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Original article

Development of benzimidazole derivatives to inhibit HIV-1 replication through protecting APOBEC3G protein

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ABSTRACT

Human APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G, A3G) is a potent restriction factor against human immunodeficiency virus type 1 (HIV-1) by inducing hypermutation of G to A in viral genome after its incorporation into virions. HIV-1 Vif (Virion Infectivity Factor) counteracts A3G by inducing ubiquitination and proteasomal degradation of A3G protein. Vif-A3G axis therefore is a promising therapeutic target of HIV-1. Here we report the screening, synthesis and SAR studies of benzimidazole derivatives as potent inhibitors against HIV-1 replication via protecting A3G protein. Based on the steep SAR of the benzimidazole scaffold, we identified compound **14** and **26** which provided the best potency, with IC_{50} values of 3.45 nM and 58.03 nM respectively in the anti-HIV-1 replication assay in H9 cells. Compound **14** and **26** also afforded protective effects on A3G protein level. Both compounds have been proved to be safe in acute toxicological studies. Taken together, we suggest that these two benzimidazole derivatives can be further developed as a new category of anti-HIV-1 leads.

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1. Introduction

Human immunodeficiency virus-1 (HIV-1) has been a worldwide epidemic for more than 30 years. Significant progress has been made in high active antiretroviral therapy (HAART) which has been the major clinical method to reduce AIDS mortality and mobility. However, the life-long cost and serious side effects of current therapeutics have been one of the obstacles to eradicate HIV-1 viruses [1–3]. Moreover, HIV-1 virus is one of the viruses undergoing the most frequent mutations, which leads to the highly frequent drug resistance for presently available drugs targeting the major viral proteins such as reverse transcriptase, protease, or integrase. Therefore, novel therapeutics targeting other viral proteins become quite attractive in pursuing new clinical therapeutics against HIV-1 [4].

Human APOBEC3 family is a host restriction factor against viruses including HIV-1 and simian immunodeficiency virus (SIV) [5–9]. In non-permissive cell such as H9 cell line where A3G is highly expressed, but not in permissive cells such as SupT1 and 293T cell line where A3G is not expressed, A3G is encapsidated into budding virions and removes the NH_2- residues from cytidines in the newly-synthesized negative strand of viral DNA. This process leaves the uridine in the negative strand, and subsequently, results in a G to A hypermutation in the plus strand of viral DNA [5,6,10]. The hypermutation causes the pre-terminations of viral protein(s) during translation or the productions of mutated viral proteins without any function, and eventually leads to inactivation of viral infectivity [5,11,12]. To neutralize the function of A3G, HIV-1 virus encodes virion infectivity factor (Vif) protein which mediates the ubiquitination of A3G in the complex with elongins B/C, cullin5,

Abbreviations: HIV-1, Human immunodeficiency virus type-1; Vif, Viral infectivity factor; A3G/APOBEC3G, (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G); ELISA, enzyme-linked immunosorbent assay; BUN, Blood urea nitrogen; CRE, Creatine; AST, Aspartate transaminase; ALT, Alanine transaminase; SPR, Surface plasmon resonance; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

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Nedd8, Rbx1, and CBF-β, and subsequently degrades A3G protein [10,13–19]. Thus, the strategy to protect A3G protein level via disruption of Vif-A3G axis opens an avenue to develop new anti-HIV-1 therapeutics.

Since the high-throughput screening (HTS) protocol was developed to efficiently screen chemical libraries for compounds specifically targeting Vif-A3G axis [20], there has been impressive progress which showed various chemical structures inhibiting HIV-1 replication by disrupting Vif-A3G axis [21–23]. We screened a drug-like chemical library by an optimized HTS work flow targeting Vif-A3G axis, and identified a hit compound **13** (Fig. 1) with benzimidazole scaffold. We further studied the SAR of this hit compound and synthesized two new compounds (**14**, **26**) with potent anti-HIV-1 activity. Our results have shown that the benzimidazole derivatives are good starting points to further develop anti-HIV-1 lead compounds that specifically protect A3G protein.

2. Results

2.1. Hit identification by high-throughput screening (HTS) and anti-HIV-1 replication assay

A cell-based high-throughput screening (Vif-A3G assay) was performed using an A3G-GFP fusion protein reporter system [20]. In brief, the Vif-expressing plasmid and A3G-GFP-expressing plasmid were co-transfected into 293T cells. These 293T cells were treated with a commercial drug-like chemical library composed of 20,155 compounds (Enamine) at 50 μM concentration. After 48 h, the wells containing GFP-positive cells were detected by an Envision system (Perkin Elmer). After the positive hits were confirmed by the same system, 372 hit compounds were identified to counteract the Vif-mediated degradation of A3G-GFP fusion protein.

These 372 hit compounds were further tested for their potential anti-HIV-1 replication activity by an anti-HIV-1 replication assay. In brief, H9 and SupT1 cells (1×10^6) were infected with the equivalent of 5 ng HIV-1 p24 for 3 h and HIV-1 replication was monitored by the detection of p24 antigen in supernatant after 4 days. The amount of p24 was quantified by ELISA. Among 372 initial hits, 155 were found to inhibit HIV-1 replication at 50 μM in H9 cells, while 22 compounds in SupT1. It is notable that H9 cells harbor APOBEC3G, while SupT1 cells does not. Most of the hits inhibit HIV-1 replication in H9 but not in SupT1, which support that the target is Vif-A3G system [24,25]. Therefore, 133 compounds were most likely to specifically inhibit HIV-1 by Vif-A3G pathway. Furthermore, we have also performed a dose response among these compounds and selected 5 compounds which showed an inhibition by more than 50% even at 5 μM (Table S1), narrowing down the hits which were more efficient. Given that the benzimidazole scaffold is widely distributed in drugs and natural products with potent biological activities and the facile methods to synthesize benzimidazole scaffold made it possible to synthesize the versatile derivatives in large quantities, compound **13** (Fig. 1) was chosen for further hit-to-lead optimization.

The work flow including both Vif-A3G assay and anti-HIV-1 replication assay was applied in the SAR studies of our synthesized benzimidazole derivatives of compound **13**. We first performed the Vif-A3G assay and the cytotoxicity test (MTS Assay) and secondly the active compounds with low toxicity identified in this step were further investigated for their inhibition activity on HIV-1 replication in the anti-HIV-1 replication assay.

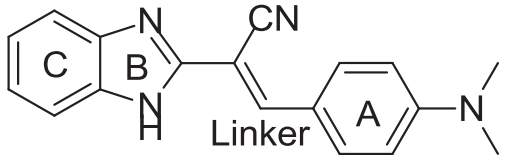


Fig. 1. The structure of the hit compound (**13**). The SAR was studied in ring A, B, C and the linker respectively.

2.2. Chemical synthesis

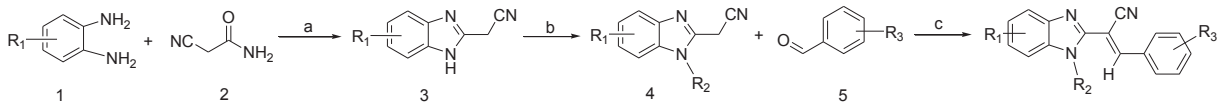
We found that the benzimidazole scaffold was shared by some hit compounds which had the IC_{50} value lower than 5.0 μM. Therefore we took compound **13** as the starting point for the hit-to-lead optimization. Syntheses of the benzimidazole substructure including ring B and C was achieved through a facile method [26], in which the substituted or unsubstituted *o*-phenylenediamine (**1**) was fused with ethylcyanoacetate (**2**) to afford compound **3**. The alkyl substitution groups were installed on the nitrogen atom of benzimidazole ring by using alkyl iodide to afford compound **4** (Scheme 1).

The syntheses of 1*H*-Benzimidazole-2-acetonitrile derivatives were achieved by following the general procedures illustrated in Scheme 1, in which the solution of compound **4** and compound **5** in ethanol with catalytic amount of piperidine was heated at 90 °C to afford the compounds for SAR studies.

2.3. SAR study

Among the active compounds identified by HTS, compound **13** ($IC_{50} = 0.51$ μM, Table 1) was selected for optimization because its benzimidazole scaffold was widely distributed in drugs and natural products with potent biological activities [27,28]. Moreover, the facile methods to synthesize benzimidazole scaffold made it possible to synthesize the versatile derivatives in large quantities [30,31]. The structure of compound **13** was divided into ring A, ring B, ring C and the linker (Fig. 1). Firstly, compounds with different alkyl groups and electro withdrawing groups in group A were synthesized (Table 1, **6**–**12**). However, none of these compounds showed better activities in the Vif-A3G assay than compound **13**. This result indicated that the *N*,*N*-alkyl groups should be a key substitution group to retain the activity. Therefore derivatives bearing different *N*,*N*-alkyl, *N*,*N*-cycloalkyl groups and nitrogen heterocyclic groups were synthesized (Table 1, **14**–**24**). The HIV-1 replication assay showed that the compound **14** with *N*,*N*-diethyl substitution had much better activity ($IC_{50} = 3.45$ nM) than the hit compound **13** (Fig. 2, A). Interestingly, compounds with cyclic alkyl groups (Table 1, **17**, **18**) lost their activities in the Vif-A3G assay, probably due to their constrained conformations in the ring structure. Compound **20** with hydroxyl groups on the *N*,*N*-alkyl groups was also synthesized to increase its water solubility. However, the hydroxyl groups significantly decreased its activity. To further study whether the *N*,*N*-alkyl substitutions could be replaced by hydroxyl or methoxy groups, compounds **25**~**32** with modifications on ring A were synthesized (Table 1). Most of the compounds were active in Vif-A3G assay, but only the compound **26** bearing both *p*-hydroxyl and *m*-methoxy groups possessed potent anti-HIV-1 activity ($IC_{50} = 58.03$ nM) (Fig. 2, B). Eventually, modifications on ring A afforded compound **14** with the highest anti-HIV-1 replication activity ($IC_{50} = 3.45$ nM).

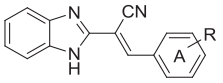
There have been reports regarding on the modifications of benzimidazole substructure to improve biological activities [27]. Ring B was further modified by adding cyclic or acyclic alkyl substitution groups on nitrogen of benzimidazole (Table 2, **33**–**35**). However, these modifications led to complete loss of the activities in Vif-A3G assay and HIV-1 replication assay. The nitrogen atom was then replaced with oxygen or sulfur atom to produce

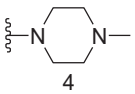
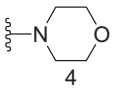
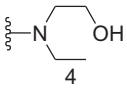
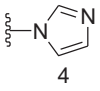
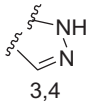
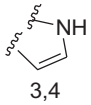


[a]Reagents and conditions: (a) 200 ºC reflux;(b) R_2-Br, DBU, DCM, 0 °C; (c) Piperidine, EtOH , reflux.

Scheme 1. Synthesis of bezimidazole derivatives.

Table 1
Influence of modifications of ring A on A3G level, cell toxicity and anti-HIV-1 replication activity.



Compd. No.	R	Vif-A3G assay	MTS assay (CC_{50}, μM)[a]	HIV-1 replication assay IC_{50} (μM)[b]
6	4-Me	–[c]	>50	>50
7	4-Et	–	>50	>50
8	4-*i*-Pr	–	>50	>50
9	4-Ph	–	>50	>50
10	4-SO_2Me	–	>50	>50
11	4-$NHCOCH_3$	–	>50	>50
12	4-COOH	–	>50	>50
13	4-$N(Me)_2$	++[d]	>50	0.51
14	4-$N(Et)_2$	+++	>50	0.00345
15	4-$N(Pr)_2$	++	>50	1.09
16	4-N(*i*-Pr)$_2$	++	>50	3.30
17		–	>50	>50
18		–	>50	>50
19		+++	>50	>50
20		+	>50	6.11
21		–	>50	>50
22	3-$N(Et)_2$	–	>50	>50
23		++	>50	0.36
24		+++	>50	0.72
25	4-OMe	++	>50	2.85
26	3-OMe, 4-OH	+++	>50	0.05803
27	3,4-OMe	+	>50	>50
28	3,4,5-OMe	+	>50	>50
29	2,4,5-OMe	+	>50	>50
30	2-OH, 4-$N(Et)_2$	+++	>50	1.50
31	2-OH, 4-N(Me)2	+++	>50	>50
32	3-OMe, 4-$N(Me)_2$	+	>50	0.29

[a] Median (50%) cytotoxic concentration.
[b] Half maximal (50%) inhibitory concentration.
[c] Test result did not show activity.
[d] The fold change of A3G-GFP mean fluorescent intensity (MFI). Calculation method was described in method. –, no or less than 1 fold change; +, 1~2 fold change; ++, 2~3 fold change; +++, >3 fold change.

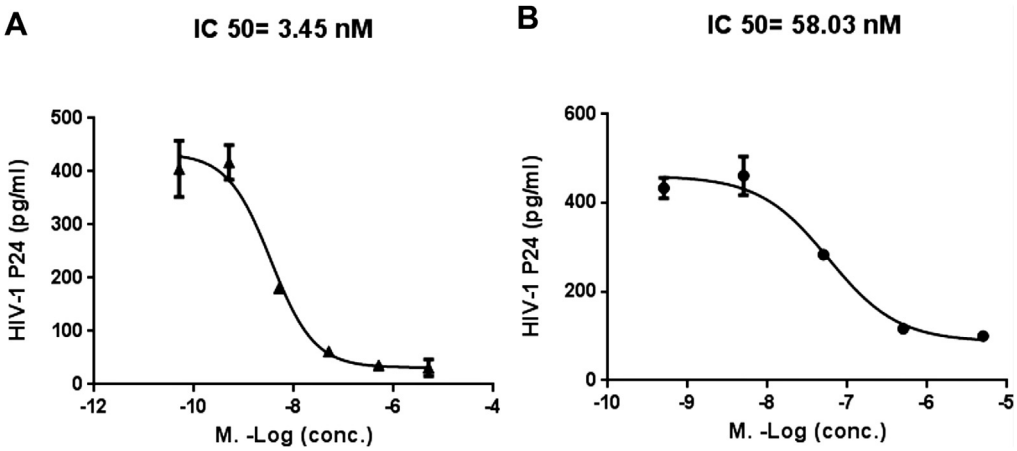
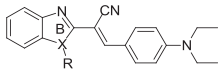


Fig. 2. Compound **14** (A) and compound **26** (B) inhibited HIV-1 replication in H9 cells with IC_{50} value of 3.45 nM and 58.03 nM respectively.

Table 2
Influence of the modifications of ring B on A3G level, cell toxicity and anti-HIV-1 replication activity.

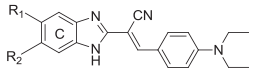


Compd. No.	X	R	Vif-A3G assay	MTS assay (CC_{50}, μM)	HIV-1 replication assay IC_{50} (μM)
33	N	Me	–	>50	>50
34	N	Et	–	>50	>50
35	N	Cyclo-Pr	–	>50	>50
36	O		++	>50	2.38
37	S		+++	>50	5.92

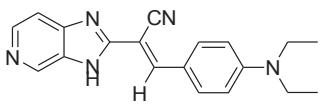
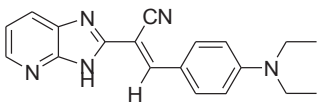
benzoxazole and benzothiazole substructures (Table 2, **36**–**37**). These modifications maintained the activities in both the Vif-A3G assay and the HIV-1 replication assay, although their activities were lower than that of compound **14**.

Ring C was modified by substitutions of electro-withdrawing groups (Table 3, **38**–**45**), alkyl groups and weak electron-donating groups (Table 3, **46**–**50**) but those compounds did not show higher activity in HIV-1 replication assay than compound **14**.

Table 3
Influence of the modifications of ring C on A3G level, cell toxicity and anti-HIV-1 activity.



Compd. ID	R_1	R_2	Vif-A3G assay	MTS assay (CC_{50}, μM)	HIV-1 replication assay (IC_{50},μM)
38	F	H	+++	>50	>50
39	Cl	H	++	>50	>50
40	Br	H	++	>50	>50
41	Cl	Cl	+++	>50	1.25
42	CF_3	H	++	>50	1.13
43	OCF_3	H	+++	>50	1.35
44	COOMe	H	++	>50	1.68
45	COOH	H	++	>50	>50
46	Me	Me	+++	>50	1.64
47	*t*-Butyl	H	+++	>50	1.04
48	OMe	H	++	>50	4.42
49	$N(Me)_2$	H	+	>50	2.68
50	SMe	H	++	>50	0.75

Compd. ID	Structure	Vif-A3G assay	MTS assay (CC_{50}, μM)	HIV-1 replication assay (IC_{50},μM)
51		++	>50	7.30
52		++	>50	3.58

Moreover, compounds with pyridine instead of benzene ring in the benzimidazole structure (Table 3, **51**, **52**) were synthesized but afforded no anti-HIV-1 activity. These results indicated that the unsubstituted benzene structure of ring C was important to maintain the activity.

In the studies of the effect of the linker on the activity, compound **53** with a rigid structure in the linker (Table 4) showed lower activity than the hit compound **14**. Compound **54** bearing a sigma bond rather than an alkene bond in the linker was synthesized (Table 4) but it was inactive in the Vif-A3G assay. These results indicated that the alkene linker was important for the anti-HIV-1 activity.

From the SAR studies described above, it has been elucidated that the *N*,*N*-alkyl substitution or *p*-hydroxyl together with *m*-methoxy substitution of ring A, benzimidazole substructure including ring B and ring C, and the alkene linker connecting ring A and ring B are required for their anti-HIV-1 activity.

2.4. Compounds 14 and 26 protected A3G protein from degradation by Vif

To determine whether compound **14** and **26** could rescue A3G protein from Vif-mediated degradation and thus increase the A3G protein level in 293T cells, compound **14** and **26** were subjected to a flow cytometry assay. 293T cells were co-transfected with Vif and A3G-GFP-expressing plasmids for 48 h, followed by the treatment of compound **14** and **26** (0.0005–5 μM). The MFI (mean fluorescent intensity) of Envision showed that the cellular A3G protein level was protected by compound **14** and **26** in a dose-dependent manner (Fig. 3A). The similar results were also observed in other hit compounds (Fig. S4). The western blot and the fluorescence microscope images (Fig. 3B) also confirmed that compounds **14** and **26** inhibited Vif-mediated degradation of A3G-GFP fusion protein in 293T cells. In primary cells (PBMCs) compound **14** and **26** could also markedly inhibit HIV-1 production (Fig. 3C). Meanwhile, compound **14** and **26** showed no effect to inhibit HIV-1 production in permissive SupT1 cells (Fig. 3D).

Surface plasmon resonance (SPR) was performed to study whether the compounds **14** and **26** could decrease the binding affinity of Vif-A3G complex and thus protected A3G from Vif-mediated degradation. The recombinant Vif-His protein (purity was confirmed by SDS-PAGE, Fig. S3) was immobilized on a CM5 Sensor Chip (carboxymethylated dextran covalently attached to a gold surface) with an amine coupling kit from GE Healthcare. The recombinant A3G-His protein (purity was confirmed by SDS-PAGE, Fig. S3, 50 μg/mL) was pre-incubated (25 °C) with (green) or without (red) compound **14** (4.0 μM) in a PBS buffer (10 mM) with 5% DMSO (Fig. 4A, up). Then the binding of these A3G proteins with Vif proteins were tested. Similar experiment was done with compound **26** (9.0 μM) (Fig. 4A, down). The SPR sensorgrams showed that the Vif-A3G binding was reduced (about 17% of max resonance unit) after the treatment of compounds **14** or **26** when compared with the untreated A3G protein. These results indicate that compound **14** and **26** could disrupt the interaction of A3G and Vif. To exclude the possibility that compound **14** and **26** could disrupt other protein–protein interactions in Vif-A3G axis, we carried out the co-IP experiments with Vif and ElonginB, ElonginC and Cullin5. We found that these two compounds did not affect the binding of Vif/ElonginB, Vif/ElonginC and Vif/Cullin5 (Fig. 4B–D). In a word, both the SPR and co-IP showed that compound **14** and **26** could disrupt the binding of A3G and Vif and then protect the A3G from Vif-mediated degradation.

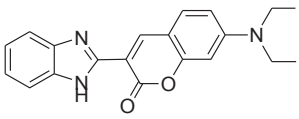
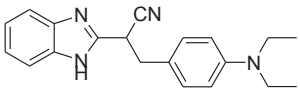
2.5. Toxicity test

The structure of benzimidazole has been widely distributed in drugs and natural products and showed potent activities including some undesired biological effects [27,28]. To address this issue, the acute toxicities of compound **14** and **26** on mice were tested. Two weeks after injected intraperitoneally with compound **14** or **26** in the dose of 0 mg/kg, 500 mg/kg, 1000 mg/kg, 5 male Balb/c mice did not show significant change of body weight. In the 14th day, the blood samples and the organs of heart, liver, lung, spleen and kidney were examined and no significant abnormity was found. The functions of the key enzymes including AST, ALT, BUN, and CRE in the histological sections of these organs were also normal (Fig. 5).

3. Conclusion and discussion

We performed the SAR study on benzimidazole derivatives for their anti-HIV-1 replication activity through protecting A3G protein level. The benzene ring (Fig. 1, ring A) and its *N*,*N*-dialkyl amine substitution group were required for the anti-HIV-1 replication activity. The *N*,*N*-dialkyl group, *m*-methoxy and *p*-hydroxyl groups were the most favored substitutions. The modifications on benzimidazole substructure and the linker (Fig. 1, ring C) also afforded compounds with higher activity than the hit compound (lowest IC_{50} value as 0.24 μM, compound **53**), which indicated the possibility to increase activity by modifications on this substructure. However, modifications on alkyl substitutions on the nitrogen atom of benzimidazole were not favored. These results indicate that the binding site of Vif-A3G complex for compounds **14** and **26** is highly strict and there could be strong interactions between *N*,*N*-dialkyl group and the key amino acids. This information could be very useful when the structure information of was applied to identify new therapeutics targeting Vif-A3G complex [29,30]. Compound **14** and **26** were two most potent compounds with the IC_{50} value of 3.45 nM and 58.03 nM respectively. Their anti-HIV-1 activity mechanism was studied and our data suggested that compound **14** and **26** protected the A3G protein from degradation by Vif in 293T cells. SPR studies showed that compounds **14** and **26** decreased, although not completely disrupted, the Vif-A3G interaction. The compound **14** and **26** showed satisfactory acute toxicity properties.

Table 4
Influence of modification of linker on A3G level, cell toxicity and anti-HIV-1 replication activity.

Compd. ID		Vif-A3G assay	MTS assay (CC_{50}, μM)	HIV-1 replication assay (IC_{50},μM)
53		+++	>50	0.24
54		–	>50	>50

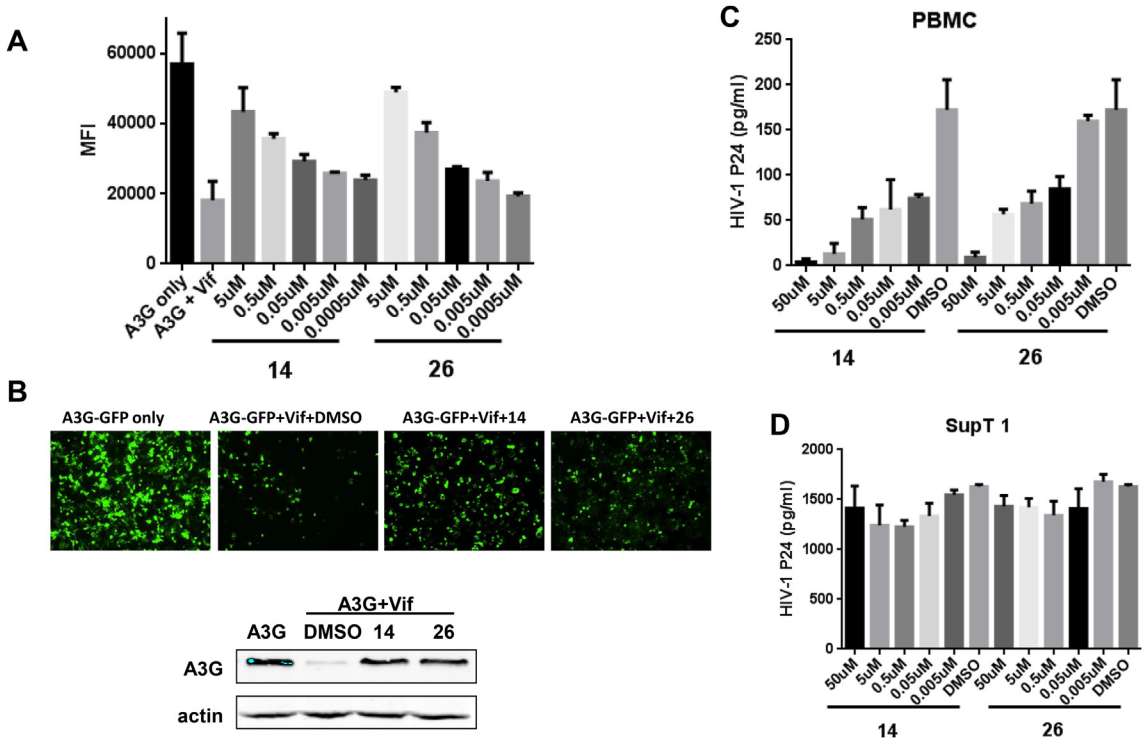


Fig. 3. Compound **14** and **26** can counteract the Vif-mediated degradation of A3G in 293T cells. (A) The MFI analysis for the effect of compound **14** or **26** on the intensity of A3G-GFP fluorescence. Vif and A3G-GFP were co-transfected into HEK293T cells and then the cells were treated with DMSO, multiple concentrations of compound **14** or compound **26** respectively. After 48 h, the fluorescence of cells was detected by Microplate Reader. (B) Fluorescence microscope images and western blot results for the effect of A3G-GFP inhibited by Vif in HEK293T cells under the treatment of compound **14** and compound **26**. (C and D) PBMCs (C) and SupT1 cells (D) were infected by HIV-1 and then treated by Compound **14** and compound **26** under varied concentrations. P24 was detected as production of HIV-1.

Taken together, our data demonstrated that the compounds **14** and **26** bearing benzimidazole sub-structure could be a good basis to develop lead compounds with higher potency to inhibit HIV-1 replication through protecting A3G protein.

In light of the essential role of Vif in the infectivity of HIV-1 viruses, the Vif-A3G axis has become an important target for developing anti-HIV-1 leads [20–23,31,32]. Therefore the working mechanisms of compound **14** and **26** should be further studied including the exact target proteins in the Vif-A3G complex and the corresponding binding sites. In our study, compound **14** and **26** protected the protein level of A3G and inhibited HIV-1 replication. Meanwhile, the protein level of Vif in 293T cells did not change significantly (Fig. S1). The stable Vif level was also observed in 293T cells treated with compound IMB25/36 [22]. However, RN-18, a potent compound targeting Vif-A3G axis, significantly decreases Vif protein level in 293FT cells [20,23]. On the contrary, Vif protein level was elevated by compound VEC-5 which may disrupt the A3G-Vif-E3 complex and inhibits hydrolysis of Vif by E3, ElonginB/C-Cullin5 complex [31]. These different responses of quantitative changes of Vif protein after different treatments suggest that these compounds may have different target sites on Vif-A3G axis or they may have profound effect on other proteins involved in the Vif-mediated A3G degradation.

4. Experimental section

4.1. General chemistry methods

All reagents and solvents were purchased from Sigma–Aldrich, J&K Chemical, Aladdin. Tetrahydrofuran and dichloromethane were freshly distilled from sodium/benzophenone under nitrogen and from CaH_2 under nitrogen respectively. Analytical thin-layer plates were obtained from Qingdao Haiyang Chemical. Flash chromatography was performed with silica gel 60. NMR spectra were recorded on a Bruker Avance 400 with the solvents indicated. Purity was analyzed by reverse-phase HPLC performed on Agilent 6130 Quadrupole and Agilent 1200 equipment, or on Agilent ProStar 218 system. The purity of compounds for the SAR study was more than 95% tested by ESI-LCMS. HRMS were recorded at the Instrumental Analysis & Research Center in Sun Yat-sen University using a Thermo Scientific LTQ-Orbitrap Elite mass spectrometer.

4.2. General procedure for the synthesis 2-(1H-benzimidazol-2-yl) acetonitrile derivatives (3, 4)

The mixture of *o*-phenylenediamine (**1**) and cyanoacetamide (**2**) were stirred in 200 °C for 30 min without any solvent. After cooling, the mixture was dissolved in ethanol. The crude product was purified by silica gel column chromatography using dichloromethane-methanol (60:1) as eluant to give the pure compound **3**.

To a solution of compound **3** in DMF was added K_2CO_3 in 0 °C and the mixture was stirred at 0 °C with adding alkyl iodide drop wise. After the mixture was stirred at 0 °C for 1 h, the solvent was removed in vacuo. The residue was diluted with H_2O and extracted with EtOAc. The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified chromatography on silica gel with solvent cyclohexane-acetone (20:1) to give the compound **4**.

4.3. 2-(1H-benzimidazol-2-yl)-3-(4-methylphenyl)acrylonitrile (6)

To the solution of 2-(1*H*-benzimidazol-2-yl)acetonitrile (80 mg,

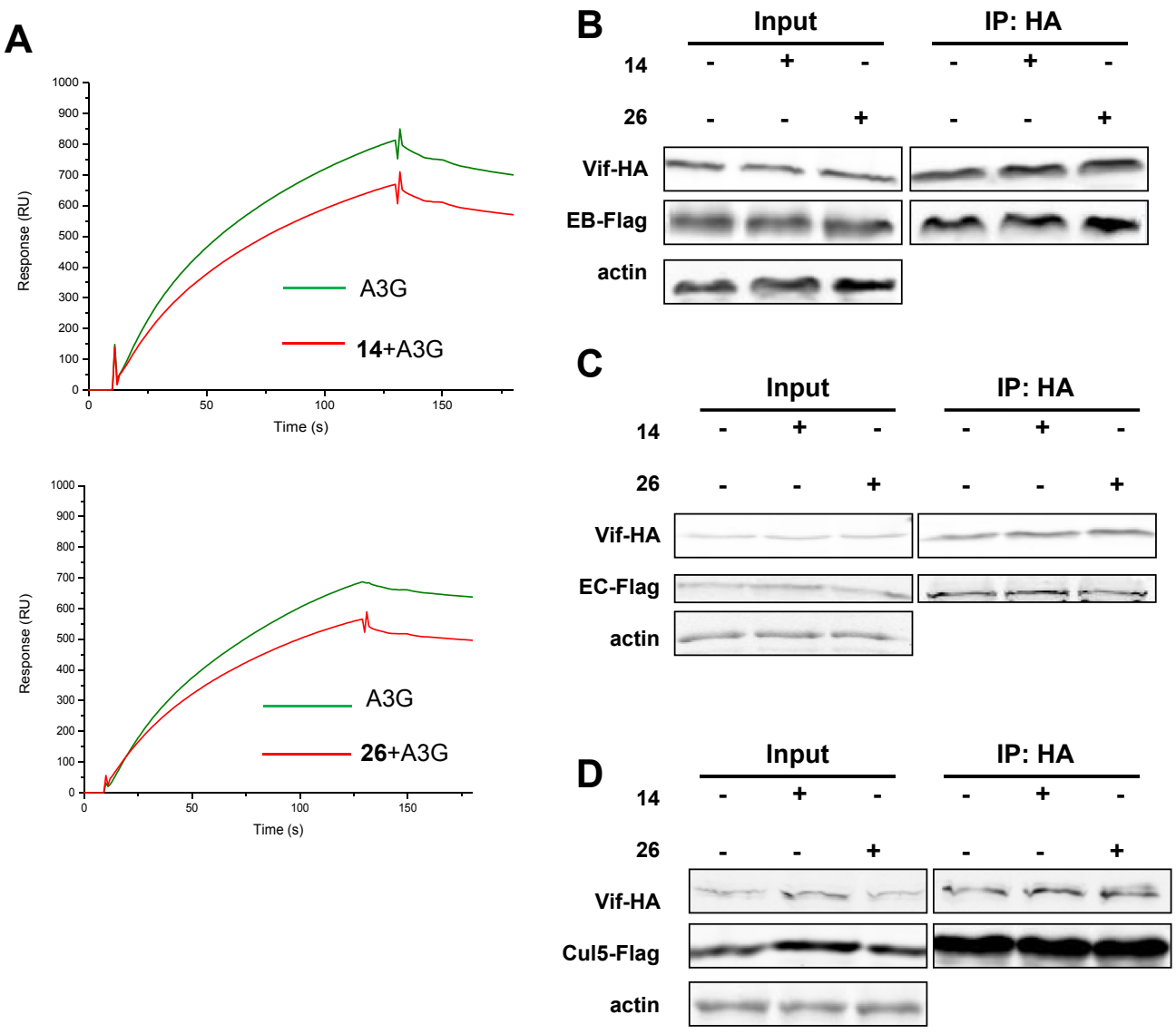


Fig. 4. (A) Surface plasmon resonance experiment showed that compound **14** (up) and compound **26** (down) reduced the binding of A3G and Vif. (B–D) HEK293T cells were transfected with pcDNA3.1-Vif-HA and pcDNA3.1-EC/EB/CUL5-Flag and then were treated with DMSO, compound **14** and compound **26** respectively. After 48 h, cells were harvested for co-IP assay.

0.51 mmol), 4-methylbenzaldehyde (60 mg, 0.50 mmol) in ethanol (1.5 mL) was added 1,4-diazacyclohexane (45 mg, 0.52 mmol). The mixture was refluxed for 4 h in room temperature. The crude product was filtered off and purified by silica gel chromatography using cyclohexane-acetone-ethyl acetate (10:1:1) as eluant to afford compound **6** (120 mg, 93%). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.30 (s, 1H), 7.91 (d, J = 8.2 Hz, 2H), 7.62 (dd, J = 5.4, 3.3 Hz, 2H), 7.42 (d, J = 8.1 Hz, 2H), 7.29–7.21 (m, 2H), 2.41 (s, 3H). ESI-MS (*m*/*z*): 260.1 $[M+H]^+$.

4.4. 2-(1H-benzimidazol-2-yl)-3-(4-ethylphenyl)acrylonitrile (7)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-ethylbenzaldehyde instead of 4-methylbenzaldehyde. Yield: 30%. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 13.03 (s, 1H), 8.31 (s, 1H), 7.93 (d, J = 8.2 Hz, 2H), 7.69 (d, J = 6.9 Hz, 1H), 7.56 (d, J = 6.8 Hz, 1H), 7.44 (d, J = 8.3 Hz, 2H), 7.27 (d, J = 8.8 Hz, 2H), 2.70 (q, J = 7.6 Hz, 2H), 1.23 (t, J = 7.6 Hz, 3H). ESI-MS (*m*/*z*): 274.1 $[M+H]^+$.

4.5. 2-(1H-benzimidazol-2-yl)-3-(4-isopropylphenyl)acrylonitrile (8)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-isopropylbenzaldehyde instead of 4-methylbenzaldehyde. Yield: 49%. ^{1}H NMR (500 MHz, DMSO-*d*6) δ 13.06 (s, 1H), 8.32 (s, 1H), 7.95 (d, J = 8.3 Hz, 2H), 7.79–7.53 (m, 2H), 7.49 (d, J = 8.2 Hz, 2H), 7.27 (s, 2H), 3.14–2.89 (m, 1H), 1.26 (d, J = 6.9 Hz, 9H). ESI-MS (*m*/*z*): 288.1 $[M+H]^+$.

4.6. 2-(1H-benzimidazol-2-yl)-3-(biphenyl-4-yl)acrylonitrile (9)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-phenylbenzaldehyde instead of 4-methylbenzaldehyde. Yield: 10%. ^{1}H NMR (500 MHz, DMSO-*d*6) δ 13.12 (s, 1H), 8.40 (s, 1H), 8.11 (d, J = 8.4 Hz, 2H), 8.01 (d, J = 8.4 Hz, 1H), 7.94 (d, J = 8.5 Hz, 2H), 7.93–7.89 (m, 1H), 7.84–7.80 (m, 2H), 7.79–7.76 (m, 1H), 7.72 (d, J = 8.3 Hz, 2H), 7.67 (d, J = 7.8 Hz, 1H), 7.59 (d, J = 7.8 Hz, 1H), 7.46 (d, J = 7.4 Hz, 2H). ESI-MS

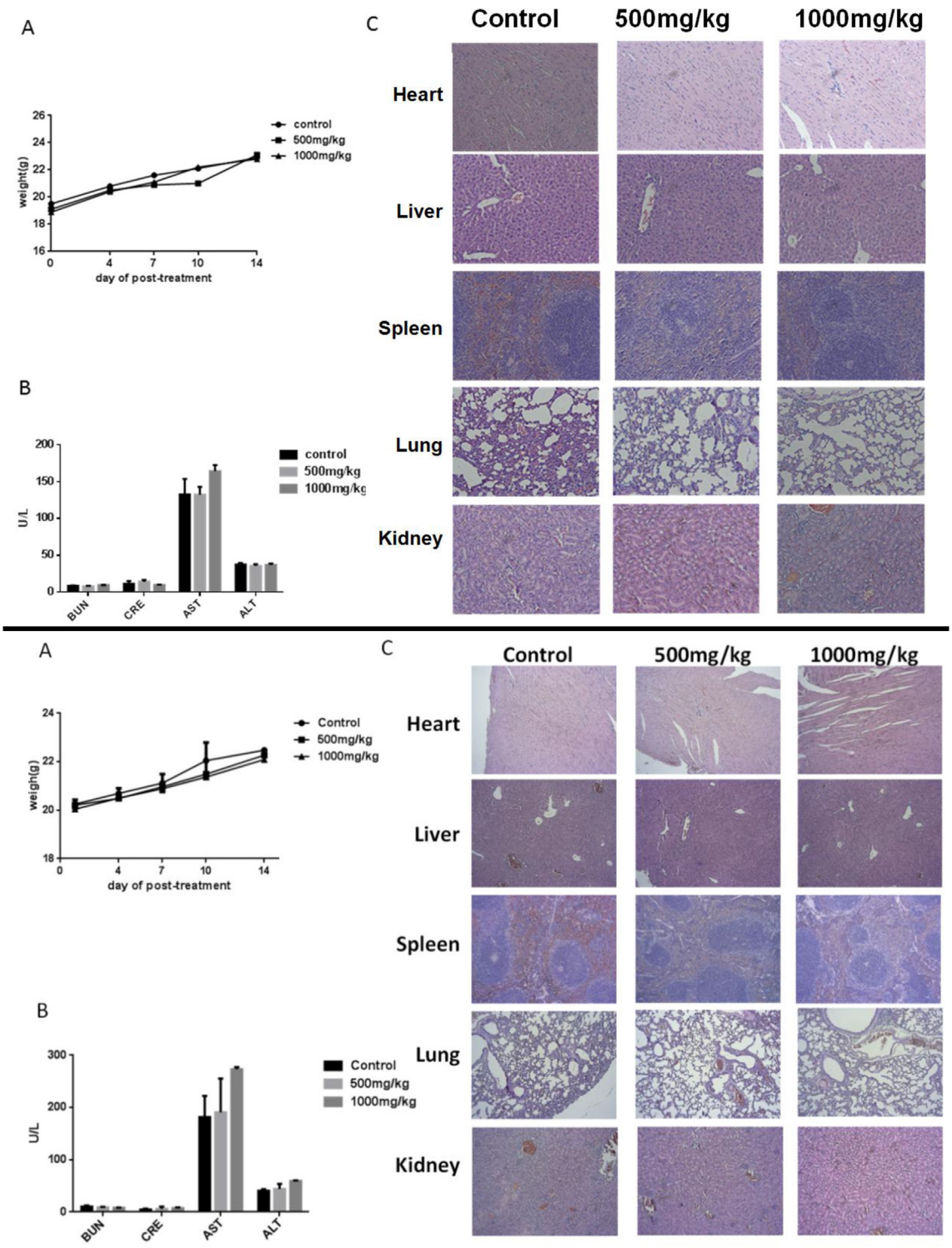


Fig. 5. The safety of compound **14** (up) and **26** (down) was examined with acute toxicological study. (A). The measurement of weight of male Balb/c mice after intraperitoneally injected with compounds. (B). The effect of compounds on the hepatic and renal function of mice (BUN: Blood urea nitrogen, CRE: Creatine, AST: Aspartate Transaminase, ALT: Alanine transaminase). C. The several vital organs from the mice treated with various doses of compounds were histologically sectioned and HE stained.

(*m*/*z*): 322.1 $[M+H]^+$.

4.7. 2-(1H-benzimidazol-2-yl)-3-(4-methylsulfonylphenyl) acrylonitrile (10)

To a solution of 2-(1*H*-benzimidazol-2-yl)acetonitrile (361 mg, 2.5 mmol) and 4-methylsulfonylbenzaldehyde (428 mg, 2.5 mmol) in ethanol (6 mL) was added with NaOH (200 mg, 5 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction mixture was poured into ice-cold water and neutralized with acetic acid. The mixture was filtered and the filtrate was concentrated and purified by silica gel chromatography using cyclohexane-acetone

(10:1) as eluant to afford compound **8** (208 mg, 26%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 (s, 1H), 8.24 (d, J = 8.5 Hz, 2H), 8.15 (d, J = 8.5 Hz, 2H), 7.65 (d, J = 10.6 Hz, 2H), 7.38–7.33 (m, 2H), 3.22 (s, 3H). ESI-MS (m/z): 324.1 $[M+H]^+$.

4.8. 2-(1H-benzimidazolyl)-3-(4-N-acetamidophenyl)acrylonitrile (11)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-acetamidobenz-aldehyde instead of 4-methylbenzaldehyde. Yield: 51%. 1H NMR (500 MHz, DMSO-*d*6): δ 13.01 (s, 1H), 10.36 (s, 1H), 8.26 (s, 1H), 7.99–7.97 (m, 2H), 7.82–7.80 (m, 2H), 7.62–7.58 (m, 2H), 7.26–7.25 (m, 2H), 2.11 (s, 3H). ESI-MS (m/z): 303.2 $[M+H]^+$.

4.9. 4-[2-(1H-benzimidazol-2-yl)-2-cyanoethenyl]benzoic acid (12)

2-(1*H*-benzimidazol-2-yl)-3-(4-carboxyl-phenyl)-acrylonitrile (80 mg, 0.27 mmol) and methanol (30 mg, 1 mmol) were dissolved in DMF (1.5 mL), followed by adding DCC (56 mg, 0.27 mmol) and DMAP (8 mg, 0.07 mmol). The mixture was stirred at room temperature for 4 h. The crude product was purified by chromatography on silica gel with solvent cyclohexane-acetone (1:1). To afford product as brown solid (68 mg, 83%).^{1}H NMR (400 MHz, DMSO-d_6): δ 8.46 (s, 1H), 8.19 (d, J = 8.5 Hz, 2H), 8.16–8.12 (m, 2H), 7.67 (s, 2H), 7.30 (dd, J = 6.1, 3.2 Hz, 2H). ESI-MS (m/z): 290.1 $[M+H]^+$.

4.10. 2-(1H-benzimidazol-2-yl)-3-(4-dimethylaminophenyl) acrylonitrile (13)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(dimethylamino)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.90 (d, J = 8.2 Hz, 2H), 7.81–7.75 (m, 1H), 7.5 (m, 1H), 7.40–7.32 (m, 1H), 7.30 (dd, J = 7.8, 3.2 Hz, 1H), 6.88 (d, J = 8.2 Hz, 2H), 3.08 (s, 6H). ESI-MS (m/z): 289.2 $[M+H]^+$.

4.11. 2-(1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl) acrylonitrile (14)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(diethylamino)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 40%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.73 (br s, 1H), 8.11 (s, 1H), 7.89 (d, J = 9.5 Hz, 2H), 7.56 (br s, 2H), 7.22–7.19 (m, 2H), 6.84 (d, J = 9.0 Hz, 2H), 3.46 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). HRMS (ESI) m/z calcd for C20H21N4 H+: 317.1761. Found: 317.1760 (Δ = −0.32 ppm).

4.12. 2-(1H-benzimidazol-2-yl)-3-(4-dipropylaminophenyl) acrylonitrile (15)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(dipropylamino)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 80%. ^{1}H NMR (500 MHz, DMSO–*d*6): δ 8.19 (s, 1H),7.91 (d, J = 9.5 Hz, 2H), 7.64 (dd, J = 3.0 and 6.0 Hz, 2H), 7.34 (dd, J = 3.0 and 6.0 Hz, 2H), 6.86 (d, J = 9.5 Hz, 2H), 3.38 (t, J = 7.5 Hz, 4H), 1.60–1.57 (m, 4H), 0.92 (t, J = 7.5 Hz 6H). ESI-LCMS (m/z): 345.3 $[M+H]^+$.

4.13. 2-(1H-benzimidazol-2-yl)-3-(4-diisopropylaminophenyl) acrylonitrile (16)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(diisopropylamino) benzaldehyde instead of 4-methylbenzaldehyde. Yield: 48%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.18 (br s, 1H), 7.89 (br s, 2H), 7.65–7.62 (m, 2H), 7.34–7.32 (m, 2H), 7.01 (br s, 2H), 4.13–4.08 (m, 2H), 1.32–1.25 (m, 12H). ESI-LCMS (m/z): 345.0 $[M+H]^+$.

4.14. 2-(1H-benzimidazol-2-yl)-3-[4-(1-pyrrolidinyl)phenyl] acrylonitrile (17)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(1-pyrrolidinyl)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 21%. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.96 (s, 1H), 7.85 (d, J = 9.0 Hz, 2H), 7.59–7.34 (m, 2H), 6.82 (d, J = 9.1 Hz, 1H), 6.55 (d, J = 8.9 Hz, 1H), 3.37 (t, J = 6.6 Hz, 4H), 2.13–1.90 (m, 4H). ESI-LCMS (m/z): 315.1 $[M+H]^+$.

4.15. 2-(1H-benzimidazol-2-yl)-3-[4-(4-methyl-1-piperazinyl) phenyl]acrylonitrile (18)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(4-methyl-1-piperazinyl) benzaldehyde instead of 4-methylbenzaldehyde. Yield: 68%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.83 (br s, 1H), 8.16 (s, 1H), 7.91 (d, J = 9.0 Hz, 2H), 7.70–7.50 (m, 2H), 7.22 (d, J = 3.0 Hz, 2H), 7.11 (d, J = 9.0 Hz, 2H), 3.40 (t, J = 5.0 Hz, 4H), 2.44 (t, J = 5.0 Hz, 4H), 2.24 (s, 3H). ESI-MS (m/z): 344.3 $[M+H]^+$.

4.16. 2-(1H-benzimidazol-2-yl)-3-(4-morpholinophenyl) acrylonitrile (19)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(4-morpholinyl)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 43%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.86 (s, 1H), 8.18 (s, 1H), 7.93 (d, J = 8.5 Hz, 2H), 7.58 (br s, 2H), 7.23–7.22 (m, 2H), 7.11 (d, J = 8.5 Hz, 2H), 3.76–3.74 (m, 4H), 3.36–3.35 (m, 4H). ESI-MS (m/z): 331.2 $[M+H]^+$.

4.17. 2-(1H-benzimidazol-2-yl)-3-{4-[ethyl(2-hydroxyethyl)amino] phenyl}acrylonitrile (20)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-[*N*-ethyl-*N*-(hydroxyethyl)amino]benzaldehyde instead of 4-methylbenzaldehyde. Yield: 68%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.17 (br s, 1H),7.90 (d, J = 9.0 Hz, 2H), 7.64–7.61 (m, 2H), 7.32 (br s, 2H), 6.90 (d, J = 9.0 Hz, 2H), 3.61 (t, J = 6.0 Hz, 2H), 3.57–3.50 (m, 4H), 1.15 (t, J = 7.0 Hz, 3H). ESI-LCMS (m/z): 333.3 $[M+H]^+$.

4.18. 2-(1H-benzimidazol-2-yl)-3-[4-(1H-imidazol-1-yl)phenyl] acrylonitrile (21)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(imidazol-1-yl)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 15%. 1H NMR (500 MHz, DMSO-*d*6): δ 13.11–13.09 (m, 1H), 8.45–8.36 (m, 2H), 8.15–8.13 (m, 2H), 7.95–7.91 (m, 3H), 7.73–7.59 (m, 2H), 7.27–7.17 (m, 3H). ESI-MS (m/z): 312 $[M+H]^+$.

4.19. 2-(1H-benzimidazol-2-yl)-3-(3-diethylaminophenyl) acrylonitrile (22)

The title compound was synthesized according to the procedure of preparing compound **6** except using 3-(diethylamino)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 35%. 1H NMR

(400 MHz, CD3OD): δ 8.17 (s, 1H), 7.63 (brs, 2H), 7.49 (s, 1H), 7.40–7.29 (m, 3H), 7.15 (d, J = 8.0 Hz, 1H), 6.93 (dd, J = 2.4 and 8.4 Hz, 1H), 3.48 (q, J = 6.8 Hz, 4H), 1.23 (t, J = 7.2 Hz, 6H) ppm; ESI-MS (m/z): 317.1 $[M+H]^+$.

4.20. 2-(1H-benzimidazol-2-yl)-3-(1H-indazol-5-yl)acrylonitrile (23)

The title compound was synthesized according to the procedure of preparing compound **6** except using 1*H*-indazole-6-acetalde hyde instead of 4-methylbenzaldehyde. Yield: 72%. 1H NMR (500 MHz, DMSO-*d*6): δ 13.48 (s, 1H), 13.03–13.00 (m, 1H), 8.47 (s, 2H), 8.33 (s, 1H), 8.09–8.07 (m, 1H), 7.78–7.76 (m, 1H), 7.63 (m, 2H), 7.27–7.26 (m, 2H). ESI-MS (m/z): 286.2 $[M+H]^+$.

4.21. 2-(1H-benzimidazol-2-yl)-3-(1H-indol-5-yl)acrylonitrile (24)

The title compound was synthesized according to the procedure of preparing compound **6** except using 1*H*-indole-6-carboxa ldehyde instead of 4-methylbenzaldehyde. Yield: 49%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.97 (s, 1H), 11.59 (s, 1H), 8.42 (s, 1H), 8.29 (s, 1H), 7.88–7.86 (m, 1H), 7.69–7.67 (m, 1H), 7.61–7.60 (m, 1H), 7.56–7.51 (m, 2H), 7.27–7.22 (m, 2H), 6.65 (s, 1H). ESI-MS (m/z): 285.2 $[M+H]^+$.

4.22. 2-(1H-benzimidazol-2-yl)-3-(4-methoxyphenyl)acrylonitrile (25)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(methoxy)benzaldehyde instead of 4-methylbenzaldehyde. Yield: 50%. ^{1}H NMR (500 MHz, DMSO-*d*6) δ 12.98 (s, 1H), 8.28 (s, 1H), 8.01 (d, J = 8.9 Hz, 2H), 7.73–7.64 (m, 1H), 7.55 (d, J = 6.7 Hz, 1H), 7.34–7.20 (m, 2H), 7.18 (d, J = 8.9 Hz, 2H), 3.88 (s, 3H). ESI-MS (m/z): 276.1 $[M+H]^+$.

4.23. 2-(1H-benzimidazol-2-yl)-3-(4-hydroxy-3-methoxyphenyl) acrylonitrile (26)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-hydroxy-3-methoxy benzaldehyde instead of 4-methylbenzaldehyde. Yield: 33%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.93 (br s, 1H), 10.21 (br s, 1H), 8.23 (s, 1H),7.74 (d, J = 2.0 Hz, 1H), 7.66 (br s, 1H), 7.54 (br s, 1H), 7.47 (dd, J = 2.0 and 8.5 Hz, 1H), 7.24 (br s, 2H), 6.98 (d, J = 8.0 Hz, 1H), 3.87 (s, 3H). HRMS (ESI) m/z calcd for C17H14O2N3 H+: 292.1080. Found: 292.1079 (Δ = −0.34 ppm).

4.24. 2-(1H-benzimidazol-2-yl)-3-(3,4-dimethoxyphenyl) acrylonitrile (27)

The title compound was synthesized according to the procedure of preparing compound **6** except using 3,4-dimethoxy benzaldehyde instead of 4-methylbenzaldehyde. Yield: 15%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.98 (s, 1H), 8.28 (s, 1H), 7.74 (d, J = 2.5 Hz, 1H), 7.68 (d, J = 8.0 Hz, 1H), 7.59 (dd, J = 2.0 and 8.5 Hz, 1H), 7.54 (d, J = 7.5 Hz, 1H), 7.30–7.18 (m, 3H), 3.88 (s, 3H), 3.86 (s, 3H). ESI-MS (m/z): 306.2 $[M+H]^+$.

4.25. 2-(1H-benzimidazol-2-yl)-3-(3,4,5-trimethoxyphenyl) acrylonitrile (28)

The title compound was synthesized according to the procedure of preparing compound **6** except using 3,4,5-trimethoxybenzaldehyde instead of 4-methylbenzaldehyde. Yield: 11%. 1H NMR (500 MHz, DMSO-*d*6): δ 13.02 (br s, 1H), 13.03–13.00 (m, 1H), 8.30 (s, 2H), 7.75–7.50 (m, 2H), 7.39 (s, 2H), 7.26 (br s, 2H), 3.87 (s, 2H), 3.78 (s, 1H). ESI-LCMS (m/z): 336.1 $[M+H]^+$.

4.26. 2-(1H-benzimidazol-2-yl)-3-(2,4,5-trimethoxyphenyl) acrylonitrile (29)

The title compound was synthesized according to the procedure of preparing compound **6** except using 2,4,5-trimethoxyben zaldehyde instead of 4-methylbenzaldehyde. Yield: 69%. 1H NMR (500 MHz, DMSO-*d*6): δ 13.01 (s, 1H), 8.51 (s, 1H), 7.89 (s, 1H), 7.70–7.50 (m, 2H), 7.24 (br s, 2H), 6.85 (s, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.80 (s, 3H). ESI-MS (m/z): 336.2 $[M+H]^+$.

4.27. 2-(1H-benzimidazol-2-yl)-3-(4-diethylamino-2-hydroxyphenyl)acrylonitrile (30)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(diethylamino)-2-hydroxybenzaldehyde instead of 4-methylbenzaldehyde. Yield: 11%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.71 (s, 1H), 8.54 (s, 1H), 7.83–7.58 (m, 2H), 7.60–7.58 (m, 1H), 7.19 (s, 2H), 6.62 (d, J = 8.0 Hz, 1H), 6.41 (s, 1H), 3.45 (q, J = 6.5 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-MS (m/z): 333.3 $[M+H]^+$.

4.28. 2-(1H-benzimidazol-2-yl)-3-[4-(dimethylamino)-2-hydroxyphenyl]acrylonitrile (31)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(dimethylamino)-2-hydroxybenzaldehyde instead of 4-methylbenzaldehyde. Yield: 20%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.76 (br s, 1H), 8.59–8.56 (m, 1H), 7.69–7.50 (m, 3H), 7.20 (br s, 2H), 6.64–6.60 (m, 1H), 6.46–6.40 (m, 1H), 3.06 (s, 6H). ESI-MS (m/z): 305.1 $[M+H]^+$.

4.29. 2-(1H-benzimidazole-2-yl)-3-(4-dimethylamino-3-methoxyphenyl)acrylonitrile (32)

The title compound was synthesized according to the procedure of preparing compound **6** except using 4-(dimethylamino)-3-methoxybenzaldehyde instead of 4-methylbenzaldehyde. Yield: 24%. 1H NMR (500 MHz, DMSO-*d*6): δ 12.90 (s, 1H), 8.22 (s, 1H), 7.71 (s, 1H), 7.67–7.64 (m, 1H), 7.55–7.49 (m, 2H), 7.30–7.20 (m, 2H), 6.95 (d, J = 9.0 Hz, 1H), 3.88 (s, 3H), 2.91 (s, 6H). ESI-MS (m/z): 319.2 $[M+H]^+$.

4.30. 2-(1-methyl-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (33)

The title compound was synthesized according to the procedure of preparing compound **14** except using 1-methyl-2-benzimida zolylacetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 51%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.89 (t, J = 6.7 Hz, 2H), 7.89–7.76 (m, 2H), 7.37 (ddd, J = 11.2, 6.7, 1.6 Hz, 2H), 7.30 (dd, J = 7.1, 2.5 Hz, 2H), 4.02 (s, 3H), 2.43 (s, 3H). ESI-LCMS (m/z): 331.2 $[M+H]^+$.

4.31. 2-(1-ethyl-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl) acrylonitrile (34)

The title compound was synthesized according to the procedure of preparing compound **14** except using 1-ethyl-2-benzimida zolylacetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 11% yield. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.14 (s, 1H), 7.96 (d, J = 8.2 Hz, 2H), 7.69 (dd, J = 11.2, 7.7 Hz, 2H), 7.41 (d, J = 8.1 Hz, 2H), 7.38–7.25 (m, 2H), 4.49 (q, J = 7.2 Hz, 2H), 2.41 (s, 3H), 1.44 (t,

J = 7.2 Hz, 3H). ESI-MS (m/z): 345.0 $[M+H]^+$.

4.32. 2-(1-clopropyl-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (35)

The title compound was synthesized according to the procedure of preparing compound **14** except using 1-cyclopropyl-2-benzimidazolylacetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 34%. 1H NMR (500 MHz, DMSO-*d6*): δ 8.07 (s, 1H), 7.98 (d, J = 9.0 Hz, 2H), 7.71–7.67 (m, 2H), 7.41–7.33 (m, 2H), 7.10 (d, J = 9.0 Hz, 2H), 3.73–3.70 (m, 1H), 3.50–3.47 (m, 4H), 1.66–1.60 (m, 6H), 1.30–1.28 (m, 2H), 1.00–0.98 (m, 2H) ppm. ESI-MS (m/z): 357.2 $[M+H]^+$.

4.33. 2-(benzoxazole-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (36)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(benzoxazolyl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 52%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.21 (s, 1H), 8.00 (d, J = 9.5 Hz, 2H), 7.75–7.70 (m, 2H), 7.41–7.38 (m, 2H), 6.84 (d, J = 9.0 Hz, 2H), 3.48 (t, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 318.2 [M + H]$^+$.

4.34. 2-(benzothiazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (37)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(benzothiazolyl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 61%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.11–8.07 (m, 2H), 8.01–7.97 (m, 3H), 7.54–7.51 (m, 1H), 7.46–7.43 (m, 1H), 6.83 (d, J = 9.0 Hz, 2H), 3.47 (q, J = 7.0 Hz, 4H), 1.14 (t, J = 6.5 Hz, 6H). ESI-LCMS (m/z): 334.1 $[M+H]^+$.

4.35. 2-(5-fluoro-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (38)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-fluoro-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 64%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.11 (s, 1H), 7.89 (d, J = 9.5 Hz, 2H), 7.59–7.56 (m, 1H), 7.41–7.37 (m, 1H),7.12–7.08 (m, 1H), 6.85 (d, J = 9.5 Hz, 2H), 3.47 (q, J = 7.5 Hz, 4H), 1.15 (t, J = 6.5 Hz, 6H). ESI-LCMS (m/z): 335.2 $[M+H]^+$.

4.36. 2-(5-chloro-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (39)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-chloro-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 54%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (s, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.61 (d, J = 2.5 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.24 (dd, J = 2.0 and 8.5 Hz, 1H), 6.847 (d, J = 9.0 Hz 2H), 3.478 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 351.2 $[M+H]^+$.

4.37. 2-(5-bromo-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (40)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-bromo-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 35%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (s, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.75 (d, J = 1.5 Hz, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.36–7.34 (m, 1H), 6.84 (d, J = 9.5 Hz, 2H), 3.48 (q, J = 7.5 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 395.2 $[M+H]^+$.

4.38. 2-(5, 6-dichloro-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (41)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5,6-dichloro-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 35%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (s, 1H), 7.89 (d, J = 8.5 Hz 1H), 7.78 (s, 1H), 6.84 (d, J = 9.5 Hz, 1H), 3.49–3.37 (m, 4H), 1.14 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 385.1 $[M+H]^+$.

4.39. 2-[5-(trifluoromethyl)-1H-benzimidazol-2-yl]-3-(4-diethylaminophenyl)acrylonitrile (42)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-trifluoromethyl-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 67%. ^{1}H NMR (500 MHz, DMSO- *d*6): δ 8.17 (s, 1H), 7.92 (d, J = 9.0 Hz, 2H) 7.89 (s, 1H), 7.73 (d, J = 8.5 Hz, 1H), 7.53 (d, J = 8.5 Hz, 1H), 6.85 (d, J = 9.5 Hz, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 384.9 $[M+H]^+$.

4.40. 2-(5-trifluoromethoxy-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (43)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-trifluoromethoxy-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 79%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.13 (s, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.62 (d, J = 9.0 Hz, 1H), 7.43 (d, J = 9.0 Hz, 1H), 7.39 (d, J = 9.0 Hz, 1H), 6.85 (d, J = 9.0 Hz, 2H), 3.47 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 401.1 $[M+H]^+$.

4.41. Methyl 2-[1-cyano-2-(4-diethylaminophenyl)vinyl]-1H-benzimidazole-5-carboxylate (44)

The title compound was synthesized according to the procedure of preparing compound **14** except using methyl 2-cyanomethyl-5-benzimidazolecarboxylate instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 59%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.16 (s, 1H), 8.13 (d, J = 1.0 Hz, 1H), 7.92 (d, J = 9.0 Hz, 2H), 7.85 (dd, J = 1.0 and 8.0 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 6.85 (d, J = 9.0 Hz, 2H), 3.87 (s, 3H), 3.47 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 6.5 Hz, 6H). ESI-LCMS (m/z): 375.2 $[M+H]^+$.

4.42. 2-[1-cyano-2-(4-diethylaminophenyl)vinyl]-3H-benzimidazol-5-carboxylic acid (45)

To a solution of Compound **44** (100 mg, 0.28 mmol) in MeOH/H_2O (1:1, 5 mL) was added NaOH (27 mg, 0.68 mmol). After the solution was stirred at room temperature overnight, the pH was adjusted to 1 with HCl solution (1N). After the solvent was removed in vacuo, the residue was purified by reverse phase preparative HPLC in a gradient increase of methanol in water solution to afford compound **45** (67 mg, 72%). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.17 (s, 1H), 8.13 (s, 1H) 7.92 (d, J = 9.5 Hz, 2H), 7.85 (dd, J = 2.0 and 9.0 Hz 1H), 7.63 (d, J = 8.0 Hz, 1H), 6.86 (d, J = 9.5 Hz, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (m/z): 361.0 $[M+H]^+$.

4.43. 2-(5,6-dimethyl-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (46)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5,6-dimethyl-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 56%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.13 (s, 1H), 7.89 (d, J = 9.0 Hz, 2H), 7.40 (s, 2H) 6.87 (d, J = 9.5 Hz, 2H), 3.49 (q, J = 7.0 Hz, 4H), 2.34 (s, 6H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 345.3 $[M+H]^+$.

4.44. 2-[5-(tert-butyl)-1H-benzimidazol-2-yl]-3-(4-diethylaminophenyl)acrylonitrile (47)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-*tert*-butyl-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 72%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.12 (s, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.54–7.51 (m, 2H), 7.39 (d, J = 8.5 Hz, 1H), 6.87 (d, J = 8.5 Hz, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.33 (s, 9H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 373.3 $[M+H]^+$.

4.45. 2-(5-methoxy-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (48)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-methoxy-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl) acetonitrile. Yield: 42%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.07 (s, 1H), 7.88 (d, J = 9.0 Hz, 2H), 7.49 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 2.0 Hz, 1H), 6.91 (dd, J = 2.0 and 9.0 Hz, 1H), 6.86 (d, J = 9.0 Hz, 2H), 3.81 (s, 3H), 3.48 (t, J = 6.5 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 347.0 $[M+H]^+$.

4.46. 2-[5-(dimethylamino)-1H-benzimidazol-2-yl]-3-(4-diethylaminophenyl)acrylonitrile (49)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-dimethylamino-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 75%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.14 (s, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.57 (d, J = 9.0 Hz, 1H), 7.16–7.14 (m, 2H), 6.88 (d, J = 9.5 Hz, 2H), 3.49 (q, J = 7.0 Hz, 4H), 3.07 (s, 6H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 360.0 $[M+H]^+$.

4.47. 2-(5-methanethionyl-1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl)acrylonitrile (50)

The title compound was synthesized according to the procedure of preparing compound **14** except using 2-(5-methanethionyl-1*H*-benzimidazol-2-yl)acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 33%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.10 (s, 1H), 7.89 (d, J = 9.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 1H), 7.44 (d, J = 1.0 Hz, 1H), 7.19 (d, J = 8.0 Hz, 1H), 6.86 (d, J = 9.0 Hz, 2H), 3.48 (q, J = 7.5 Hz, 4H), 2.53 (s, 3H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 363.0 $[M+H]^+$.

4.48. 2-(3H-imidazo[4,5-c]pyridin-2-yl)-3-(4-diethylaminophenyl) acrylonitrile (51)

The title compound was synthesized according to the procedure of preparing compound **14** except using 3*H*-imidazo[4,5-*c*]pyridine-2-acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 46%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.84 (d, J = 0.5 Hz, 1H), 8.23–8.21 (m, 1H), 7.91 (d, J = 8.5 Hz, 2H), 7.55–7.53 (m, 1H), 6.84 (d, J = 9.5 Hz, 2H), 3.50–3.40 (m, 4H), 1.17–1.11 (m, 6H). ESI-LCMS (*m*/*z*): 318.2 $[M+H]^+$.

4.49. 2-(3H-imidazo[4,5-b]pyridin-2-yl)-3-(4-diethylaminophenyl) acrylonitrile (52)

The title compound was synthesized according to the procedure of preparing compound **14** except using 3*H*-imidazo[4,5-*b*]pyridine-2-acetonitrile instead of 2-(1*H*-benzimidazol-2-yl)acetonitrile. Yield: 43%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.29 (dd, J = 1.5 and 5.0 Hz, 1H), 8.17 (s, 1H), 7.94 (dd, J = 1.5 and 8.0 Hz, 1H), 7.89 (d, J = 9.0 Hz, 2H), 7.20 (dd, J = 5.0 and 8.0 Hz, 1H), 6.85 (d, J = 9.0 Hz, 2H), 3.47 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ESI-LCMS (*m*/*z*): 318.2 $[M+H]^+$.

4.50. 7-diethylamino-3-(benzimidazol-2-yl)coumarin (53)

The title compound is a byproduct of the synthesis of compound **30**. Compound **53** was eluted out when the reaction mixture was purified by silica gel chromatography using cyclohexane-acetone-ethyl acetate (10:1:1). Yield: 15%. 1H NMR (500 MHz, DMSO- *d*6): δ 12.28 (s, 1H), 8.93 (s, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.65–7.58 (m, 2H), 7.17 (t, J = 4.5 Hz, 2H), 6.83 (dd, J = 2.0 and 9.0 Hz, 1H), 6.67 (d, J = 2.0 Hz, 1H), 3.48 (q, J = 6.5 Hz, 4H), 1.17 (t, J = 7.0 Hz, 6H). ESI-MS (*m*/*z*): 334.1 $[M+H]^+$.

4.51. 2-(1H-benzimidazol-2-yl)-3-(4-diethylaminophenyl) propanenitrile (54)

To a solution of Compound **14** (80 mg, 0.25 mmol) in MeOH (5 mL) was added 5% Pd/C (10 mg, 10% wt) under hydrogen atmosphere at room temperature for 12 h. The reaction mixture was filtered through celite and the solvent was concentrated in vacuo. The crude product was purified by reverse phase preparative HPLC in a gradient increase of methanol in water solution to afford compound **54**. Yield: 42%. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 12.68 (br s, 1H), 7.62–7.51 (m, 2H),7.20 (br s, 2H),7.05 (d, J = 8.5 Hz, 2H), 6.57 (d, J = 8.5 Hz, 2H), 4.74 (dd, J = 6.5 and 9.0 Hz, 1H), 3.28 (q, J = 6.0 Hz, 4H), 3.20 (dd, J = 5.0 and 9.5 Hz, 2H), 1.04 (t, J = 6.0 Hz, 6H). ESI-LCMS (*m*/*z*): 319.2 $[M+H]^+$

4.52. Plasmids and cells

The sequence for full-length *vif* was amplified with PCR from an infectious HIV-1 clone pNL4-3 and inserted into pcDNA3.1-intron with a fused hemagglutinin (HA) tag at the N terminus. The cDNA fragment coding for hA3G was cloned into pEGFP-C1, as described previously [31].The 293T cells were maintained in the conditioned Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), plus 100 μg/mL penicillin and streptomycin. The H9 and SupT1 cells were cultured in conditioned RPMI 1640 supplemented with 10% FBS and 100 μg/mL penicillin and streptomycin.

4.53. High-throughput screen

Vif-HA and A3G-GFP plasmids were co-transfected into 293T cells in 96-well plate (Corning, Costar) and Lipofectamine 2000 (Invitrogen) by following the instructions of manufacturer. After transfection, 20,155 drug-like set compounds (Enamine, Monmouth Jct, NJ) were added using a Tecan Freedom EVO150 (Tecan, Männedorf, Schweiz) with a final concentration of 50 μM. Column 12 received only DMSO instead of any compounds. In addition, column 1 was only transfected with the plasmid expressing A3G-GFP as a control. The GFP expression was detected with a PE

Envision (Perkin–Elmer) at 48 h post-transfection [20]. The fold change of mean fluorescent intensity (MFI) was calculated as: MFI of cells with compound treatment together with transfection of A3G-GFP and Vif/MFI of cells only with transfection of A3G-GFP and Vif.

4.54. Co-immunoprecipitation (Co-IP)

HEK293T cells were transfected with pcDNA3.1-Vif-HA and pcDNA3.1-EC/EB/CUL5-Flag and were lysed 48 h later with IP lysis buffer. Then the lysate were incubated with anti-HA beads (Sigma) overnight at 4 °C. Then IP products were centrifuged and washed three times with lysis buffer. Western blot was conducted to analyze the immunoprecipitated samples with the following primary antibodies: anti-FLAG (rabbit polyclonal, MBL) or anti-HA (mouse monoclonal, MBL) [33].

4.55. PBMCs isolation

The peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors through Ficoll gradient centrifugation, followed by culturing in the RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml of penicillin and 100 μg/mL of streptomycin (Invitrogen).

4.56. HIV-1 production, infection, and IC50 assay

HIV-1 infectious clone, pNL4-3 (X4) was amplified with HB101 competent cells (Promega). To generate viruses, 293T cells were transfected with 10 μg of infectious clones and Lipofectamine 2000 (Invitrogen) by following the instructions of manufacturer. Culture supernatants were harvested at 48 h post-transfection and stored at −80 °C. To normalize viral inputs, the amount of p24 was measured by HIV-1 p24 enzyme-linked immunosorbent assay (ELISA). The target cells (1×10^6) were infected with the equivalent of 5 ng HIV-1 p24 in 1 mL for 3 h at 37 °C. The virus-containing supernatants were then removed by washing 3 times with PBS. The cells were maintained in conditioned RPMI 1640 medium and HIV-1 replication was monitored by p24 detection [5].

4.57. Cell toxicity assay

Cell toxicity assay was performed with the CellTiter-Glo Luminescent Cell Viability Assay Kit which was purchased from Promega Company. The instructions of manufacturer were followed. Luminescence was recorded with a Promega plate reader [34,35].

4.58. Protein purification

The plasmid pET32a harboring His-tagged Vif or His-tagged A3G genes were transformed into E. Coli BL21 competent cells (Novagen) respectively. After the expression of proteins was induced by 1 mM isopropylthio-β-D-galactoside, the bacterial cells were lysed by sonication. The insoluble fraction was pelleted at 10,000 × g for 10 min, and the supernatant was applied to a Ni-conjugated agarose bead column (GE). After washing, the bound His fusion proteins were eluted with 500 μM Imidazole. Then the proteins were suspended in PBS buffer and the concentration was measured by the Bradford method. The samples were then aliquoted and frozen at −80 °C [36].

4.59. Surface plasmon resonance

The measurements were carried out with a Biacore T100 instrument (GE Healthcare). A Biacore CM5 Sensor Chip and an amine coupling kit were purchased directly from GE Healthcare. The suitable pH value of 4.0 for Vif-His immobilization (50 μg/mL in 10 mM acetate buffer) was determined first. The CM5 censor chip was activated and then injected with Vif-His (100 μg/mL, in 10 mM acetate buffer, pH 4.0) for 7 min. The residual activated groups on the surfaces were blocked with an injection of ethanolamine HCl (1M) for 7 min A3G-His protein was pre-incubated with DMSO, compound **14**, or compound **26** for 30 min and then was injected (30 μl/min). Binding to the Vif-His protein was monitored for about 120 s. The dissociate time were 200 s for compound **14** and 150s for **26** with running buffer in per cycle.

4.60. Acute toxicological assay

Male Balb/c mice, 4–6weeks, were purchased from Laboratory Animal Center in Sun Yat-Sen University, Guangzhou, China. These mice were randomly divided into three groups and were then intraperitoneally injected with compounds at different doses. After two weeks, mice were sacrificed. The organs including heart, liver, spleen, lung, and kidney were fixed in 4% formaldehyde at room temperature for hematoxylin and eosin staining and the blood samples were subjected to the analysis of hepatic or renal functions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.050.

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