Biochemical and Biophysical Research Communications 403 (2010) 40–45

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of a novel Vpr-binding compound that inhibits HIV-1 multiplication in macrophages by chemical array

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ARTICLE INFO

Article history:
Received 2 October 2010
Available online 29 October 2010

Keywords:
HIV-1 inhibitor
Vpr
Chemical array
Vpr-binding compound
Macrophages
Small-molecule affinity beads

ABSTRACT

Although HIV-1 replication can be controlled by highly active anti-retroviral therapy (HAART) using protease and reverse transcriptase inhibitors, the development of multidrug-resistant viruses compromises the efficacy of HAART. Thus, it is necessary to develop new drugs with novel targets. To identify new anti-HIV-1 compounds, recombinant Vpr was purified from transfected COS-7 cells and used to screen compounds by chemical array to identify those that bound Vpr. From this screen, 108 compounds were selected as positive for Vpr binding. Among these, one structurally similar group of four compounds showed anti-HIV activity in macrophages. In particular, compound SIP-1 had high inhibition activity and reduced the levels of p24 by more than 98% in macrophages after 8 or 12 days of infection. SIP-1 had no cytotoxic effects and did not disrupt cell cycle progression or induce apoptosis of Molt-4 and HeLa cell lines as measured by MTT assay, flow-cytometry analysis, and a caspase-3 assay. In addition, SIP-1 specifically bound to Vpr as assessed by photo-cross-linked small-molecule affinity beads. These results suggest that Vpr is a good target for the development of compounds that could potentially inhibit HIV-1 replication. Collectively, our results strongly suggest that chemical array is a useful method for screening anti-viral compounds.

1. Introduction

HIV-1 infection can be controlled with combinations of anti-retroviral drugs. One approach, termed highly active anti-retroviral therapy uses protease and reverse transcriptase inhibitors, which can decrease viremia below the limit of detection and stop disease progression [1,2]. However, due to problems with these drugs, including the development of viral escape mutants [3], the persistence of viral reservoirs [4–6], poor patient compliance due to complex drug regimens [7], and toxic side effects [8], the need for new drugs with novel targets has become apparent. Many steps in the HIV replication cycle, such as viral adsorption, viral entry, virus-cell fusion, viral assembly and disassembly, proviral integration, viral mRNA transcription, and nuclear import of the viral genome, are potential targets for intervention [9]. In addition to specific steps in the viral life cycle, the HIV-1 accessory gene products Vpr, Vif, Nef, and Vpu have recently been highlighted as potential targets for inhibiting HIV-1 infection. In particular, Vpr is an ideal target due to its unique functions, including nuclear import [10], induction of cell cycle arrest at the G_2 phase [11], apoptosis regulation [11–14] and splicing inhibition [15,16]. The identification of interactions between these accessory gene products and critical host factors that are required for HIV-1 replication may provide novel targets for the development of compounds that are potentially capable of inhibiting HIV-1 replication, thereby decreasing the viral burden in cases of drug-resistant HIV-1 infection.

Macrophages are a major target of HIV-1 and serve as a viral reservoir that releases a small number of viral particles in symptomatic carriers [9,17]. Several studies have shown that Vpr is important for nuclear import of the pre-integration complex (PIC) in macrophages [18–21]. Our studies have shown that Vpr is targeted to the nuclear envelope and then transported into the nucleus by importin α alone, in an importin β-independent manner [19,22]. Recently, we also demonstrated that a compound which suppresses the interaction between Vpr and importin α resulted

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doi:10.1016/j.bbrc.2010.10.107

in a decrease in HIV-1 replication in macrophages [23,24]. Thus, the inhibition of function of Vpr may targets for development of compounds that are potentially inhibiting HIV-1 replication.

Chemical array represent one of the most promising and high-throughput approaches for screening ligands of proteins of interest [25], and we recently succeeded in obtaining novel anti-viral compounds using this method [26]. In the present study, we screened for Vpr-binding compounds using a chemical array. This screen resulted in the discovery of novel anti-HIV-1 compounds that block viral replication in macrophages.

2. Materials and methods

2.1. Cell culture

COS-7, 293T, HeLa or Molt-4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (SIGMA) or RPMI1640 (Invitrogen) containing penicillin, streptomycin and glutamine (PSG, GIBCO) and 10% fetal bovine serum (FBS, SIGMA) at 37 °C for 2 days in 5% CO_2 as previously described [26].

2.2. Construction of expression plasmids

The pCAGGS mammalian vectors [27] encoding Flag-mRFP-Flag-Vpr or Flag-mRFP were constructed as follows: the cDNAs corresponding to full-length HIV-1 Vpr, which encodes a 96-residue protein, and the Flag-mRFP gene were amplified by PCR using the primers Xho-Flag-mRFP-F, 5′-AAACTCGAGATGGATTACAAGGACGACGATGACAAGATGGCCTCCTCCGAGGACGTCATC-3′; Not-Vpr-R, TTTGCGGCCGCCTAGGATCTACTGGCTCCATTTC; and Not-mRFP-R, 5′-AAAGCGGCCGCTTAGGCGCCGGTGGAGTG-3′ (restriction enzyme sites are underlined). PCR amplification was performed with KOD Plus Ver.2 (TOYOBO) using mRFP-Flag-Vpr/pCS2+ as a template. After purification of the products using a MinElute PCR Purification Kit (QIAGEN), the DNA was digested with the appropriate restriction enzymes and cloned into pCAGGS. DNA sequencing was performed, and the predicted amino acid sequence of Vpr completely corresponded to the previous reported sequence of pNL432 [28] (GenBank ID: M19921).

2.3. Expression and purification of Flag-fusion proteins

COS-7 cells (1×10^6 cells) were transfected with 10 μg of the pCAGGS mammalian vector encoding Flag-mRFP-Flag-Vpr (Vpr) or Flag-mRFP (mRFP) using FuGene HD transfection reagent (Roche). Two days after transfection, expressed proteins were purified using ANTI-FLAG M2 agarose (SIGMA) as described previously [26].

2.4. SDS–PAGE and immunoblot analysis

Purified proteins were separated by 15% SDS–PAGE under reducing conditions and stained with Coomassie brilliant blue (CBB). Immunoblot analysis was performed using standard methods as described previously [29] with an anti-Flag monoclonal antibody (MAb), followed by incubation with an anti-mouse IgG-alkaline phosphatase antibody (SIGMA).

2.5. Screening for Vpr-binding compounds by chemical array

The chemical arrays were prepared according to our previous reports [25,30]. Solutions of the 8800 compounds (2.5 mg/ml in DMSO) in the NPDepo (RIKEN Natural Products Depository) were arrayed onto five separate photoaffinity-linker-coated glass slides with a chemical arrayer developed at RIKEN. Screening for Vpr-binding compounds was performed by a chemical array screening method as described previously [26,30].

2.6. Macrophage preparation

Human peripheral blood mononuclear cells (PBMCs) were collected from three healthy donors (Nos.1–3) and isolated on a Ficoll gradient (Immuno-Biological Laboratories). Monocytes were selected from PBMC using MACS CD14 MicroBeads (Miltenyi Biotec) and a MACS Separation column (Miltenyi Biotec) with a Quandro MACS Separation Unit (Miltenyi Biotec) as previously described [19]. Monocytes were cultured for 10 days in RPMI 1640 containing 10% FBS, 5% AB serum and 10 ng/ml of human macrophage-colony stimulating factor (PeproTech EC) to promote differentiation into mature macrophages.

2.7. Viral infection of macrophages

HIV-1 was introduced into 293T cells by transfection of macrophage-tropic pNF462 viruses encoding either wild-type Vpr [31] or a deficient form of the protein [32] as described above. After filtration with a 0.45 μM Millipore filter, viral stocks were titrated by an enzyme-linked immunosorbent assay (ELISA) to determine the relative p24 values as described previously [33]. Differentiated primary macrophages (2×10^5 cells/wells) were infected with HIV-1 (a total of 1 ng of p24) at 37 °C for 1 h. After washing three times with RPMI, the cells were cultured in RPMI containing serial 10-fold dilutions of the different compounds ranging in concentrations from 0 to 50 μM or from 0 to 10 μM. Cell supernatants were collected 4, 8 and 12 days after infection, and the p24 values were calculated by ELISA.

2.8. MTT cell viability assay

Molt-4 cells (1×10^5 cells/well) were cultured in 24 well plate at 37 °C for 2 days in the RPMI1640 containing serially diluted compounds ranging from 0 to 10 μM. MTT analysis was performed using a standard method as described previously [26].

2.9. Cell cycle analysis

HeLa cells (1.6×10^6 cells) were harvested at 48 h in the presence of 10 μM of compound and analyzed by flow cytometry for DNA content as described previously [34].

2.10. Analysis of caspase-3 activation

HeLa cells (2×10^6 cells) were harvested 24 h after the addition of 10 μM of compound, and caspase-3 activity was then determined using a caspase-3/CPP32 fluorometric assay kits (Bio Vision) as described previously [34].

2.11. Photo-cross-linked small-molecule affinity beads assay

Compound was cross-linked to Sepharose beads as described previously [35]. Briefly, *N*-hydroxysuccinimide-activated beads were washed three times with 1 mM aq. HCl and coupling solution (0.1 M $NaHCO_3$, 50% dioxane mixture). A solution of photoaffinity linker in coupling solution was then added to the beads, and the beads were incubated at 37 °C for 2 h on a rotator. After washing five times with coupling solution, the beads were blocked with 1 M ethanolamine in 0.1 M Tris–HCl (pH 8.0) buffer at 37 °C for 1 h on a rotator. Beads were then washed three times with milli-Q water and methanol on a spin column and transferred to a glass sample vial. A methanol solution of the compound was added to beads and the mixture was concentrated and dried *in vacuo*. The

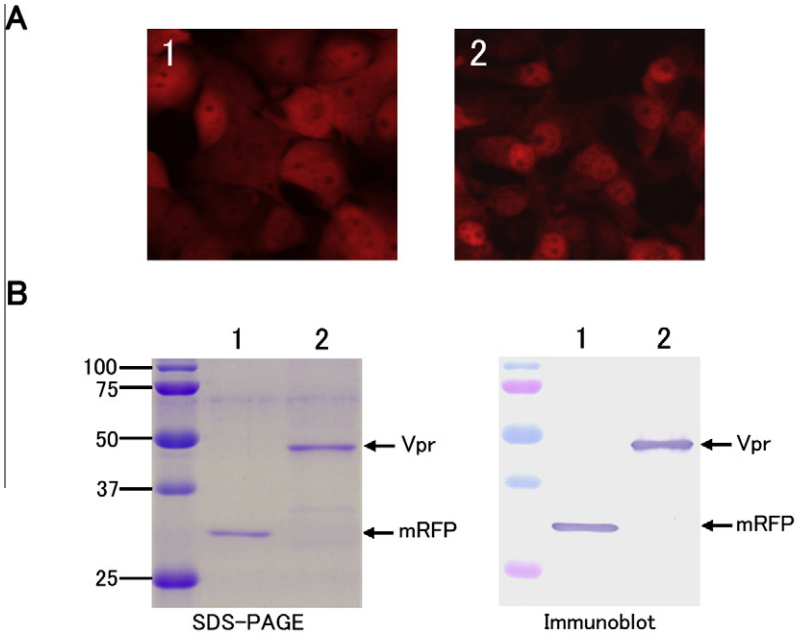
beads were irradiated at 365 nm (4 J/cm^2) with a UV cross-linker and washed with methanol to yield compound cross-linked affinity beads.

Recombinant Vpr or mRFP was incubated with compound cross-linked affinity beads at 4 °C for 16 h. After centrifugation, the beads were washed three times with buffer containing 10 mM Tris (pH 7.8), 150 mM NaCl and 0.05% NP-40. The protein that bound to the compound was then separated by 15% SDS–PAGE and detected by immunoblot analysis using an anti-Flag MAb.

3. Results and discussion

To search for compounds that bound to HIV-1 Vpr using a photo-cross-linked chemical array, plasmid was constructed that express chimeric Vpr protein containing an N-terminal Flag tag and mRFP. As a negative control, an expression vector encoding recombinant Flag-mRFP was constructed. To produce chimeric Vpr and mRFP, recombinant plasmids were transfected to COS-7 cells, and the expression of each protein was examined using confocal microscopy. As shown in Fig. 1A, control mRFP was located both in the nucleus and the cytoplasm (Panel 1). In contrast, Vpr mainly localized in the nucleus (Panel 2), indicating that the recombinant Vpr retained its nuclear transport activity. The expressed proteins were purified using Flag-affinity beads and were examined using SDS–PAGE and immunoblot analysis. Purified mRFP and Vpr were clearly detected as single protein bands with apparent molecular masses consistent with their predicted sequences (Fig. 1B left panel). Immunoblot analysis using an anti-Flag MAb also indicated that the Flag-fusion proteins were purified appropriately (Fig. 1B right panel).

The purified proteins were used to screen Vpr-binding compounds by chemical arrays. Flag-mRFP fusion Vpr or Flag-mRFP were incubated with 8800 compounds from a NPDepo that were immobilized on glass slides in duplicate. "Hit ligands" were detected by merged display analysis as described previously [25,26]. As a result of this analysis, a total of 108 compounds were specifically selected as Vpr binding molecules. Among these, 65 compounds were classified into 11 groups by the similarities in their chemical structures (Fig. 1C). First, we investigated whether compounds typical of these 11 groups could block HIV-1 replication in macrophages. Primary macrophages derived from healthy donor No.1 were infected with the macrophage-tropic pNF462 HIV-1 viruses and cultured in RPMI1640 containing serial 10-fold dilutions of different compounds ranging in concentration from 0 to 50 μM. After 4 and 8 days of infection, viral replication was assayed by p24 ELISA. Among the compounds tested, only one



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	Groups by chemical structures											Others
	1	2	3	4	5	6	7	8	9	10	11	
Number of derivative	6	9	15	11	4	4	3	3	4	3	3	43
Inhibition of HIV-1 replication	–	–	–	–	–	+	–	–	–	–	–	not tested

Fig. 1. Purification of recombinant Vpr protein and chemical array screening. (A) Confocal laser-scanning analysis of recombinant protein localization. COS-7 cells were transfected with pCAGGS mammalian vectors encoding Flag-mRFP (mRFP, Panel 1) or Flag-mRFP-Flag-Vpr (Vpr, Panel 2). (B) SDS–PAGE and immunoblot analysis of purified recombinant proteins. Samples were separated by 15% SDS–PAGE and stained by CBB (left panel). After transferring to a membrane, they were detected by anti-Flag MAb and alkaline phosphatase staining (right panel). Lane 1, purified mRFP; lane 2, purified Vpr. (C) Summary of the screening of compounds by chemical array and inhibitory activities of the compounds. Compounds were classified into 11 groups and the others by the similarities in their chemical structures. Inhibitory activities of the compounds against HIV-1 in macrophages derived from healthy donor No. 1 is shown by + and no activity is shown by –.

belonging to group 6 reduced virus replication efficiently (Fig. 1C) in a dose-dependent manner both on 4 and 8 days after infection (data not shown). This compound showed no cytotoxic effects on Molt-4 cells by MTT assay (data not shown), indicating that the compound has anti-HIV-1 activity.

This compound was designated SIP-1 since it had a basic structure of spiro[1H-indole-3,2′-pyrrolidine]-2-one (Fig. 2A). And we then tested the effect of the other three compounds in the SIP group on HIV-1 replication (Fig. 2B). Primary macrophages derived from healthy donor No. 2 were infected with HIV-1 and cultured in the absence or presence of the compounds at the indicated concentrations for 4, 8 and 12 days. At all time points, macrophages infected with the Vpr^+ virus showed higher p24 values than the macrophages infected with the Vpr^- virus. However, difference of p24 value between two types of viruses was decreased after 12 days infection, suggesting that the Vpr play an important role in early stage of infection in macrophage. As a result, all four compounds inhibited HIV-1 replication on 10 μM treatment at all time points after infection (Fig. 2B). In particular, SIP-1 strongly inhibited viral replication than the other three compounds and reduced the p24 value by approximately 80% at 10 μM. The 50% inhibition concentration (IC_{50}) of SIP-1, SIP-2, SIP-3 and SIP-4 was 5.5, >10, 6.8 and 8.9 μM, respectively, at 8 days post-infection (Table 1). All compounds had no effect of host cell viability as observed by microscopy. In addition, these compounds had no cytotoxic effect on Molt-4 cells as measured by MTT assay (Fig. 2C, Table 1).

To examine the inhibition activity of SIP-1 in more detail, macrophages derived from healthy donor No. 3 were infected with HIV-1. SIP-1 inhibited HIV production by more than 98% at 8 and 12 days post-infection at 10 μM, and at 1 μM reduced viral production by about 80% and 66% at 8 and 12 days post-infection, respectively (Fig. 3A). The IC_{50} of SIP-1 in this assay was 0.5 μM (Table 1) at 8 days post-infection. Different inhibitory effects obtained in Figs. 2 and 3 may be due to distinction of each healthy donor. SIP-1 had no effect on host cell cycle progression as assayed by flow-cytometry analysis (Fig. 3B), or on apoptosis as determined by measurements of caspase-3 activity (Fig. 3C). Collectively, the inhibition of viral replication in infected cells by SIP-1 is due to an ability to inhibit HIV-1 rather than some non-specific cell toxicity since SIP-1 had no advance effect on the cell growth and viability of the cells at 10 μM treatment reaching inhibition levels up to 98%.

Table 1
Summary of the binding and inhibitory activities of the compounds.

Compound[a]	Binding activity to Vpr		IC_{50} (μM)[b]	Cytotoxicity
	Array	Affinity beads		
SIP-1	+[c]	+	5.5 (0.5)	–
SIP-2	+	Not tested	>10	–
SIP-3	+	Not tested	6.8	–
SIP-4	+	Not tested	8.9	–

[a] The Purity of the compounds was greater than 88%.
[b] The IC_{50} compounds after 8 days infection in Fig. 2 is indicated and those of IC_{50} in Fig. 3 is shown in a parenthesis.
[c] Positive activity is shown by + and no activity is shown by –.

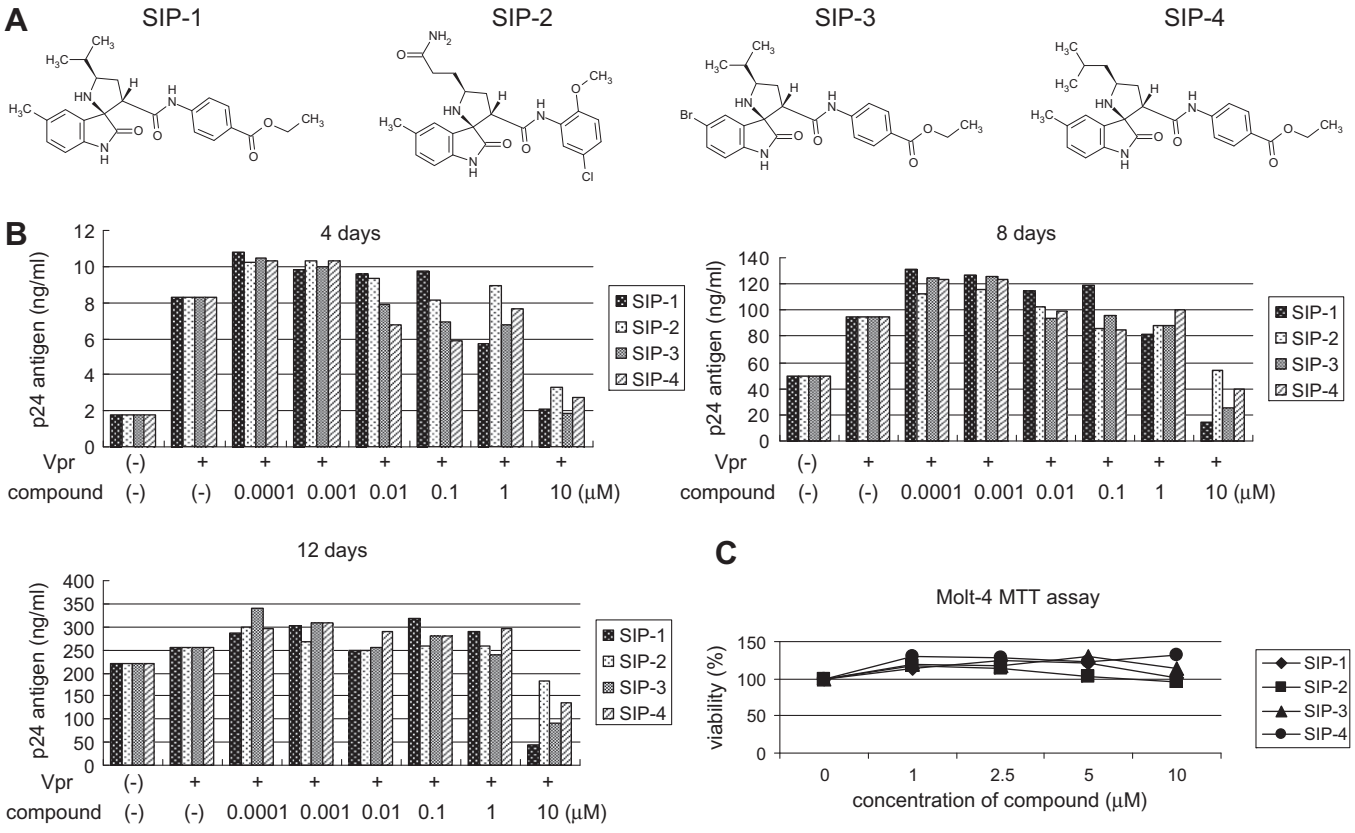

Fig. 2. The effect of different SIP compounds on HIV-1 replication in macrophages and on Molt-4 cell viability. (A) Chemical structures of the compounds screened by chemical arrays. (B) Reduction of viral replication by different SIP compounds. Terminally differentiated primary macrophages derived from healthy donor No.2 were infected by HIV-1 and incubated with serial 10-fold dilutions of compounds ranging in concentration from 0 to 10 μM. The level of virus in the culture supernatants was measured at 4, 8 and 12 days after inoculation by p24 antigen ELISA. The data represent the average p24 value from two wells. (C) The viability of Molt-4 cells was determined by MTT assay following incubation with the SIP compounds. The data represent the average from three wells.

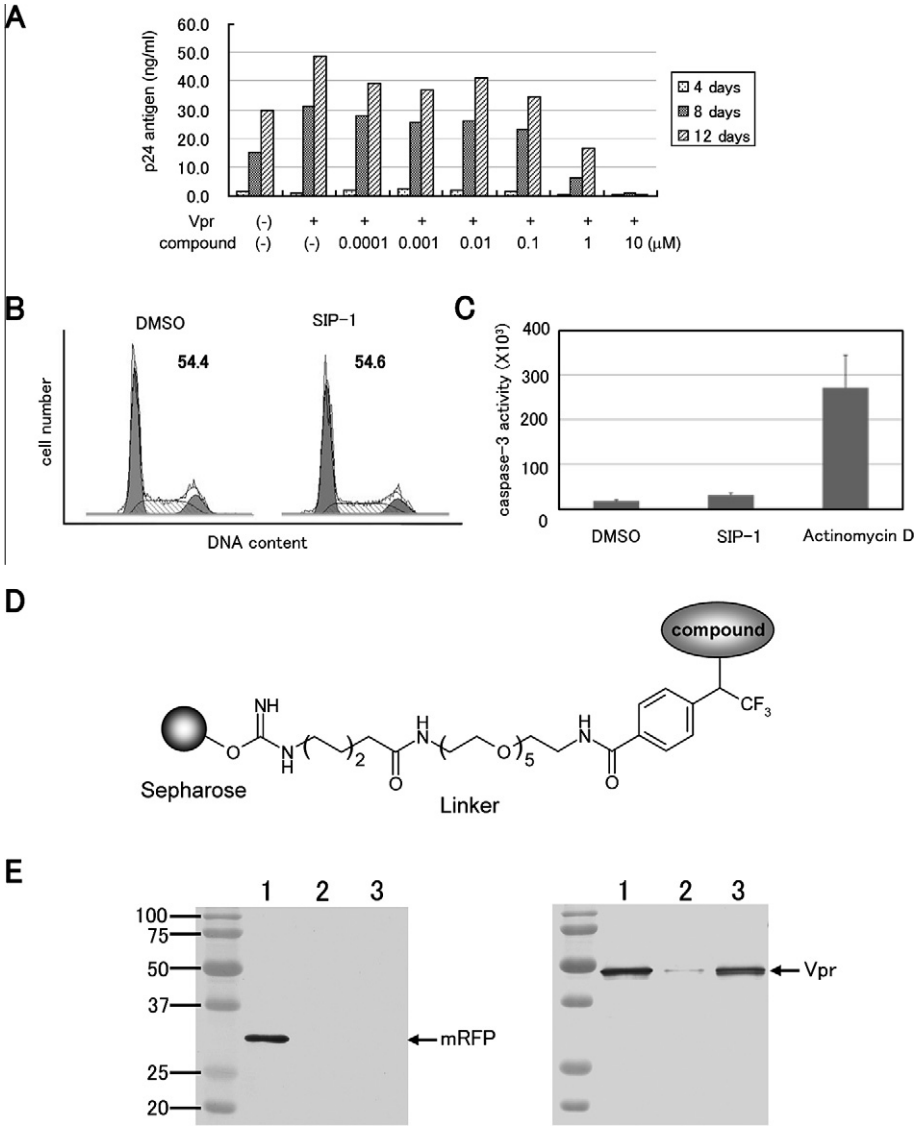

Fig. 3. The effect of SIP-1 on viral replication and normal cell function, and the ability of SIP-1 to bind Vpr. (A) SIP-1-mediated inhibition of viral replication in macrophages. Terminally differentiated primary macrophages derived from healthy donor No. 3 were infected with HIV-1 and then incubated in serial 10-fold dilutions of SIP-1 ranging in concentration from 0 to 10 μM. The levels of virus in the culture supernatants were measured at 4, 8 and 12 days after inoculation by p24 antigen ELISA. The data represent the average p24 value from two wells. (B) Effect of SIP-1 on cell cycle progression. HeLa cells were cultured for 48 h in the presence of 10 μM SIP-1 or DMSO, and cell cycle profiles were analyzed by flow cytometry. The percentage of cells in G1 phase was calculated using ModFit LT Software (Verity Software House) and shown by black bold on the right of each panel. (C) The effect of SIP-1 on apoptosis. HeLa cells were cultured for 24 h in the presence of 10 μM SIP-1, DMSO or Actinomycin D, and caspase-3 activity was determined using a caspase-3/CPP32 fluorometric assay kit (Bio Vision). (D and E) Photo-cross-linked small-molecule affinity beads assay of compound-Vpr binding. A model structure of SIP-1-linked Sepharose beads is shown (D). (E) Purified mRFP (left panel) or Vpr (right panel) was mixed with SIP-1 that had been photo-cross-linked to affinity beads, and then the beads were incubated at 4 °C for 16 h. The proteins that bound to the compound were detected by immunoblot analysis using an anti-Flag MAb followed by incubation with an alkaline phosphatase conjugated antibody. Lane 1, 5% of the total input protein used for the binding assay; lane 2, control beads containing only linker; lane 3, compound-linked beads.

To determine whether SIP-1 directly interacted with Vpr, we performed binding assay using photo-cross-linked small-molecule affinity beads (Fig. 3D and E). Purified Vpr or mRFP was applied to compound-cross-linked affinity beads, and the beads were incubated at 4 °C for 16 h. After sedimentation of the beads, bound proteins were detected by immunoblotting using an anti-Flag MAb. In this assay, recombinant Vpr coprecipitated with compound-linked affinity beads (Fig. 3E lane 3, right panel), whereas control mRFP did not (Fig. 3E left panel), indicating that Vpr specifically binds to the compound.

Together, these results suggest that SIP-1 inhibits Vpr function via binding to its functional domain, thereby inhibiting HIV-1 replication. However, this SIP-1 inhibits which particular function of Vpr is not understood. Therefore, in ongoing studies, we seek to clarify the mechanism of action of SIP group compounds by analyzing the crystal structure of the SIP–Vpr complex. Detailed descriptions of these interactions will provide new therapeutic strategies for rational drug design. Indeed, as the IC_{50} of SIP-1 is approximately 0.5 μM at 8 days after infection, new compound derivatives could potentially develop novel anti-HIV drugs.

4. Conclusions

First, a screen was conducted to identify Vpr-binding compounds using high-throughput chemical arrays. Next, we examined the effect of these compounds on the replication of the macrophage-tropic pNF462 HIV-1 virus in primary macrophages derived from three different human donors. All four SIP group

compounds, which had a basic structure of spiro[1H-indole-3,2′-pyrrolidine]-2-one, inhibited HIV-1 replication in macrophages at 8 or 12 days post-infection and had no cytotoxic effects, indicating that SIP-1-mediated inhibition of viral replication is Vpr-dependent. Finally, assays using photo-cross-linked small-molecule affinity beads indicated that SIP-1 directly binds Vpr, thereby inhibiting HIV-1 replication.

Acknowledgments

We thank Dr. T. Saito for supplying compounds from the NPDepo. This work was supported in part by a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan (Research on HIV/AIDS) and in part by the Chemical Biology Research Project (RIKEN).

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