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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Modification and biological evaluation of novel 4-hydroxy-pyrone derivatives as non-peptidic HIV-1 protease inhibitors

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Abstract In this study, we have modified 4-hydroxy-pyran-2-ones, especially introduced heteroatoms (***S*** or ***O***) into the substituents, and detected their interactions with the binding pockets of HIV-1 protease (PR). The results indicated that the ethoxyl groups at C-2′ and C-5′ of the phenyl ring could enhance the affinities to the $\mathbf{S_1}'$ and $\mathbf{S_2}'$ pockets and improve the inhibitory activities. The most potent compound **10f** with an IC_{50} of 3.5 nM in enzymatic assay also exhibited good antiviral activity at the cellular level; it exhibited an EC_{50} value of 2.9 μM in Simian immunodeficiency virus-infected CEM cells and suppressed the PR activity in 293T cells using western blot analysis.

Keywords 4-Hydroxy-pyran-2-ones · HIV-1 protease inhibitors · Anti-SIV activity

Introduction

Human immunodeficiency virus type 1 (HIV-1) protease (PR), that cleaves the Gag and Gag/Pol polyproteins, is essential for the formation of mature and infectious virions (Redshaw, 1994). In an attempt to develop new anti-HIV agents, numerous new and potent HIV PR inhibitors have been identified (Huff, 1991; Meek, 1992; Thaisrivongs, 1994; Leung *et al.*, 2000), but most of them are peptidomimetic compounds and often associates with a low bioavailability and rapid clearance (West and Fairlie, 1995; Lebon and Ledecq, 2000). Thus, it is important to design and identify new non-peptidic structural inhibitors.

Different research groups have synthesized series of 4-hydroxy-pyran-2-one derivatives as HIV-1 PR inhibitors (Romines and Chrusciel, 1995; Vara Prasad *et al.*, 1999; Tummino *et al.*, 1996; Thaisrivongs *et al.*, 1996; Lunney *et al.*, 1994; Skulnick *et al.*, 1995; Tait *et al.*, 1997; Sun *et al.*, 2005). The pyran-2-one group, 4-hydroxyl group, and substitution at the 3-position were all necessary for the HIV-1 PR inhibitory activity. X-ray structural study showed that there were four internal four pockets ($\mathbf{S_2}$, $\mathbf{S_1}$, $\mathbf{S_1}$, $\mathbf{S_2}$) within HIV-1 PR for the 4-hydroxy-pyran-2-ones to fill (Thaisrivongs *et al.*, 1996; Lunney *et al.*, 1994; Skulnick *et al.*, 1995).

Great efforts were focused on adding thiosubstitutes to the C-3 position of 4-hydroxy-pyran-2-ones. As a result, compound **1** (Fig. 1) was identified to be a potent HIV PR inhibitor with a very low IC_{50} value of 1.1 nM (Vara Prasad *et al.*, 1999). But it showed poor antiviral activity (EC_{50} = 15 μM). Compound **2** was the most active one in vitro (IC_{50} = 5 nM) because of the hydrogen bond formed between –COOH and PR residues. However, it exhibited low antiviral activity in cellular assay due to the high polarity of the carboxylic acid (Tait *et al.*, 1997). Our previous study (Sun *et al.*, 2005) has revealed some 4-hydroxy-3-thiosubstituted-pyran-2-ones (compound **3** in Fig. 1) which showed good antiviral activities in Simian immunodeficiency virus (SIV)-induced syncytium in hybrid human B-/T-cell lines (CEM174) (Mahlknecht *et al.*, 2000).

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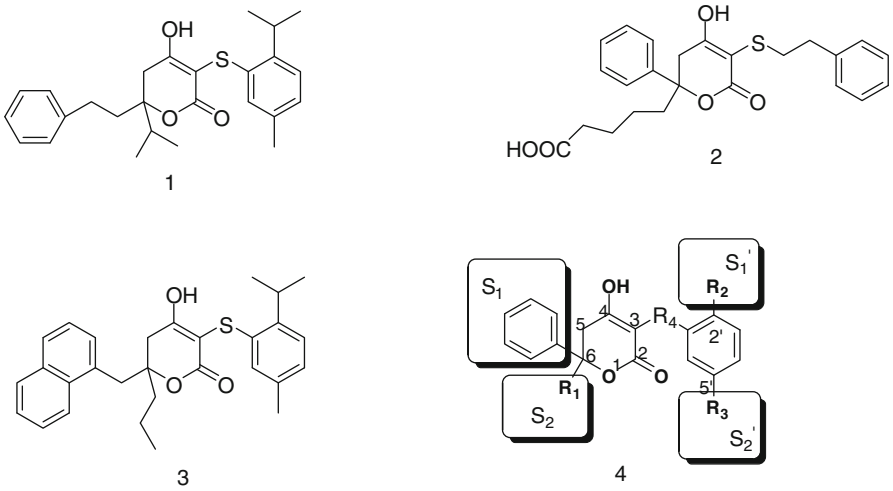
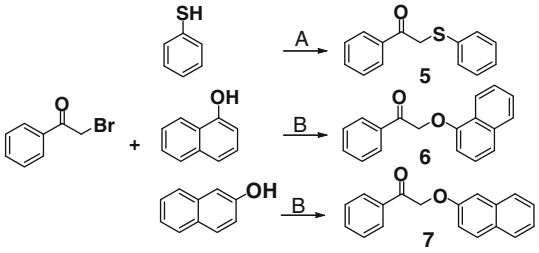


Fig. 1 4-Hydroxy-3-thiosubstituted-pyran-2-ones and four binding pockets in HIV-1 PR

Based on those results, we designed the template **4** (Fig. 1). The propyl group at C-6 position was replaced with a phenyl group which could improve the activity as previously reported (Tait *et al.*, 1997). For the other substituent at the C-6 position of the pyrone ring, we inserted ***O*** or ***S*** between the aryl group and the heterocycle. We expected that the heteroatom could form hydrogen bond and provide suitable polarity to improve the activity. We also modified at the C-2′ and C-5′ positions of the phenyl group located at the C-3 position of the pyrone ring. Besides various alkyl groups, we introduced ***O***-containing group to this position, which was rarely studied before.

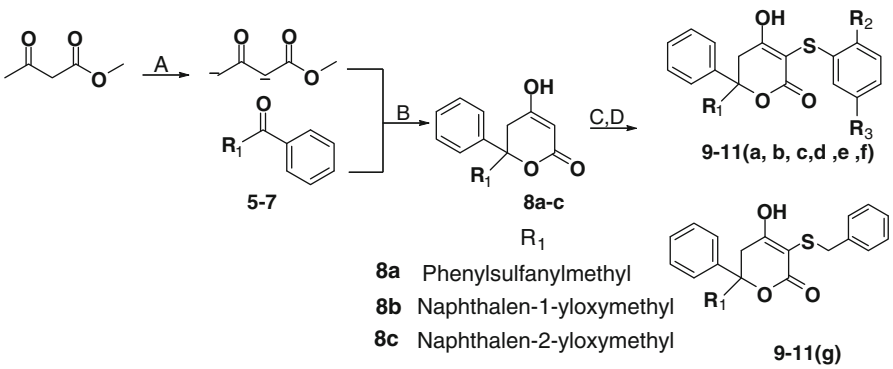


Scheme 1 Reagents and conditions: (A) K_2CO_3, EtOH, rt; (B) K_2CO_3, acetone, reflux

Overall, we have synthesized a number of 4-hydroxy-pyran-2-ones derivatives and tested their inhibitory activities. Furthermore, the structures were docked to the binding sites of HIV-1 PR using AutoDock3.0 program (Morris *et al.*, 1998; Huang *et al.*, 2002), to investigate the interaction characteristics between respective compound and HIV-1 PR.

Synthesis

α-Bromoacetophenone was prepared by α-bromination of acetophenone with $CuBr_2$ (Peter *et al.*, 1998), and then reacted with thiophenol or naphthol (alpha- or beta-) in the presence of potassium carbonate, to get the appropriate ketone (**5–7** in Scheme 1) (Wang *et al.*, 1998; Seshadri *et al.*, 1981). The ketone reacted with the dianion of methyl acetoacetate to produce compound **8** in Scheme 2. The method for the synthesis of the dihydropyrone targets (**9–11** in Scheme 2) involved preparation of the 3-bromodihydropyrone derivatives with NBS in the dark and then displacement of the bromide by the appropriately substituted thiophenol.



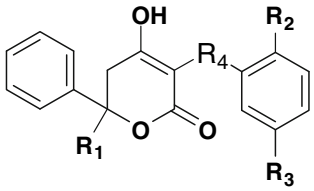
Scheme 2 Regeants and conditions: (A) (i) NaH, (ii) *n*-BuLi, dry THF, followed by ketone addition, 0°C; (B) (i) NaOH, CH_3OH, (ii) H^+, 50–70% (for two steps); (C) NBS, dry *t*-BuOH, reflux; (D) corresponding thiophenol, piperidine, CH_2Cl_2, 50–65% (for two steps)

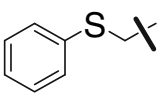
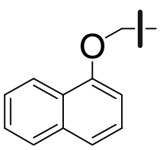
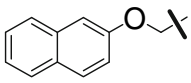
Results and discussion

The inhibitory activities for HIV PR (Nashed *et al.*, 1989) were tested and results were listed in Table 1. To further investigate their inhibitory activities at the cellular level, we also evaluated the antiviral activities of the compounds against SIV-induced syncytium in CEM174 cells (Mahlknecht *et al.*, 2000). Their $EC5_0$, TC_{50}, and TI values are listed in Table 2. The compounds **9a–d** to **11a–d** with phenylthiomethyl or naphthalenyloxymethyl at the C-6 position of the pyrone had high IC_{50}, some of which even had IC_{50} higher than 200 nM. Since the phenyl group at the C-6 position of the pyrone ring was reported to improve the inhibitory activity, we assumed that the observed impressed activity could be due to the inserted heteroatoms. The compounds were docked into the binding site of HIV-1 PR structure using AutoDock3.0 program (Morris *et al.*, 1998; Huang *et al.*, 2002). Figure 2 from docking result showed that the phenyl group at C-6 position of the pyrone ring could fit the $\mathbf{S_1}$ pocket very well as predicted. On the other hand, though the other group at C-6 position of the pyrone ring could insert into the $\mathbf{S_2}$ pocket, there was no hydrogen bond formed by the ***O*** or ***S*** atom as the –COOH group did. Instead, the affinity was weakened because of the polarity caused by the heteroatom when it overlaid into the $\mathbf{S_2}$ pocket which is hydrophobic.

To explore the interaction with $\mathbf{S_1}'$ and $\mathbf{S_2}'$ pockets, we modified at the **2′** and **5′** positions of the phenyl ring located at the C-3 position of the pyrone ring. The data in Table 1 showed that the activities were enhanced when the bulk of the alkyl group at the **2′** and **5′** positions was increased. Substitution of the phenyl ring at the **2′** position with ***Cl*** group also resulted in an enhanced enzymatic

Table 1 The modification and IC_{50} values of 4-hydroxy-pyrones



Entry[a]	R_1	R_2	R_3	R_4^b	IC_{50}^c (nM)
9a		H	H	S	258
9b		CH_3	H	S	240
9c		C_2H_5	H	S	238
9d		Cl	H	S	70
9e		CH_3	CH_3	S	196
9f		CH_3CH_2O	CH_3CH_2O	S	24
9g		H	H	SCH_2	32
10a		H	H	S	159
10b		CH_3	H	S	64
10c		C_2H_5	H	S	100
10d		Cl	H	S	59
10e		CH_3	CH_3	S	126
10f		CH_3CH_2O	CH_3CH_2O	S	3.5
10g		H	H	SCH_2	128
11a		H	H	S	126
11b		CH_3	H	S	61
11c		C_2H_5	H	S	22
11d		Cl	H	S	89
11e		CH_3	CH_3	S	65
11f		CH_3CH_2O	CH_3CH_2O	S	9.2
11g		H	H	SCH_2	73

[a] All compounds were racemic mixtures. IDV was used as positive control

[b] The ***S*** atom was attached to the pyrone ring and CH_2 was linked to the phenyl group when R_4 was SCH_2

[c] IC_{50} is the effective concentration at which 50% of HIV-1 PR activity is inhibited and is the average of at least two runs

Table 2 Antiviral activity and cytotoxicity of 4-hydroxy-pyrones

Entry	EC_{50}^a (μM)	TC_{50}^b (μM)	TI[c]
9a	12.4	18.6	1.5
9b	13.2	19.8	1.5
9c	12.6	25.0	2.0
9d	7.2	25.4	3.5
9e	10	>100	>10
9f	5.8	>100	17.2
9g	4.8	>100	>26
10a	11.7	72.0	6.1
10b	7.1	17.3	2.4
10c	11	20.9	1.9
10d	6.8	16.9	2.4
10e	8.7	56.9	6.5
10f	2.9	17.1	5.9
10g	11	>100	>9.1
11a	9.8	>100	>10
11b	7.1	16.5	2.3
11c	4.3	58.9	13.7
11d	7.6	22.8	3.0
11e	7.5	>100	13.3
11f	4.3	35.2	8.2
11g	7.9	40.7	5.2
IDV	<0.1	>100	>1000

[a] EC_{50} is the effective concentration at which 50% of the CEM cells are protected from SIV infection and is the average of at least two runs

[b] TC_{50} is the concentration that elicits cytotoxicity in 50% of uninfected CEM cells

[c] TI, therapeutic index = TC_{50}/EC_{50}

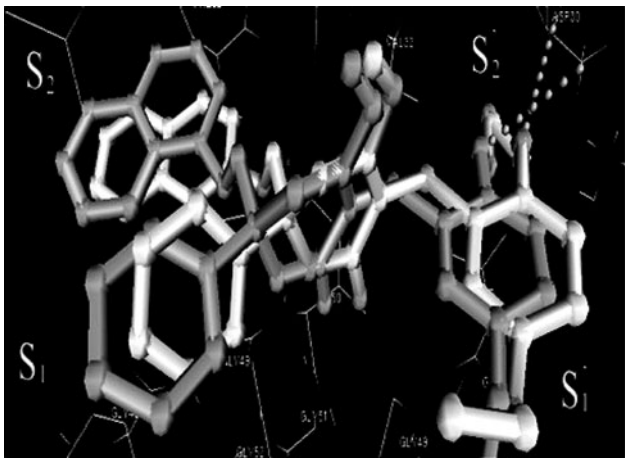


Fig. 2 Molecular modeling of compounds **10f** and **11f** docked in HIV-1 PR. Compound **10f** was *white* and **11f** was *gray*. Program AutoDock3.0 automatically selected the best confirmation among the stereo isomers

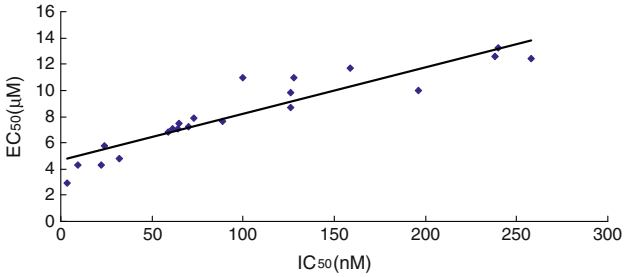


Fig. 3 The antiviral activity of the compounds is correlated with their PR inhibitory activity

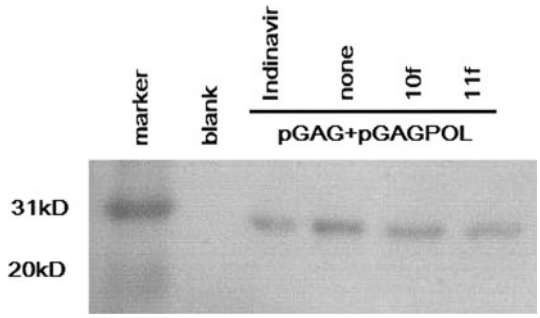


Fig. 4 Western blot assay with anti-p24 antibody showed convincingly reduced expression of p24 in 293T cells co-transfected with pGAGPOL and pGAG which encoded PR and its substrate, respectively, in the cases of **10f** and **11f** treatments. The cells without co-transformation were used as blank and IDV was used as positive control

activity (**10d** versus **10a**, **11d** versus **11a**). The most significant modification that dramatically improved the inhibitory activities was the ethoxyl groups at **2′** and **5′** positions (**9f**, **10f**, and **11f**). Figure 2 from molecular modeling displayed that the active conformation of **10f** nearly coincided with **11f**. Their 2′-ethoxyl group and 5′-ethoxyl group effectively filled the $\mathbf{S_1}'$ and the $\mathbf{S_2}'$ pockets and they could form hydrogen bonds with the residue **Asp30** in $\mathbf{S_2}'$ pocket. These interactions reduced their docking energy and promoted their inhibitory activities. Therefore, even with the heteroatom-containing substituents on the pyrone, the derivatives **10f** and **11f** exhibited good inhibitory activity with the IC_{50} below 10 nM.

We also inserted a methylene group between the ***S*** atom and the phenyl group (**9g–11g**) (Table 1). The prolonged linker yielded a modest enhancement in the binding affinity with the enzyme relative to compounds **9a–11a**.

As shown in Table 2, Compound **10f** with an enzyme IC_{50} of 3.5 nM exhibited a significant antiviral activity ($EC_{50} = 2.9$ μM), whereas the majority of the derivatives showed modest or low EC_{50} values (Table 2). Furthermore, we calculated the correlation between PR inhibitory activity and antiviral activity, which showed a correlation value of 0.85 ($P < 0.05$).

To test whether these compounds are indeed targeting viral PR (Fig. 3), we co-transfected 293T cells with HIV Gag/Pol and Gag expressing plasmids (pGAGPOL and pGAG), in the presence of different compounds. Since, during HIV morphogenesis, the Gag and Gag–Pol precursor need to be cleaved by viral PR to generate different structural and enzymatic proteins including Gagp24, we can easily test the effect of different compounds on PR's activity by measuring Gagp24 level in the cells by anti-p24 western bolt analysis (Chien *et al.*, 2006). As shown in Fig. 4, the p24 protein expression level was decreased in the presence of **10f** and **11f**, indicating that the activity of PR was inhibited by these compounds in 293T cells. These results provide evidence that compounds **10f** and **11f** would be a promising HIV-1 PR inhibitor because of potent enzyme inhibitory activity and antiviral activity.

Conclusion

In conclusion, we modified the 4-hydroxy-pyran-2-one, especially introduced heteroatoms into the substituents to detect the interaction with the binding pockets. The results indicated that the heteroatom (***S*** or ***O***) at the C-6 position of the pyrone was disadvantageous to fit the $\mathbf{S_2}$ pocket, whereas ethoxyl groups at **C-2′** and **C-5′** of the phenyl ring could enhance the affinities to the $\mathbf{S_1}'$ and $\mathbf{S_2}'$ pockets and improve the inhibitory activity. The most potent compound **10f** which inhibited HIV-1 PR in vitro ($IC_{50} = 3.5$ nM) also exhibited significant antiviral activity in a cell-based SIV infection assay with an EC_{50} value at 2.9 μM and inhibited the PR activity in 293T cells.

Experimental section

General

All reactions were performed with commercially available reagents and they were used without further purification. Solvents were dried by standard methods and stored over molecular sieves. All reactions were monitored by thin-layer chromatography and viewed with UV light. Melting points were determined on an XA-4 instrument. All the title compounds were characterized by ^{1}H NMR spectra on a Varian 300 MHz photometer using the solvents described. Chemical shifts were reported in parts per million relative to tetramethylsilane for deteriorated water (D_2O). Signals were quoted as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were measured on a VG-ZAB-HS spectrometer or an ABI QSTAR spectrometer.

1-Phenyl-2-(phenylthio)ethanone (**5**)

α-Bromoacetophenone (5.6 g, 28 mmol) was stirred in ethanol (100 ml) with anhydrous K_2CO_3 (4.2 g, 30 mmol). Benzenethiol (3.3 g, 30 mmol) was added, and stirring was continued for 1 h. The reaction mixture was filtered and diluted with $CHCl_3$ (200 ml), and the organic layer was washed with H_2O (4× 50 ml). The $CHCl_3$ solution was dried (Na_2SO_4), filtered, and concentrated on a rotary evaporator under reduced pressure. The product was recrystallized (petroleum ether/ethyl acetate = 50:1) to give 5.5 g (87% yield) of the title compound. mp 53–55°C. (Seshadri *et al.*, 1981)

2-(Naphthalen-1-yloxy)-1-phenylethanone (**6**)

α-Bromoacetophenone (5.6 g, 28 mmol) was stirred in acetone (100 ml) with anhydrous K_2CO_3 (4.2 g, 30 mmol). naphthalen-1-ol (4.4 g, 30 mmol) was added, and the mixture was refluxed for 2 h. The reaction mixture was quenched with water and allowed to reach room temperature. The aqueous layer was extracted with Et_2O (4× 50 ml). The combined organic fraction was washed (1 N NaOH), dried (Na_2SO_4), filtered, and concentrated on a rotary evaporator under reduced pressure. The product was recrystallized to give 6.2 g (85% yield) of the title compound. mp 71–73°C (Wang *et al.*, 1998).

2-(Naphthalen-3-yloxy)-1-phenylethanone (**7**)

Compound **7** was prepared in the same way as **6**, yield = 83%, mp 101–104°C (Wang *et al.*, 1998).

5,6-Dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**8a**)

Methyl acetoacetate (3.4 ml, 32 mmol) was added dropwise to a slurry of hexane-washed sodium hydride (0.84 g, 35 mmol) in anhydrous THF at 0°C and the reaction stirred at 0°C for 15 min. *n*-Butyllithium (15 ml, 2.4 mol/l) was then added at 0°C and the reaction stirred at 0°C for 15 min. Compound **5** (3.42 g, 15 mmol) in THF was added to the dianion, and the reaction was stirred at 0°C for 30 min, and then allowed to warm to room temperature. The mixture was stirred for 16 h at room temperature. Water or 0.1 N NaOH was added to the reaction mixture and stirred for 30 min. After extracting with Et_2O, the aqueous layer was cooled to 0°C and acidified with 6 N HCl to pH 1–2. The aqueous layer was extracted with EtOAc (3× 30 ml). The organic extracts were combined, dried over $MgSO_4$, and concentrated. After being purified by column chromatography (silica gel, petroleum ether/ethyl acetate = 5:1), **8a** was obtained as an off-white solid, 2.04 g, mp 122–124°C, yield = 43.5%. EI-MS *m/z*: 312 (M^+), 189, 123. ^{1}H NMR($CDCl_3$) δ3.26 (1H, d, J = 22.5 Hz, CH_2), 3.28 (1H, d, J = 22.5 Hz, CH_2), 3.33 (1H, d, J = 10.5 Hz, CH_2), 3.36 (1H, d, J = 10.5 Hz, CH_2), 3.55 (1H, d, J = 17.4 Hz, SCH_2), 3.57 (1H,d, J = 17.4 Hz, SCH_2), 7.19–7.42 (10H, m, Ar–H).

5,6-Dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**8b**)

Compound **8b** was prepared in the same way as **8a**, yield = 62%, mp 154–156°C. EI-MS *m/z*: 346 (M^+), 158, 144. ^{1}H NMR ($CDCl_3$) δ3.42 (1H, d, J = 17.4 Hz, CH_2), 3.81 (1H, d, J = 17.4 Hz, CH_2), 4.23 (1H, d, J = 10.2 Hz, OCH_2), 4.46 (1H, d, J = 10.2 Hz, OCH_2), 5.51 (1H, s, CH=COH), 6.73 (1H, d, J = 7.2 Hz, Ar–H), 7.27–7.59 (9H, m, Ar–H), 7.79–7.82 (1H, m, Ar–H), 8.17–8.20 (1H, m, Ar–H).

5,6-Dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**8c**)

Compound **8c** was prepared in the same way as **8a**, yield = 67%, mp 100–102°C. EI-MS *m/z*: 346 (M^+), 158, 144. ^{1}H NMR ($CDCl_3$) δ3.32 (1H, d, J = 17.4 Hz, CH_2), 3.79 (1H, d, J = 17.4 Hz, CH_2), 4.14 (1H, d, J = 10.2 Hz, OCH_2), 4.39 (1H, d, J = 10.2 Hz, OCH_2), 5.73 (1H, s, CH=COH), 7.05–7.77 (12H, m, Ar–H).

5,6-Dihydro-4-hydroxy-6-phenyl-3-(phenylthio)-6-((phenylthio)methyl)pyran-2-one (**9a**)

Compound **8a** (624 mg, 2 mmol) was stirred in *t*-BuOH (50 ml) with NBS (356 mg, 2 mmol) in the dark. The

reaction mixture was refluxed for 2 h. The solvent was evaporated, and the residue was partitioned between $CHCl_3$ and water. The aqueous layer was extracted with $CHCl_3$ (4× 30 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The crude product was dissolved in $CHCl_3$ (50 ml). To the solution, benzenethiol (230 mg, 2.1 mmol) was added. The mixture was cooled to 0°C (ice bath) and piperidine (0.2 ml) was added. The reaction mixture was stirred at room temperature for 2 h. Water was added and the aqueous layer was extracted with $CHCl_3$ (2× 15 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. After being purified by column chromatography (silica gel, dichloromethane/methanol = 100:1), **9a** was obtained as an off-white solid, 353 mg, yield = 42%, mp 173–175°C. EI-MS *m/z*: 420 (M^+), 312, 105. ^{1}H NMR ($CDCl_3$) δ: 3.38 (1H, d, J = 14.1 Hz, CH_2), 3.43 (1H, d, J = 17.7 Hz, SCH_2), 3.58 (1H, d, J = 17.7 Hz, SCH_2), 3.64 (1H, d, J = 14.1 Hz, CH_2), 6.55 (2H, d, J = 7.5 Hz, Ar–H), 6.93–7.19 (3H, m, Ar–H), 7.19–7.50 (10H, m, Ar–H).

3-(*o*-Tolylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9b**)

The compound was obtained in the same way from 2-methylbenzenethiol and compound **8a** in the same procedure for **9a**. **9b** was obtained as an off-white solid, 443 mg, yield = 51%, mp 162–163°C. EI-MS *m/z*: 434 (M^+), 325, 311. ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 3H, CH_3), 3.36 (d, 1H, J = 13.8 Hz, CH_2), 3.43 (d, 1H, J = 17.4 Hz, SCH_2), 3.57 (d, 1H, J = 17.4 Hz, SCH_2), 3.62 (d, J = 13.8 Hz, CH_2), 6.58 (s, 1H, Ar–H), 6.96 (s, 1H, Ar–H), 7.05 (d, 1H, J = 7.2 Hz, Ar–H), 7.15–7.58 (m, 11H, Ar–H).

3-(2-Ethylphenylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9c**)

9c was obtained as an off-white solid, 488 mg, yield = 54%, mp 105–107°C. MS (EI) *m/z*: 448 (M^+), 325, 311. ^{1}H NMR ($CDCl_3$) δ 1.22 (3H, t, J = 7.5 Hz, $CH_2\underline{CH_3}$), 2.68–2.75 (2H, m, $\underline{CH_2}CH_3$), 3.38 (1H, d, J = 14.1 Hz, CH_2), 3.44 (1H, d, J = 17.4 Hz, SCH_2), 3.60 (1H, d, J = 17.4 Hz, SCH_2), 3.65 (1H, d, J = 14.1 Hz, CH_2), 6.58 (1H, s, Ar–H), 6.98 (1H, d, J = 6.6 Hz, Ar–H), 7.05 (1H, d, J = 6.6 Hz, Ar–H), 7.17–7.31 (5H, m, Ar–H), 7.33–7.47 (6H, m, Ar–H).

3-(2-Chlorophenylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9d**)

9d was obtained as an off-white solid, 368 mg, yield = 40%, mp 83–85°C. EI-MS *m/z*: 454 (M^+), 331, 311. ^{1}H NMR ($CDCl_3$) δ 3.36 (1H, d, J = 13.8 Hz, CH_2), 3.42 (1H, d, J = 17.4 Hz, SCH_2), 3.59 (1H, d, J = 17.4 Hz, SCH_2), 3.62 (1H, d, J = 13.8 Hz, CH_2), 6.65 (1H, t, J = 6.6 Hz, Ar–H), 6.90 (1H, t, J = 6.6 Hz, Ar–H), 7.10–7.52 (12H, m, Ar–H).

3-(2,5-Dimethylphenylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9e**)

9e was obtained as an off-white solid, 466 mg, yield = 52%, mp 156–158°C. EI-MS *m/z*: 448 (M^+), 325. ^{1}H NMR ($CDCl_3$) δ 1.91 (3H, s, CH_3), 2.28 (3H, s, CH_3), 3.38 (1H, d, J = 13.8 Hz, CH_2), 3.41 (1H, d, J = 17.4 Hz, SCH_2), 3.62 (1H, d, J = 13.8 Hz, CH_2), 3.64 (1H, d, J = 17.4 Hz, SCH_2), 6.78 (1H, d, J = 7.8 Hz, Ar–H), 6.98 (1H, d, J = 7.8 Hz, Ar–H), 7.21–7.48 (11H, m, Ar–H).

3-(2,5-Diethoxyphenylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9f**)

9f was obtained as an off-white oil, 588 mg, yield = 54%. EI-MS *m/z*: 508 (M^+), 464, 385. ^{1}H NMR ($CDCl_3$) δ 1.34–1.42 (6H, m, $\underline{CH_3}CH_2O$), 3.18 (1H, d, J = 17.4 Hz, SCH_2), 3.34 (1H, d, J = 14.1 Hz, CH_2), 3.47 (1H, d, J = 17.4 Hz, SCH_2), 3.53 (1H, d, J = 14.1 Hz, CH_2), 3.87–3.99 (4H, m, $CH_3\underline{CH_2}O$), 6.74 (2H, d, J = 2.7 Hz), 6.99 (2H, d, J = 2.7 Hz), 7.14–7.49 (9H, m).

3-(Benzylthio)-5,6-dihydro-4-hydroxy-6-phenyl-6-((phenylthio)methyl)pyran-2-one (**9g**)

9g was obtained as an off-white solid, 373 mg, yield = 43%, mp 136–138°C EI-MS *m/z*: 434 (M^+), 311. ^{1}H NMR ($CDCl_3$) δ 3.05 (1H, d, J = 17.4 Hz, SCH_2), 3.33 (2H, s, SCH_2), 3.49 (1H, d, J = 7.5 Hz, CH_2), 3.54 (1H, d, J = 7.5 Hz, CH_2), 3.72 (1H, d, J = 17.4 Hz, SCH_2), 6.55 (2H, d, J = 6.6 Hz, Ar–H), 7.17–7.50 (13H, m, Ar–H).

5,6-Dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenyl-3-(phenylthio)pyran-2-one (**10a**)

Compound **8b** (692 mg, 2 mmol) was stirred in *t*-BuOH (50 ml) with NBS (356 mg, 2 mmol) in the dark. The reaction mixture was refluxed for 2 h. The solvent was evaporated, and the residue was partitioned between $CHCl_3$ and water. The aqueous layer was extracted with $CHCl_3$ (4× 30 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The crude product was dissolved in $CHCl_3$ (50 ml). To the solution, benzenethiol (230 mg, 2.1 mmol) was added. The mixture was cooled to 0°C (ice bath) and piperidine (0.2 ml) was added. The reaction mixture was stirred at room temperature for 2 h. Water was added and the aqueous layer was extracted

with $CHCl_3$ (2× 15 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. After being purified by column chromatography (silica gel, dichloromethane/methanol = 150:1), **10a** was obtained as an off-white solid, 505 mg, yield = 55%, mp 164–166°C. EI-MS *m/z*: 454 (M^+), 346, 144. ^{1}H NMR ($CDCl_3$) δ 3.10 (d, 1H, J = 20.4 Hz, CH_2), 3.46 (d, 1H, J = 20.4 Hz, CH_2), 3.59 (d, 1H, J = 17.4 Hz, OCH_2), 3.89 (d, 1H, J = 17.4 Hz, OCH_2), 6.60 (d, 1H, J = 7.2 Hz, Ar–H), 6.74–6.78 (m, 1H, Ar–H), 6.96–7.06 (m, 2H, Ar–H), 7.30–7.36 (m, 1H, Ar–H), 7.46–7.57 (m, 10H, Ar–H), 7.63 (m, 1H, Ar–H), 7.80 (m, 1H, Ar–H).

3-(*o*-Tolylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10b**)

Yield = 55%. mp 151–153°C. EI-MS *m/z*: 468 (M^+), 311, 144. ^{1}H NMR ($CDCl_3$) δ 2.38 (3H, s, CH_3), 3.61 (1H, d, J = 17.7 Hz, CH_2), 3.90 (1H, d, J = 17.7 Hz, CH_2), 4.28 (1H, d, J = 9.9 Hz, OCH_2), 4.57 (1H, d, J = 9.9 Hz, OCH_2), 6.60 (1H, s, Ar–H), 6.77 (1H, d, J = 7.5 Hz, Ar–H), 6.96 (1H, d, J = 7.2 Hz, Ar–H), 7.05 (1H, d, J = 7.2 Hz, Ar–H), 7.38 (1H, t, J = 7.8 Hz, Ar–H), 7.41–7.59 (7H, m, Ar–H), 7.64–7.67 (3H, m, Ar–H), 7.79–7.82 (1H, m, Ar–H).

3-(2-Ethylphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10c**)

Yield = 57%. mp 102–104°C. EI-MS *m/z*: 482 (M^+), 339, 325. ^{1}H NMR ($CDCl_3$) δ 1.27 (3H, t, J = 7.5 Hz, $CH_2\underline{CH_3}$), 2.72–2.80 (2H, m, $\underline{CH_2}CH_3$), 3.62 (1H, d, J = 17.7 Hz, CH_2), 3.91 (1H, d, J = 17.7 Hz, CH_2), 4.30 (1H, d, J = 9.9 Hz, OCH_2), 4.55 (1H, d, J = 9.9 Hz, OCH_2), 6.59 (1H, t, J = 7.5 Hz, Ar–H), 6.79 (1H, d, J = 7.5 Hz, Ar–H), 6.98–7.03 (1H, Ar–H), 7.05 (1H, d, J = 7.5 Hz, Ar–H), 7.38 (2H, t, J = 8.1 Hz, Ar–H), 7.47–7.55 (6H, m, Ar–H), 7.67–7.70 (3H, m, Ar–H), 7.81–7.84 (1H, m, Ar–H).

3-(2-Chlorophenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10d**)

Yield = 39%. mp 173–175°C. EI-MS *m/z*: 488 (M^+), 345, 331. ^{1}H NMR ($CDCl_3$) δ 3.11 (1H, d, J = 20.4 Hz, CH_2), 3.46 (1H, d, J = 20.4 Hz, CH_2), 4.25 (1H, d, J = 10.2 Hz, OCH_2), 4.54 (1H, d, J = 10.2 Hz, OCH_2), 6.78 (1H, d, J = 7.5 Hz, Ar–H), 7.03–7.09 (2H, m, Ar–H), 7.27–7.34 (2H, m, Ar–H), 7.47–7.54 (10H, m, Ar–H), 7.78–7.85 (1H, m, Ar–H).

3-(2,5-Dimethylphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10e**)

Yield = 56%. mp 176–178°C, EI-MS *m/z*: 482 (M^+), 325, 339. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.92 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 3.58 (d, 1H, J = 17.7 Hz, CH_2), 3.92 (d, 1H, J = 17.7 Hz, CH_2), 4.25 (d, 1H, J = 9.9 Hz, OCH_2), 4.50 (d, 1H, J = 9.9 Hz, OCH_2), 6.74–6.80 (m, 2H, Ar–H), 6.95 (d, 1H, J = 7.2 Hz, Ar–H), 7.36–7.41 (m, 2H, Ar–H), 7.45–7.52 (m, 6H, Ar–H),7.66 (d, 2H, J = 6.6 Hz, Ar–H), 7.76–7.81 (m, 2H, Ar–H).

3-(2,5-Diethoxyphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10f**)

Yield = 51%. mp 142–144°C. EI-MS *m/z*: 542 (M^+), 346, 498. ^{1}H NMR ($CDCl_3$) δ 1.35–1.43 (6H, m, $\underline{CH_3}CH_2O$), 3.37 (1H, d, J = 17.4 Hz, CH_2), 3.74 (1H, d, J = 17.4 Hz, CH_2), 3.90–3.99 (4H, m, $CH_3\underline{CH_2}O$), 4.20 (1H, d, J = 9.9 Hz, OCH_2), 4.42 (1H, d, J = 9.9 Hz, OCH_2), 6.70–6.79 (3H, m, Ar–H), 7.07 (1H, d, J = 2.7 Hz, Ar–H), 7.28–7.38 (4H, m, Ar–H), 7.43–7.54(6H, m, Ar–H), 7.77–7.80 (1H, m, Ar–H).

3-(Benzylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-1-yloxy)methyl)-6-phenylpyran-2-one (**10g**)

Yield = 45%. mp 167–169°C. EI-MS *m/z*: 468 (M^+), 144, 91. ^{1}H NMR ($CDCl_3$) δ 3.22 (1H, d, J = 17.4 Hz, CH_2), 3.56 (2H, m, SCH_2), 3.80 (1H, d, J = 17.4 Hz, CH_2), 4.17 (1H, d, J = 9.3 Hz, OCH_2), 4.41 (1H, d, J = 9.3 Hz, OCH_2), 6.72 (1H, d, J = 7.2 Hz, Ar–H), 6.90 (2H, s, Ar–H), 7.19–7.32 (6H, m, Ar–H), 7.44–7.60 (7H, m, Ar–H), 7.81 (1H, s, Ar–H).

5,6-Dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenyl-3-(phenylthio)pyran-2-one (**11a**)

Compound **8c** (692 mg, 2 mmol) was stirred in *t*-BuOH (50 ml) with NBS (356 mg, 2 mmol) in the dark. The reaction mixture was refluxed for 2 h. The solvent was evaporated, and the residue was partitioned between $CHCl_3$ and water. The aqueous layer was extracted with $CHCl_3$ (4× 30 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The crude product was dissolved in $CHCl_3$ (50 ml). To the solution, benzenethiol (230 mg, 2.1 mmol) was added. The mixture was cooled to 0°C (ice bath) and piperidine (0.2 ml) was added. The reaction mixture was stirred at room temperature for 2 h. Water was added and the aqueous layer was extracted

with $CHCl_3$ (2× 15 ml). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated. After being purified by column chromatography (silica gel, dichloromethane/methanol = 150:1), **11a** was obtained as an off-white solid, 488 mg, yield = 53%. mp 182–184°C. EI-MS *m/z*: 454 (M^+), 311, 297. ^{1}H NMR ($CDCl_3$) δ 3.46 (1H, d, J = 17.7 Hz, CH_2), 3.88 (1H, d, J = 17.7 Hz, CH_2), 4.17 (1H, d, J = 10.2 Hz, OCH_2), 4.46 (1H, d, J = 10.2 Hz, OCH_2), 6.63 (2H, d, J = 7.2 Hz, Ar–H), 6.97–7.08 (4H, m, Ar–H), 7.15–7.19 (1H, m, Ar–H), 7.35–7.48 (5H, m, Ar–H), 7.60–7.78 (5H, m, Ar–H).

3-(*o*-Tolylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11b**)

Yield = 51%. mp 92–94°C. EI-MS *m/z*: 468 (M^+), 346, 144. ^{1}H NMR ($CDCl_3$) δ 2.33 (3H, s, CH_3), 3.48 (1H, d, J = 17.7 Hz, CH_2), 3.90 (1H, d, J = 17.7 Hz, CH_2), 4.18 (1H, d, J = 10.2 Hz, OCH_2), 4.48 (1H, d, J = 10.2 Hz, OCH_2), 6.64 (1H, t, J = 7.2 Hz, Ar–H), 6.95 (1H, t, J = 7.2 Hz, Ar–H), 7.03–7.09 (2H, m, Ar–H), 7.17–7.21 (1H, m, Ar–H), 7.36–7.50 (5H, m, Ar–H), 7.62–7.70 (4H, m, Ar–H), 7.75–7.79 (2H, m, Ar–H).

3-(2-Ethylphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11c**)

Yield = 56%. mp 150–152°C. EI-MS *m/z*: 482 (M^+), 346, 325. ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, J = 7.5 Hz, $CH_2\underline{CH_3}$), 2.70–2.78 (2H, m, $\underline{CH_2}CH_3$), 3.47 (1H, d, J = 17.7 Hz, CH_2), 3.89 (1H, d, J = 17.7 Hz, CH_2), 4.18 (1H, d, J = 10.2 Hz, OCH_2), 4.46 (1H, d, J = 10.2 Hz, OCH_2), 6.60 (1H, t, J = 7.2 Hz, Ar–H), 6.99–7.20 (4H, m, Ar–H), 7.35–7.49 (5H, m, Ar–H), 7.61–7.78 (6H, d, Ar–H).

3-(2-Chlorophenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11d**)

Yield = 41%. mp 73–75°C. EI-MS *m/z*: 488 (M^+), 346, 144. ^{1}H NMR ($CDCl_3$) δ 3.47 (1H, d, J = 17.7 Hz, CH_2), 3.90 (1H, d, J = 17.7 Hz, CH_2), 4.17 (1H, d, J = 10.2 Hz, OCH_2), 4.47(1H, d, J = 10.2 Hz, OCH_2), 6.71 (1H, s, Ar–H), 7.00–7.24 (6H, m, Ar–H), 7.36–7.47 (5H, m, Ar–H), 7.62–7.79 (4H, m, Ar–H).

3-(2,5-Dimethylphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11e**)

Yield = 60%. mp 73–75°C. EI-MS *m/z*: 482 (M^+), 346, 425. ^{1}H NMR ($CDCl_3$) δ 1.92 (3H, s, CH_3), 2.30 (3H, s, CH_3), 3.47 (1H, d, J = 17.7 Hz, CH_2), 3.91 (1H, d, J = 17.7 Hz, CH_2), 4.15 (1H, d, J = 10.2 Hz, OCH_2), 4.44 (1H, d, J = 10.2 Hz, OCH_2), 6.79 (1H, d, J = 7.8 Hz, Ar–H), 6.95 (1H, d, J = 7.8 Hz, Ar–H), 7.08 (1H, s, Ar–H), 7.16–7.20 (1H, m, Ar–H), 7.27–7.49 (5H, m, Ar–H), 7.61–7.79 (6H, m, Ar–H).

3-(2,5-Diethoxyphenylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11f**)

Yield = 53%. mp 135–137°C. EI-MS *m/z*: 542 (M^+), 498, 385. ^{1}H NMR ($CDCl_3$) δ 1.35–1.44 (6H, m, $\underline{CH_3}CH_2O$), 3.24 (1H, d, J = 17.7 Hz, CH_2), 3.73 (1H, d, J = 17.7 Hz, CH_2), 3.89–3.99 (4H, m, $CH_3\underline{CH_2}O$), 4.10 (1H, d, J = 10.2 Hz, CH_2), 4.34 (1H, d, J = 10.2 Hz, CH_2), 6.71–6.77 (2H, m, Ar–H), 7.02–7.12 (3H, m, Ar–H), 7.33–7.42 (5H, m, Ar–H), 7.48–7.51 (3H, m, Ar–H), 7.65–7.77 (2H, m, Ar–H).

3-(Benzylthio)-5,6-dihydro-4-hydroxy-6-((naphthalen-2-yloxy)methyl)-6-phenylpyran-2-one (**11g**)

Yield = 52%. mp 137–139°C. EI-MS *m/z*: 468 (M^+), 311, 144. ^{1}H NMR ($CDCl_3$) δ 3.13 (1H, d, J = 17.7 Hz, CH_2), 3.57 (2H, m, SCH_2), 3.76 (1H, d, J = 17.7 Hz, CH_2), 4.10 (1H, d, J = 9.9 Hz, OCH_2), 4.35 (1H, d, J = 9.9 Hz, OCH_2), 6.72 (1H, d, J = 9.9 Hz, Ar–H), 6.84–6.87 (2H, m, Ar–H), 7.05–7.17 (7H, m, Ar–H), 7.43–7.48 (5H, m, Ar–H), 7.50–7.53 (2H, m, Ar–H).

PR inhibition assay

The tested compounds were dissolved in 4.0 µl DMSO and incubated with HIV-1 PR in 200 µl buffer (0.1 M MES, 0.2 M NaCl, 5 mM EDTA, pH5.5) for 10 min. Enzymatic assays were initiated by the addition of PR substrate (Lys-Ala-Arg-Val-Leu-Phe(NO_2)-Glu-Ala-Met).The hydrolysis of the substrate resulted in a linear increase in UV absorbance at 310 nm over a 30-min period (Nashed *et al.*, 1989). The IC_{50} was defined as the compounds concentration required to inhibit the final absorbance by 50% and was the average of at least two runs. Indinavir (IDV) was used as the positive control.

Anti-SIV assay

Inhibition of SIV-induced syncytium in CEM174 cell culture was measured in a 96-well microplate containing 2×10^5 CEM cells/ml infected with 100 $TCID_{50}$ of SIV per well and containing appropriate dilutions of the tested compounds. After 5 days of incubation at 37°C in 5% CO_2-containing humidified air, CEM giant (syncytium) cell

formation was examined microscopically. The EC_{50} was defined as the concentration required to protect CEM cells against the cytopathogenicity of SIV by 50%. The TC_{50} was the concentration that elicits cytotoxicity in 50% of uninfected CEM cells under some conditions.

Western immunoblot assay

The confluent 293T cells were trypsinized and seeded onto a 6-well plate 24 h prior to transfection with plasmids pGAG and pGAGPOL (Chien *et al.*, 2006). 12 h after transfection by standard calcium phosphate precipitation method, the culture medium was removed and the cells were washed twice with phosphate-buffered saline. Then fresh medium was added together with diluted compounds at a final concentration of 20 μM. After 48 h post-transfection, the medium was collected and ultra-filtrated (cutting off molecular weight: 30 kDa). The filtration was mixed with the cells which were scratched form the plate on ice base. The equal amounts of samples were subjected to SDS–PAGE and were transferred onto nitrocellulose membrane. The membrane was blocked with 5% gelatin in *tris*-buffered saline containing 0.05% Tween 20 (TBST), followed by incubation with the HRP-conjugated anti-p24 monoclonal antibody (Beijing Perfect Biotechnology, Ltd., Beijing, People's Republic of China) at a 1:500 dilution in 5% gelatin-TBST for 1 h on a rocking platform at room temperature. The membranes were then washed thrice (10 min each) with TBST. The HRP activity was determined with diaminobenzidine. IDV was used as positive control.

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