H, t,

J = 2.4 Hz, ArH-2), 4.18 (2H, s, SCH_2), 3.82 (3H, s, 3′-OCH_3); MS m/z 240 (M+1, 100).

5.1.12. 3-(3-Hydroxyphenyl)-2-thioxothiazolidin-4-one (12d)

Starting with 3-hydroxyaniline (545 mg, 5 mmol) to afford 720 mg of **12d**, pale-yellow solid, 64% yield. Mp 192–194 °C; ^{1}H NMR δ ppm 8.89 (1H, s, OH), 7.34 (1H, t, J = 8.0 Hz, ArH-5), 6.98 (1H, dd, J = 2.4 and 8.0 Hz, ArH-6), 6.69–6.65 (2H, m, ArH-2, 4), 4.17 (2H, s, SCH_2); MS m/z 226 (M+1, 100).

5.1.13. 3-(3-Bromophenyl)-2-thioxothiazolidin-4-one (12e)

Starting with 3-bromoaniline (688 mg, 4 mmol) to afford 820 mg of **12e**, pale-yellow solid, 71% yield. Mp 176–180 °C; ^{1}H NMR δ ppm 7.64 (1H, d, J = 8.0 Hz, ArH-6), 7.41 (1H, t, J = 8.0 Hz, ArH-5), 7.38 (1H, s, ArH-2), 7.17 (1H, d, J = 8.0 Hz, ArH-4), 4.20 (2H, s, SCH_2); MS m/z 285.6 (M−1, 100).

5.1.14. 3-(3-Nitrophenyl)-2-thioxothiazolidin-4-one (12f)

Starting with 3-nitroaniline (552 mg, 4 mmol) to afford 884 mg of **12f**, pale-yellow solid, 87% yield. Mp 166–168 °C; ^{1}H NMR δ ppm 8.38 (1H, d, J = 8.4 Hz, ArH-4), 8.17 (1H, t, J = 2.0 Hz, ArH-2), 7.76 (1H, t, J = 8.4 Hz, ArH-5), 7.61 (1H, d, J = 8.4 Hz, ArH-6), 4.27 (2H, s, SCH_2); MS m/z 253 (M−1, 100).

5.1.15. 3-(4-Bromophenyl)-2-thioxothiazolidin-4-one (12j)

Starting with 4-bromoaniline (860 mg, 5 mmol) to afford 643 mg of **12j**, pale-yellow solid, 45% yield. Mp 162–164 °C; ^{1}H NMR δ ppm 7.67 (2H, d, J = 6.8 Hz, ArH-2, 6), 7.09 (2H, d, J = 6.8 Hz, ArH-3, 5), 4.19 (2H, s, SCH_2); MS m/z 288 (M+1, 100).

5.1.16. 3-(4-(Trifluoromethyl)phenyl)-2-thioxothiazolidin-4-one (12h)

A solution of 4-(trifluoromethyl)phenyl isothiocyanate (609 mg, 3.0 mmol), 2-mercaptoacetic acid (300 mg, 3.25 mmol) in methanol (10 mL), and water (20 mL) was heated to reflux for 4 h. After cooling to room temperature, the mixture was poured into water, and the solid was filtered out and purified by flash silica column [gradual eluant: EtOAc/petroleum ether, 0–40%] to afford 450 mg of **12h**, pale-yellow solid, 54% yield. Mp 140–142 °C; ^{1}H NMR δ ppm 7.82 (d, 2H, J = 8.4 Hz, ArH-2, 6), 7.38 (d, 2H, J = 8.4 Hz, ArH-3, 5), 4.23 (s, 2H, SCH_2); MS m/z 276 (M−1, 100).

5.1.17. 3-Benzyl-2-thioxothiazolidin-4-one (12k)

To a solution of benzylamine (5 mL, 45.8 mmol) in ether was dropwise added carbon disulfide (2.7 mL, 45.8 mmol, mole ratio 1:1) in ether in ice-bath for 10 min with stirring. After another 1 h, the precipitate ammonium dithiocarbamates was collected, washed with ether, and dried at room temperature. The mixture of produced ammonium dithiocarbamates (6.42 g) and bromoacetic acid (3.2 g, 23 mmol) in ethanol was heated to reflux for 5 h, and then poured into ice water. The product was extracted with EtOAc three times, removed solvent under reduced pressure, and purified by flash silica column [gradual eluant: EtOAc/petroleum ether, 0–40%] to afford 4.18 g of **12k**, pale-yellow solid, 41% yield, mp 72–74 °C; ^{1}H NMR δ ppm 7.39–7.22 (5H, m, ArH), 4.59 (2H, s, NCH_2Ar), 4.00 (2H, s, SCH_2); MS m/z 91 ($PhCH_2^+$, 100), 224 (M+1, 50).

5.1.18. 3-Phenylethyl-2-thioxothiazolidin-4-one (12l)

The preparation was the same as that of compound **12k**. Starting with 3-trifluoromethylbenzylamine (1 mL, 8 mmol) and carbon sulfide (0.5 mL, 8.3 mmol) to obtain 1.23 g of 3-trifluoromethylbenzylammonium dithiocarbamate, following the reaction with bromoacetic acid (560 mg, 4 mmol) to afford 700 mg of **12l**, white solid, 37% yield (calculated by amine). Mp 86–88 °C; ^{1}H NMR δ ppm 7.35–7.22 (5H, m, ArH), 4.22 (2H, t, J = 8.0 Hz, N–CH_2), 3.93 (2H, s, 5-H), 2.93 (2H, t, J = 8.0 Hz, CH_2-Ar); MS m/z 238 (M+1, 100).

5.1.19. General procedure for preparation of 13a–l

A mixture of **6** and 3-aryl-2-thioxothiazolidine-4-one (**13a–l**, mole ratio 1:1) in the presence of NH_4OAc (100 mg) in toluene (20 mL) and methanol (10 mL) was heated to reflux for 3–5 h. After the reaction was finished, ethyl acetate was added to the solution, and organic phase was washed with water and brine, successively, and dried over Na_2SO_4. After removal of solvent under reduced pressure, the residue was purified by flash silica column [gradual eluant: EtOAc/petroleum ether with AcOH (3%), 0–60%] to give the pure product.

5.1.20. 3-Phenyl-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13a)

Starting with **6** (133 mg, 0.5 mmol) and **12a** (105 mg, 0.5 mmol) to afford 132 mg of **13a**, yellow solid, 58% yield. Mp 184–188 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.21 (1H, d, J = 8.0 Hz, ArH-4), 7.98 (1H, s, ArH), 7.84 (1H, t, J = 8.0 Hz, ArH-5), 7.75 (1H, s, =CH), 7.66 (1H, d, J = 8.0 Hz, ArH-6), 7.56–7.38 (5H, m, ArH × 5), 6.38 (1H, s, PyH-4), 2.23 and 2.09 (each 3H, s, CH_3 × 2); MS m/z 457 (M−1, 100); HPLC purity 98.28%.

5.1.21. 3-(3-Methoxyphenyl)-5-((1-(3-(1*H*-tetrazol-5-yl) phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13b)

Starting with **6** (133 mg, 0.5 mmol) and **12b** (120 mg, 0.5 mmol) to afford 151 mg of **13b**, yellow solid, 62% yield. Mp 148–150 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.22 (1H, d, J = 7.6 Hz, ArH-4), 7.97 (1H, s, ArH-2), 7.85 (1H, t, J = 7.6 Hz, ArH-5), 7.73 (1H, s, =CH), 7.66 (1H, d, J = 7.6 Hz, ArH-6), 7.46 (1H, t, J = 8.0 Hz, ArH-5′), 7.09 (1H, dd, J = 2.4 and 8.0 Hz, ArH-6′), 7.02–6.93 (2H, m, ArH), 6.37 (1H, s, PyH-4), 3.79 (3H, s, OCH_3), 2.23 and 2.09 (each 3H, s, CH_3 × 2); MS m/z 487 (M−1, 100); HPLC purity 98.39%.

5.1.22. 3-(3-(Trifluoromethyl)phenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13c)

Starting with **6** (133 mg, 0.5 mmol) and **12c** (140 mg, 0.5 mmol) to afford 151 mg of **13c**, yellow solid, 57% yield. Mp 92–94 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.19 (1H, d, J = 7.6 Hz, ArH-4), 7.94–7.72 (7H, m, =CH, ArH), 7.50 (1H, d, J = 7.2 Hz, ArH-4′), 6.38 (1H, s, PyH-4), 2.23 and 2.08 (each 3H, s, CH_3 × 2); MS m/z 525 (M−1, 100); HPLC purity 98.91%.

5.1.23. 3-(3-Hydroxyphenyl)-5-((1-(3-(1*H*-tetrazol-5-yl) phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13d)

Starting with **6** (133 mg, 0.5 mmol) and **12d** (113 mg, 0.5 mmol) to afford 145 mg of **13d**, yellow solid, 61% yield. Mp 268–270 °C (dec); ^{1}H NMR (DMSO-d_6) δ ppm 9.82 (1H, s, OH), 8.21 (1H, d, J = 8.0 Hz, ArH-4), 7.97 (1H, s, ArH-2), 7.83 (1H, t, J = 8.0 Hz, ArH-5), 7.72 (1H, s, =CH), 7.64 (1H, d, J = 8.0 Hz, ArH-6), 7.33 (1H, t, J = 7.6 Hz, ArH-5′), 6.90 (1H, d, J = 7.6 Hz, ArH-6′), 6.73 (2H, m, ArH), 6.36 (1H, s, PyH-4), 2.23 and 2.09 (each 3H, s, CH_3 × 2); MS m/z 473 (M−1, 100); HPLC purity 98.80%.

5.1.24. 3-(3-Bromophenyl)-5-((1-(3-(1*H*-tetrazol-5-yl) phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13e)

Starting with **6** (133 mg, 0.5 mmol) and **12e** (144 mg, 0.5 mmol) to afford 160 mg of **13e**, yellow solid, 60% yield. Mp 108–110 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.13 (1H, d, J = 8.0 Hz, ArH-4), 7.81 (1H, s, ArH-2), 7.75–7.71 (3H, m, =CH, ArH), 7.61 (1H, t, J = 8.0 Hz, ArH-5), 7.53 (1H, t, J = 8.0 Hz, ArH-5′), 7.47–7.45 (1H, m, ArH), 7.28–7.26 (1H, m, ArH), 6.35 (1H, s, PyH-4), 2.22 and 2.07 (each 3H, s, CH_3 × 2); MS m/z 535 (M−1, 100); HPLC purity 99.24%.

5.1.25. 3-(3-Nitrophenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13f)

Starting with **6** (133 mg, 0.5 mmol) and **12f** (127 mg, 0.5 mmol) to afford 150 mg of **13f**, yellow solid, 60% yield. Mp 140–142 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.46 (1H, s, ArH-2′), 8.39 (1H, d, J = 8.0 Hz, ArH-4′), 8.23 (1H, d, J = 8.0 Hz, ArH-4), 7.98–7.95 (2H, m, ArH), 7.87 (1H, t, J = 8.0 Hz, ArH-5′), 7.84 (1H, t, J = 8.0 Hz, ArH-5), 7.76 (1H, s, =CH), 7.65 (1H, d, J = 8.0 Hz, ArH-6), 6.40 (1H, s, PyH-4), 2.45 and 2.09 (each 3H, s, $CH_3 \times 2$); MS m/z 502 (M−1, 100); HPLC purity 97.04%.

5.1.26. 3-(4-Methoxyphenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13g)

Starting with **6** (133 mg, 0.5 mmol) and **12g** (120 mg, 0.5 mmol) to afford 180 mg of **13g**, yellow solid, 73% yield. Mp 256–258 °C (dec); ^{1}H NMR (DMSO-d_6) δ ppm 8.22 (1H, d, J = 8.0 Hz, ArH-4), 7.97 (1H, s, ArH-2), 7.84 (1H, t, J = 8.0 Hz, ArH-5), 7.73 (1H, s, =CH), 7.64 (1H, d, J = 8.0 Hz, ArH-6), 7.30 (2H, d, J = 8.8 Hz, ArH-2′, 6′), 7.09 (2H, d, J = 8.8 Hz, ArH-3′, 5′), 6.37 (1H, s, PyH-4), 3.82 (3H, s, OCH_3), 2.23 and 2.09 (each 3H, s, $CH_3 \times 2$); MS m/z 487 (M−1, 100); HPLC purity 99.15%.

5.1.27. 3-(4-(Trifluoromethyl)phenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13h)

Starting with **6** (133 mg, 0.5 mmol) and **12h** (140 mg, 0.5 mmol) to afford 145 mg of **13h**, yellow solid, 55% yield. Mp 156–158 °C (dec); ^{1}H NMR (DMSO-d_6) δ ppm 8.22 (1H, d, J = 7.8 Hz, ArH-4), 7.97 (3H, m, ArH), 7.83 (1H, t, J = 7.8 Hz, ArH-5), 7.76 (1H, s, =CH), 7.71 (2H, d, ArH-3′, 5′), 7.66 (1H, d, J = 7.8 Hz, ArH-6), 6.39 (1H, s, PyH-4), 2.24 and 2.09 (each 3H, s, $CH_3 \times 2$); MS m/z 525 (M−1, 100); HPLC purity 95.96%.

5.1.28. 3-(4-Hydroxyphenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13i)

Starting with **6** (133 mg, 0.5 mmol) and **12i** (113 mg, 0.5 mmol) to afford 161 mg of **13i**, yellow solid, 68% yield. Mp 156–158 °C (dec); ^{1}H NMR (DMSO-d_6) δ ppm 9.87 (1H, s, OH), 8.22 (1H, d, J = 8.0 Hz, ArH-4), 7.96 (1H, s, ArH-2), 7.85 (1H, t, J = 8.0 Hz, ArH-5), 7.72 (1H, s, =CH), 7.65 (1H, d, J = 8.0 Hz, ArH-6), 7.15 (2H, d, J = 8.8 Hz, ArH-2′,6′), 6.89 (2H, d, J = 8.8 Hz, ArH-3′, 5′), 6.36 (1H, s, PyH-4), 2.22 and 2.08 (each 3H, s, $CH_3 \times 2$); MS m/z 473 (M−1, 100); HPLC purity 95.59%.

5.1.29. 3-(4-Bromophenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13j)

Starting with **6** (133 mg, 0.5 mmol) and **12j** (144 mg, 0.5 mmol) to afford 183 mg of **13j**, yellow solid, 68% yield. Mp 274–276 °C (dec); ^{1}H NMR (DMSO-d_6) δ ppm 8.22 (1H, d, J = 8.0 Hz, ArH-4), 7.97 (1H, s, ArH-2), 7.78–7.74 (3H, m, =CH, ArH), 7.66 (1H, d, J = 8.0 Hz, ArH-6), 7.40 (2H, d, ArH-3′, 5′), 6.38 (1H, s, PyH-4), 2.23 and 2.09 (each 3H, s, $CH_3 \times 2$); MS m/z 537 (M−1, 100); HPLC purity 97.36%.

5.1.30. 3-Benzyl-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13k)

Starting with **6** (133 mg, 0.5 mmol) and **12k** (112 mg, 0.5 mmol) to afford 210 mg of **13k**, yellow solid, 90% yield. Mp 180–182 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.21 (1H, d, J = 8.0 Hz, ArH-4), 7.95 (1H, s, ArH-2), 7.82 (1H, t, J = 8.0 Hz, ArH-5), 7.78 (1H, s, =CH), 7.63 (1H, d, J = 8.0 Hz, ArH), 7.51–7.32 (5H, m, ArH × 5), 6.29 (1H, s, PyH-4), 4.70 (2H, s, $ArCH_2$), 2.20 and 2.05 (each 3H, s, $CH_3 \times 2$); MS m/z 471 (M−1, 100); HPLC purity 97.25%.

5.1.31. 3-(Phenylethyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-2-thioxothiazolidin-4-one (13l)

Starting with **6** (133 mg, 0.5 mmol) and **12l** (112 mg, 0.5 mmol) to afford 192 mg of **13n**, yellow solid, 79% yield. Mp 148–150 °C; ^{1}H NMR (DMSO-d_6) δ ppm 8.22 (1H, d, J = 8.0 Hz, ArH-4), 7.96 (1H, s, ArH-2), 7.85 (1H, t, J = 8.0 Hz, ArH-5), 7.70 (1H, s, =CH), 7.65 (1H, d, J = 8.0 Hz, ArH-6), 7.33–7.21 (5H, m, ArH × 5), 6.31 (1H, s, PyH-4), 4.23 (2H, t, J = 8.0 Hz, N-CH_2), 2.95 (2H, t, J = 8.0 Hz, CH_2-Ar), 2.21 and 2.06 (each 3H, s, $CH_3 \times 2$); MS m/z 485 (M−1, 100); HPLC purity 99.72%.

5.1.32. Ethyl 2-(3-(4-methoxyphenyl)ureido)acetate (14a)

Triethylamine (17 mL) in dichloromethane (30 mL) was slowly added to a mixture of ethyl glycinate hydrochloride (4.2 g, 30 mmol) and triphosgene (3.0 g, 10 mmol) in dichloromethane (20 mL) at −10 °C for 30 min. The mixture was warmed to 0 °C with stirring for 1 h and then added dropwise to a solution of 4-methoxyphenylamine (3.7 g, 30 mmol) in 15 mL dichoromethane at 0 °C for 20 min with continued stirring at room temperature for 6 h. The solid precipitate was filtered out, and the filtrate was washed with water, 5% HCl aq, and brine, successively, and then dried over Na_2SO_4. After removal of solvent under reduced pressure, the crude product was purified by flash silica column [gradual eluant: EtOAc/petroleum ether, 0–30%] to afford 4.54 g of **14a**, white solid, 60% yield. Mp 122–124 °C; ^{1}H NMR δ ppm 7.23 (2H, d, J = 6.8 Hz, ArH-2, 6), 6.86 (2H, d, J = 6.8 Hz, ArH-3, 5), 6.72 (1H, br s, NH), 5.42 (1H, br s, NH), 4.21 (2H, q, J = 7.6 Hz, CH_2CH_3), 4.03 (2H, d, J = 5.6 Hz, CH_2N), 3.79 (3H, s, OCH_3), 1.27 (3H, t, J = 7.6 Hz, CH_2CH_3); MS m/z 253 (M+1, 100), 275 (M+23, 60).

5.1.33. Ethyl 2-(3-(3-(trifluoromethyl) phenyl)ureido) acetate (14b)

The preparation was the same as **14a**. Starting with 3-trifluoromethylphenylamine (4.8 g, 30 mmol) to afford 5.3 g of **14b** with a 61% yield, white solid. Mp 100–102 °C; ^{1}H NMR δ ppm: 7.60 (1H, s, ArH-2), 7.41 (1H, d, J = 8.4 Hz, ArH-6), 7.30 (2H, m, ArH-5, NH), 7.23 (1H, d, J = 8.0 Hz, ArH-4), 5.73 (1H, br s, NH), 4.25 (2H, q, J = 7.2 Hz, CH_2CH_3), 4.03 (2H, d, J = 5.6 Hz, CH_2N), 1.30 (3H, t, J = 7.2 Hz, CH_2CH_3); MS m/z 291 (M+1, 100).

5.1.34. 3-(4-Methoxyphenyl)imidazolidine-2,4-dione (15a)

To a suspension of **14a** (1.08 g, 4 mmol) in 10 mL water was added 4 mL of 10% NaOH aq and heated to reflux for 1 h. After cooling to room temperature, the solution was acidified by 36% HCl to pH 1–2 and heated again to reflux overnight. When staying at room temperature, the solid precipitate was collected and dried to afford 530 mg of **15a**, white needle solid, 64% yield. Mp 208–210 °C; ^{1}H NMR δ ppm 7.31 (2H, d, J = 8.8 Hz, ArH-2, 6), 7.01 (2H, d, J = 8.8 Hz, ArH-3, 5), 6.16 (1H, br s, CONH), 4.13 (2H, s, NCH_2), 3.84 (3H, s, OCH_3); MS m/z 207 (M+1, 100), 224 (M+18, 70), 229 (M+23, 80).

5.1.35. 2,3-(3-(Trifluoromethyl)phenyl)imidazolidine-2,4-dione (15b)

The preparation was the same as **15a**. Starting with **14b** (1.16 g, 4 mmol) to afford **15b** with 40% yield, white needle solid, mp 112–114 °C; ^{1}H NMR δ ppm 7.75 (1H, s, ArH-2), 7.67–7.59 (3H, m, ArH), 6.06 (1H, br s, CONH), 4.18 (2H, s, NCH_2); MS m/z 243 (M−1, 100).

5.1.36. 3-(4-Methoxyphenyl)-5-((1-(3-(1*H*-tetrazol-5-yl)phenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene) imidazolidine-2,4-dione (16a)

A mixture of **6** (133 mg, 0.5 mmol) and **15a** (105 mg, 0.5 mmol) in ethanol (10 mL) in the presence of piperidine (0.2 mL) was heated to reflux for 8 h and then poured into ice water, and pH

was adjusted with 5% HCl to 2–3, stirring overnight. The yellow solid was filtered out and dried to afford 183 mg of **16a**, pale-yellow solid, 80% yield. Mp 190–192 °C; ^{1}H NMR (DMSO-d_6) δ ppm 10.41 (1H, s, CONH), 8.19 (1H, d, J = 8.0 Hz, ArH-4), 7.93 (1H, s, ArH-2), 7.81 (1H, t, J = 8.0 Hz, ArH-5), 7.61 (1H, d, J = 8.0 Hz, ArH-6), 7.35 (2H, d, J = 8.8 Hz, ArH-2′, 6′), 7.05 (2H, d, J = 8.8 Hz, ArH-3′, 5′), 6.79 (1H, s, PyH-4), 6.61 (1H, s, =CH), 3.80 (3H, s, OCH_3), 2.14 and 2.05 (each 3H, s, $CH_3 \times 2$); MS m/z 454 (M−1, 100); HPLC purity 98.28%.

5.1.37. 3-(3-(Trifluoromethyl)phenyl)-5-((1-(3-(1H-tetrazol-5-yl)phenyl)-2, 5-dimethyl-1H-pyrrol-3-yl)methylene) imidazolidine-2,4-dione (16b)

The preparation was the same as **16a**. Starting with **6** (133 mg, 0.5 mmol) and **15b** (125 mg, 0.5 mmol) to afford 210 mg of **16b**, pale-yellow solid, 85% yield. Mp 174–176 °C; ^{1}H NMR (DMSO-d_6) δ ppm 10.57 (1H, s, CONH), 8.19 (1H, d, J = 8.0 Hz, ArH-4), 7.93 (1H, s, ArH-2), 7.84–7.76 (4H, m, ArH), 7.59 (1H, d, J = 8.0 Hz, ArH-6), 6.82 (1H, s, PyH-4), 6.66 (1H, s, =CH), 2.15 and 2.05 (each 3H, s, $CH_3 \times 2$); MS m/z 492 (M−1, 100); HPLC purity 97.36%.

5.2. Sandwich ELISA for detecting the gp41 6-HB formation

A sandwich ELISA as previously described[27] was used to test the inhibitory activity of the compounds on gp41 six-helix bundle formation. Briefly, peptide N36 (2 μM) was preincubated with the compound at graded concentrations at 37 °C for 30 min, followed by addition of C34 (2 μM). In the control experiments, N36 was preincubated with C34 or PBS at 37 °C for 30 min in the absence of the compounds tested. After incubation at 37 °C for 30 min, the mixture was added to wells of a 96-well polystyrene plate which were precoated with IgG (10 μg/mL) purified from rabbit antisera directed against the gp41 six-helix bundle. Then, the mAb NC-1, biotin-labeled goat-anti-mouse IgG, SA-HRP, and TMB were added sequentially. Absorbance at 450 nm was read, and the percentage of inhibition by the compounds was calculated as previously described.[27] All the samples were tested in triplicate.

5.3. Determination of the inhibitory activity of the compounds on HIV-1 replication

The inhibitory activity of compounds on HIV-1IIIB replication in MT-2 cells was determined as previously described.[12,28] Briefly, 1×10^4 MT-2 cells were infected with an HIV-1_{IIIB} strain (100 $TCID_{50}$) in 200 μL of RPMI 1640 medium containing 10% PBS in the presence or absence of a test compound at graded concentrations overnight. Next, the culture supernatants were removed, and fresh media containing no test compounds were added. On the fourth day post-infection, 100 μL of culture supernatants were collected from each well, mixed with equal volumes of 5% Triton X-100, and assayed for p24 antigen, which was quantitated by ELISA. Briefly, wells of polystyrene plates (Immulon 1B, Dynex Technology, Chantilly, VA) were coated with HIV immunoglobulin (HIVIG), which was prepared from plasma of HIV-seropositive donors with high neutralizing titers against HIV-1_{IIIB}, in 0.085 M carbonate–bicarbonate buffer (pH 9.6) at 4 °C overnight, followed by washing with buffer (0.01 M PBS containing 0.05% Tween-20) and blocking with PBS containing 1% dry fat-free milk (Bio-Rad, Inc., Hercules, CA). Virus lysates were added to the wells and incubated at 37 °C for 1 h. After extensive washes, anti-p24 mAb (183-12H-5C), biotin-labeled anti-mouse IgG1 (Santa Cruz Biotechnolgy, Santa Cruz, CA), streptavidin-labeled horseradish peroxidase (Zymed, S. San Francisco, CA), and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) were added sequentially. Reactions were terminated by addition of 1 N H_2SO_4. Absorbance at 450 nm was recorded in an ELISA reader (Ultra 386, TECAN, Research Triangle Park, NC). Recombinant protein p24 purchased from United States Biological (Swampscott, MA) was included to establish standard dose–response curves. Each sample was tested in triplicate. The percentage of inhibition of p24 production was calculated as previously described.[29] EC_{50} values were calculated using the computer program CalcuSyn, kindly provided by Dr. T. C. Chou (Sloan-Kettering Cancer Center, New York).

5.4. Assessment of in vitro cytotoxicity

The in vitro cytotoxicity of compounds on MT-2 cells was measured by XTT assay.[28] Briefly, 100 μL of the test compound at graded concentrations was added to equal volumes of cells (5×10^5/mL) in wells of 96-well plates. After incubation at 37 °C for 4 days, 50 μL of XTT solution (1 mg/mL) containing 0.02 μM of phenazine methosulphate (PMS) was added. After 4 h, the absorbance at 450 nm was measured with an ELISA reader. The CC_{50} (concentration for 50% cytotoxicity) values were calculated using the CalcuSyn program.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.047. These data include MOL files and InChiKeys of the most important compounds described in this article.

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