SJP-L-5 inhibits HIV-1 polypurine tract primed plus-strand DNA elongation, indicating viral DNA synthesis initiation at multiple sites under drug pressure

Xing-Jie Zhang1,2,3, Rui-Rui Wang1,4, Huan Chen3,3, Rong-Hua Luo3, Liu-Meng Yang3, Jing-Ping Liu5, Han-Dong Sun5, Hong-Bin Zhang2, Wei-Lie Xiao2,5 & Yong-Tang Zheng1,3,6

In a previous study the small molecule SJP-L-5 that inhibits HIV replication, has been shown to block uncoating of the viral capsid. Continued study showed that SJP-L-5 might hinder HIV capsid uncoating by blocking the completion of reverse transcription. However, to date, the mechanism has not been fully elucidated. Here, the effects of SJP-L-5 for reverse transcription were explored via quantitative PCR, DIG-labelled ELISA, fluorescent resonance energy transfer, and Southern blot assays. We also analyzed the resistance profile of this compound against reverse transcriptase. Our results show that SJP-L-5 preferentially inhibits PPT primed plus-strand DNA synthesis (EC50 = 13.4 ± 3.0 μM) over RNA primed minus-strand DNA synthesis (EC50 > 3,646 μM), resulting in formation of five segmented plus-strand DNA and loss of HIV DNA flap, suggesting failure of both nuclear import and integration. Moreover, resistance study evidenced that SJP-L-5 requires the amino acid residues Val108 and Tyr181 to exert an inhibitory effect. These results indicate SJP-L-5 as a new non-nucleoside reverse transcriptase inhibitor that inhibits HIV-1 polypurine tract primed plus-strand DNA synthesis, initiating HIV-1 down-stream plus-strand DNA synthesis at multiple sites under drug pressure.

Retroviruses (i.e., human immunodeficiency virus, HIV) are single-stranded RNA viruses that infect eukaryotic cells. The retroviral life cycle is characterized by reverse transcription (RT) of the single-stranded plus RNA genome and integration of the complementary DNA (cDNA) into the host genome. RT is a key step in HIV replication, and this process is responsible for the synthesis of a double-strand DNA from the viral single-strand RNA genome1.

RT is a complex process in which reverse transcriptase (RTase) has three functions and makes two jumps2. These three RTase functions include: (1) RNA-dependent DNA polymerization (RDDP) activity, converting single-stranded viral RNA to minus DNA; (2) DNA-dependent DNA polymerization (DDDP) activity, converting minus DNA to plus DNA; (3) RNase H activity, digesting RNA from RNA/DNA hybrids3. The first RTase jump is triggered by a minus-strand strong-stop DNA (−sssDNA), which is used as a primer to synthesize a large minus-DNA fragment. The second jump is triggered by the plus-strand strong-stop DNA (+sssDNA) near the 3′

1Key Laboratory of Bioactive Peptides of Yunnan Province/Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China. 2Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education and Yunnan Province, Yunnan University, Kunming, Yunnan, 650091, China. 3Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, Yunnan, 650204, China. 4College of Pharmaceutical Science, Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan, 650500, China. 5State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, 650201, China. 6KIZ-SU Joint Laboratory of Animal Models and Drug Development, College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu, 215006, China. Xing-Jie Zhang and Rui-Rui Wang contributed equally to this work. Correspondence and requests for materials should be addressed to W.-L.X. (email: xiaowei@ynu.edu.cn) or Y.-T.Z. (email: zhengyt@mail.kiz.ac.cn)
end of the RNA genome, synthesized from the 3′ polypurine tract (PPT), which is used as a primer. After these two jumps, three types of viral DNA have been synthesized: linear DNA, long-terminal repeat (LTR) DNA, and 2-LTR DNA (Fig. 1). Unlike other retroviruses (i.e., MMV or AMV), HIV, as a lentivirus, has a PPT sequence in the center of the RNA genome (central PPT or cPPT), as well as in the integrase gene. Previous studies suggested that the cPPT forms a gap called flap in the center of the linear DNA during RT. Thus, plus DNA of the HIV genome is discrete and holds a triple DNA structure in the center that is essential for importing the pre-integrated complex into the nucleus. Hence, this DNA flap is a potential target of anti-HIV drugs; however, such inhibitors are rarely reported. A DNA flap inhibitor could also help understanding the late process of reverse transcription, as well as the early steps of nuclear import.
Since the first RTase inhibitor, zidovudine (AZT), was approved by the FDA three decades ago, RTase has become a major target in highly active antiretroviral therapy (HAART) against HIV infection. Unlike nucleoside RTase inhibitors (NRTIs), non-nucleoside RTase inhibitors (NNRTIs) bind to the hydrophobic bag and inhibit its polymerase activity by an allosteric effect. Normally, NNRTIs inhibit both RNA- and DNA-dependent DNA polymerization activities, but not the ribonuclease H (RNase H) activity.

Our previous study showed that SJP-L-5 (Fig. 2), a nitrogen-containing biphenyl compound, whose synthesis was based on dibenzocyclooctadienelignan, gomisin M2 (SM-10), blocks the nuclear entry of the HIV pre-integrated complex by inhibiting capsid uncoating. However, the mechanism with which SJP-L-5 blocks the uncoating of the viral capsid remains unknown. Our data (unpublished) suggested that SJP-L-5 may inhibit the RTase DNA-dependent DNA polymerase function. Therefore, we hypothesize that SJP-L-5 inhibits the viral plus-strand DNA synthesis by hindering full-length plus-strand DNA maturation.

Taking our hypothesis into account, we examined the molecular mechanisms of SJP-L-5 inhibition. Here, we present new data on SJP-L-5 and its inhibitory activity on the three RTase functions. We also analyze the effect of SJP-L-5 on the synthesis of viral plus- and minus-strand DNA. Finally, we show a viral resistance profile of SJP-L-5 against HIV-1.

Results

SJP-L-5 does not block the initiation of reverse transcription, but partially decreases late-RT products in HIV-1-infected C8166 cells. In our previous study, we showed that the antiviral target of SJP-L-5 is the uncoating of the capsid, based on single-round pseudotyped virus investigation. Completion of RT is believed to trigger capsid disassembling. To explore the stage at which this compound inhibits multi-round replicative viruses, real-time qPCR assays were carried out. The -ssDNA is a signal of RT initiation, which involves RTase RDDP activity. On the other hand, late-RT DNA is a signal of late RT, which involves the RTase DDDP activity. Dead-end circular 2-LTR DNA is a nuclear import signal.

In the first 4 h, -ssDNA levels were not reduced by SJP-L-5, at a concentration sufficient to block the HIV-1 infection in cell-based assays (100 μM) (Fig. 3a). However, late-RT DNA products were partially reduced by this compound from 4 to 8 h (Fig. 3b), and 2-LTR levels were totally inhibited by SJP-L-5 from 8 to 12 h (Fig. 3c). The positive control (EFV) reduced the levels of the three viral DNA forms, suggesting that it inhibits the early steps of RT. Taken together, these results show that SJP-L-5 blocks HIV-1 infection during the late process of RT.

SJP-L-5 inhibits DDDP activity in a dose-dependent response. Based on the data shown in Fig. 3, we predicted that SJP-L-5 would target the DDDP. To evaluate this assumption, we measured the three functions of the RTase. We conducted a DIG-labelled dUTP ELISA to evaluate the RDDP activity, and a fluorescent-labelled dUTP assay to detect the DDDP or the RNase H activities.

As shown in Fig. 4a, the RTase RDDP activity was barely inhibited at the highest concentration of SJP-L-5 (inhibitory rate <25% at 3,646 μM) but was efficiently blocked by NVP with an EC₅₀ of 3.98 ± 2.26 μM in a...
dose-dependent manner, suggesting that the RDDP activity is not the main target of SJP-L-5. However, the DDDP activity was inhibited by both SJP-L-5 and NVP in a dose-dependent manner, displaying an EC_{50} of 13.4 ± 3.0 μM and 342 ± 35 nM, respectively (Fig. 4b). This result shows that SJP-L-5 is an inhibitor of the RTase DDDP activity and, thus, may interfere with viral plus-DNA synthesis. Neither SJP-L-5 nor NVP inhibited the RNase H activity (Fig. 4c).

SJP-L-5 accumulates different-length viral downstream plus-strand DNA delaying viral DNA maturation. Next, we assessed the impact of SJP-L-5 on viral plus-strand DNA synthesis and DDDP activity by treating the cells with this inhibitor. DNA flap is a viral plus-strand DNA that is discrete when the RT just finished. Based on this feature, Southern blot analysis in denaturing conditions was carried out. Two different probes were used, namely, a double-stranded DNA probe within the RTase gene next to the genomic 5′ end and a downstream plus-strand DNA probe within the gp41 gene next to the genomic 3′ end.

The upstream half-genome of the plus- and minus-strand DNA was detected with a double-stranded DNA probe in SJP-L-5- or NVP-treated cells. Both plus- and minus-strand DNA levels remained unaltered at increasing concentrations of SJP-L-5 (Fig. 5c). Moreover, upstream plus-strand DNA was higher than the control, suggesting that SJP-L-5 would not block upstream plus-strand DNA synthesis. On the other hand, plus- and minus-strand DNA levels decreased with NVP in a dose-dependent manner, suggesting that NVP inhibits both types of DNA (plus and minus). Downstream plus-strand DNA levels, detected with a plus-strand DNA probe, accumulated with SJP-L-5 in a dose-dependent manner (Fig. 5c), exhibiting at least five different DNA lengths (Fig. 5b), suggesting that SJP-L-5 induces multiple initiation sites to start the downstream plus-strand DNA synthesis while usually only the cPPT site is used as a primer. These results demonstrate that SJP-L-5 delays viral downstream plus-strand DNA maturation, disturbing formation of a triple-DNA flap.

SJP-L-5 induces mutation of the RTase gene in genotypic resistance assays. To further investigate the characteristics of SJP-L-5 inhibition of the RTase, we selected resistant viruses from HIV-1_{HxB} infected C8166 cells exposed to increasing concentrations of SJP-L-5, and sequenced the RTase gene for a genotypic assay. Since it is very difficult to introduce a mutation in a live virus, we used the plasmid pNL4-3 (the RTase

---

**Figure 3.** Real-time qPCR assays of HIV-1 viral DNA levels in SJP-L-5-treated cells. HIV-1_{HXB}-infected C8166 cells were co-cultured with SJP-L-5 (100 μM), EFV (200 nM) or DMSO (0.5%, v/v) during different time points up to 24 h. Viral DNA was isolated and quantified by real-time qPCR. (a) sssDNA formation was not inhibited with SJP-L-5, and these molecules significantly accumulated at 1 and 4 h after HIV-1 infection (P < 0.05). (b) Late-RT quantities were significantly reduced by SJP-L-5 at 4, 6, and 8 h (P < 0.05). (c) 2-LTR quantities were significantly reduced with SJP-L-5 at 8, 12, and 24 h (P < 0.05).
DNA sequence of IIIB and NL4-3 are highly similar with a similarity >99%) as a tool to perform site-directed mutations. SJP-L-5 concentrations were increased from 0.9 to 122 μM during 11 passages to select resistant HIV-1 IIIB viruses (Fig. 6a). After a 96-day selection, an SJP-L-5 resistant strain was obtained: HIV-1 L-5resi. The EC50 of SJP-L-5 against HIV-1 L-5resi was above 608 μM (1240-fold higher than the wild-type, Fig. 6b) and higher than the 122 μM value obtained in the virus selection assay, suggesting we successfully obtained a SJP-L-5-resistant virus strain. Afterwards, the DNA of HIV-1 L-5resi-infected C8166 cells was extracted and the full-length RTase sequence (1680 bp) was analyzed. The sequence analysis revealed that seven amino acids were mutated within the RTase gene, namely, V108I, E138K, Y181C, and L214F within the DNA polymerase motif, and N447S, R461K, and A508T within the RNase H motif (Fig. 6c). These results show that SJP-L-5 induces gene mutations and supports the idea which the RTase is a target of this inhibitor.

SJP-L-5 exhibits diminished activity against NNRTIs resistant mutants, but is sensitive to NRTIs resistant mutants in phenotypic resistance assays. To understand the interactions between the RTase and SJP-L-5, we investigated whether SJP-L-5 would be less sensitive to RTase mutants. Three single-site mutants (V108I, E138K, and Y181C, located in the polymerase domain), which were indicated from genotypic resistance assays, were used for a phenotypic resistance assay with SJP-L-5. Additionally, other known resistant viruses (HIV-1 A17, HIV-1 74V, and HIV-1 A018) were tested. As shown in Table 1, SJP-L-5 was resistant to NNRTI mutations, including V108I, E138K, Y181C, and “K103N + Y181C”, with fold changes (FC) ranging from 25 to 158. However, SJP-L-5 was sensitive to NRTI mutations (HIV-1 L74V and HIV-1 A018) with FC of 0.8 and 1.4, respectively. NVP displayed similar antiviral characteristics to SJP-L-5 against mutants and resistant viruses. In summary, our results suggest that SJP-L-5 is an NNRTI. However, it exerts a novel mechanism as SJP-L-5 preferentially inhibits the RTase DDDP activity instead of the RDDP.

**Discussion**

To date, there are five NNRTIs in clinical use, including nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine. These drugs inhibit viral minus-strand DNA synthesis. Here, we describe a novel compound, SJP-L-5, which inhibits the plus-strand DNA elongation rather than minus-strand DNA synthesis, presenting a different mechanism from that utilized by marketed drugs.
The synthesis of the minus-strand DNA is initiated by a tRNA primer, which binds to the primer-binding site (PBS) near the 5′ end of the viral genomic RNA. Initiation of RT is conducted by RTase RDDP activity from the tRNA primer, and the synthesis of -ssssDNA is one of the first products of this process. Real-time qPCR assays showed that SJP-L-5 does not reduce the -ssssDNA products in the first 4 h, but reduces late-RT and 2-LTR DNA levels (Fig. 3a–c). These results demonstrate that SJP-L-5 blocks HIV-1 infection through RT inhibition, although it does not work early in this process. This mechanism is quite different from those previously reported since all FDA-approved NNRTIs target the RDDP activity while SJP-L-5 inhibits the DDDP activity.

Recently, the emergence of drug resistance has become a major concern in antiviral therapy. To overcome this issue, on one hand, a series of novel structural compounds were designed, such as rilpivirine and dolutegravir, on the other hand, new drug targets are gaining more attention, for example, RNase H of RTase. To understand the mechanisms with which SJP-L-5 blocks RTase, we analyzed the three functions of the viral enzyme with methods that include ELISA and FRET. SJP-L-5 inhibited the RTase DDDP activity in a dose-dependent manner with an EC50 of 13.4 ± 3.0 μM. Strikingly, this compound showed little inhibition of the RDDP activity, which is distinctive from other NNRTIs. RNase H inhibition was not observed either (Fig. 4c). Cell-based qPCR assays suggested that SJP-L-5 preferentially inhibits plus-strand DNA synthesis rather than minus-strand DNA formation. These interesting results prompted the question of the mechanism of this inhibition. We next

Figure 5. Structural analysis of viral DNA via Southern blot. HIV-1-infected C8166 cells were co-cultured with SJP-L-5 (50, 5, and 0.5 μM) or NVP (1000, 100, and 10 nM) for 48 h. Viral DNA was extracted using Hirt’s method and denatured by boiling in a water bath. The DNA samples were digested with Pst I, subjected to electrophoresis in 0.7% agarose gel with 0.5× TBE and hybridized using DIG-labelled probes. (a) Southern blot with double-stranded DNA detection. The upstream half-genome levels of the plus-strand DNA were not reduced with SJP-L-5 but were reduced with NVP. (b) Southern blot with downstream plus-strand DNA-specific detection. The downstream half-genome levels of the plus-strand DNA were accumulated with SJP-L-5, possibly due to a delay in full-length viral DNA maturation, displaying a different mechanism from that observed with NVP. RAL (1 μM) was used to prevent integration of viral DNA into host chromosomes in all groups. Full-length blots are presented in Supplementary Figure S1. (c) Gray analysis of down-stream plus-strand DNA probe hybridization. Half genomic plus-strand DNA of SJP-L-5 accumulated in a dose-responsive manner, but was maintained at a base level of NVP at different concentrations. Furthermore, the total amounts of viral DNA were accumulated via SJP-L-5 in treatment with 50 and 5 μM. The genomic or 1/2LTR plus-strand DNAs were not affected by SJP-L-5 in a series of concentrations. The total amounts of viral DNA and genomic or 1/2LTR plus-strand DNAs decreased by NVP in a dose-responsive manner.
focused on the HIV DNA flap, a special DNA structure that exists in lentiviruses and is formed by discontinuous plus-strand DNA synthesis.

Two decades ago, it was reported that HIV reverse-transcribed DNA is discontinuous and bears two initiation sites to synthesize the viral plus-strand DNA \(^4,9,10\). Later, researchers found that a triple-DNA structure formed by the discontinuous plus-strand, called DNA flap, contributes to the nuclear import of the HIV-1 genome \(^5\). DNA flap was then believed as a viral promoting element for the uncoating of HIV-1 at the nuclear pore \(^23\). In recent years, RT and uncoating seem to be sequential processes since RT influences uncoating kinetics. DNA-RNA hybrid molecules may form a more rigid extended structure, which in turn could provide the outward force to destabilize the capsid during the early steps of RT \(^8\). Our published data demonstrated that SJP-L-5 blocks viral DNA nuclear entry by interrupting capsid disassembling \(^7\). In this paper, we analyzed the late process of RT by

**Figure 6.** Induction of an SJP-L-5-resistant virus strain. A resistant virus strain to SJP-L-5 was selected by adding increasing concentrations of SJP-L-5 (0.9 to 122 μM) to HIV-1\(_{\text{IIIB}}\)-infected C8166 cells. (a) SJP-L-5 concentrations during the experiment. After 96 days of induction, a SJP-L-5-resistant virus strain was selected. (b) Antiviral activity of SJP-L-5 against the induced resistant virus. The selected resistant virus showed an IC\(_{50}\) 1240-fold higher than the wild-type value. (c) Genotypic patterns of HIV-1\(_{\text{IIIB}}\) selected with SJP-L-5. The viral RNA was reverse transcribed, cloned into a TA cloning vector, and sequenced. Seven mutation sites were discovered; three of which are hot spots (V108I, L214F, and N447S).

**Table 1.** Anti-HIV-1 activity of SJP-L-5 against mutated viruses\(^a\).\(^b\). Abbreviations: WT, wild-type; NVP, nevirapine. \(^a\) Compounds listed in Table 1 are presented with standard deviations, \(n \geq 3\). \(^b\) EC\(_{50}\)s are exhibited by means ± standard deviations, \(n \geq 3\). \(^c\) FC, fold change (calculated as the mean EC\(_{50}\) of mutants divided by the wild-type HIV-1 NL4-3 value). \(^d\) NA, not applicable. \(^e\) TAMS, thymidine analog mutations, containing azidothymidine-resistant sites.
Southern blot. DNA hybridization data showed that SJP-L-5 does not inhibit the minus-strand DNA formation but impairs downstream plus-strand DNA elongation (Fig. 5). These results suggest that SJP-L-5 is a special RTase inhibitor that blocks the central DNA flap to be linearized by retaining the viral downstream plus-strand DNA at half genomic length. In answer to our previous question, we described the viral capsid uncoating delay in the SJP-L-5 treatment group. Due to the novel mechanism underlying SJP-L-5 inhibition, it was not possible to discover its role in our previous study. From our experience, we strongly suggest that the three functions of the RTase are evaluated during antiviral drug screening.

NNRTIs are small hydrophobic molecules that inhibit the HIV-1 RTase by an allosteric effect. They bind to a pocket located about 10 Å away from the dNTP binding site. We used resistance assays, including resistance selection and mutant sensitivity assays, to explore the interactions between SJP-L-5 and HIV-1 RTase. Since this compound did not inhibit RNaseH, we did not further evaluate the RNaseH mutations (N447S, R461K, and A508T) with SJP-L-5. Moreover, L214F occurs naturally as a polymorphism and does not cause NNRTI resistance. The results showed that SJP-L-5 is resistant to V108I, E138K, Y181C, and "K103N + Y181C", which are traditional resistance sites of NNRTIs; the main sites being V108I and Y181C, based on genotypic and phenotypic assays. These results suggest that SJP-L-5 may bind to Val108 and Tyr181 more firmly than to other amino acids. Therefore, SJP-L-5 binds to the HIV-1 RTase hydrophobic bag in the same way as NNRTIs. However, our study shows that SJP-L-5 preferentially inhibits RTase DDDP activity. RT needs to be further investigated and, to this regard, SJP-L-5 could be a valuable tool to understand this process.

Multiple segmented plus-strand DNA is rarely reported in HIV but was observed in another retrovirus: the avian sarcoma-leukosis virus (ASLV), which is an alpharetrovirus genus virus. Previous studies showed that only one gap (a triple DNA flap) exists in the center of HIV DNA genome. Our results suggest that more than one gap may exist under the treatment of SJP-L-5. This phenomenon provides insight into the details of HIV reverse transcription. However, why segmented plus-strand DNA only formed under the pressure of SJP-L-5 needs to be further investigated.

Based on our results, we suggest a model of how SJP-L-5 inhibits HIV-1 replication (Fig. 7). Typically, the HIV-1 DNA genome is formed during reverse transcription with a triple-DNA flap at the center. Later, the viral DNA genome enters the nucleus and is integrated into the host DNA genome. In response to SJP-L-5 treatment, both cPPT and PPT primed plus-strand DNA synthesis are blocked, leading to a cPPT gap and a PPT gap in the center and the 3’ end of HIV-1 DNA genome. Under the pressure of this compound, HIV-1 uses multiple sites to initiate its plus-strand DNA synthesis within downstream plus-strand DNA, forming five segmented plus-strand DNA bands and two PPT gaps (cPPT and PPT). Due to a lack of matured DNA (a triple-DNA flap), the impeded viral DNA fails to enter the host cell nucleus and cannot integrate into the host DNA genome.

**Figure 7.** A Model of how SJP-L-5 inhibits PPT-primed HIV-1 plus-DNA synthesis. HIV-1 plus-strand DNA synthesis is initiated by cPPT or PPT in the central genome or 3’ end, leading to formation of a triple-strand flap in the center. Later, the viral DNA genome enters the nucleus and is integrated into the host DNA genome. In response to SJP-L-5 treatment, both cPPT and PPT primed plus-strand DNA synthesis are blocked, leading to a cPPT gap and a PPT gap in the center and the 3’ end of HIV-1 DNA genome. Under the pressure of this compound, HIV-1 uses multiple sites to initiate its plus-strand DNA synthesis within downstream plus-strand DNA, forming five segmented plus-strand DNA bands and two PPT gaps (cPPT and PPT). Due to a lack of matured DNA (a triple-DNA flap), the impeded viral DNA fails to enter the host cell nucleus and cannot integrate into the host DNA genome.
results indicate SJP-L-5 as a new NNRTI that inhibits HIV-1 polypurine tract (PPT) primed plus-strand DNA synthesis, while initiating HIV-1 down-stream plus-strand DNA synthesis at multiple sites under drug pressure.

Materials and Methods

Compounds and reagents. SJP-L-5, with a molecular weight of 329.13, was provided by Professor Handong Sun (Kuning Institute of Botany, Chinese Academy of Sciences). Nevirapine (NVP) and efavirenz (EFV) were purchased from US Pharmacopeia (USA). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at –20 °C. DMSO, Tris, KCl, MgCl₂, 3-[(3-cholamidopropyl) dimethylammonio]–1-propane sulfonate (CHAPS), diethiothreitol (DTT), and ethylene glycol tetra-acetic acid (EGTA) were purchased from Sigma-Aldrich (USA). dUTP-AF555 was purchased from Invitrogen (USA), and dATP, dCTP, and dGTP were purchased from Takara (China).

Cells and viruses. C8166 cells were kindly provided by the AIDS Reagent Project, the UK Medical Research Council (MRC). C8166 are human T-lymphoblastoid cells sensitive to HIV infection, exhibiting a rapid and pronounced cytopathic effect. C8166 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) with 100 U/mL penicillin G and 100 μg/mL streptomycin in a humidified incubator with 5% CO₂ at 37 °C. Laboratory-adapted strains (HIV-1 NL4-3) and RT-inhibitors resistant strains (HIV-1 A17, HIV-1 74V, and HIV-1.A018) were obtained from the NIH AIDS Research and Reference Program (Bethesda, MD, USA). All viruses were propagated in C8166 cells.

Quantitative PCR of viral reverse transcripts. C8166 cells were seeded at a density of 1 × 10⁶ cells/well in 24-well culture plates and infected with HIV-1_vpr at M.O.I. 0.5 for 2 h under gentle rocking every 15 min at 4 °C. After 2 h of virus adsorption, the cells were washed and synchronized. Then, the cells were incubated in presence or absence of 100 μM SJP-L-5, 200 nM EFV, or 0.5% (v/v) DMSO. Cells were collected at 0, 1, 2, 4, 6, 8, 12, and 24 h of incubation with the drugs. Total cellular DNA was isolated using Blood Gen Mini Kit (CWBio, China). The -ssDNA was amplified using hRU5-F2 (5′-GCC TCA ATA AAG CTT GCC TTGA-3′) and hRU5-R (5′-TGA CTA AAA GGG TCT GAG GGA TCT-3′) primers with the hRU5-P probe (5′-FAM-AGA GTC ACA CAA CAG AGG GCC ACA CAC TA-TAMRA-3′). Reverse transcription product U5v (late-RT) was amplified with MH531 (5′-GCC TCA ATA AAG CTG TGG TTG A-3′) and MH532 (5′-GCC TCA ATA AAG CTG TGG TTG A-3′) primers and LRT-P probe (5′-FAM-CAG TGG CGC CCG AAC AGG GA-TAMRA-3′). For 2-LTR circle, the LTR-R3 primer (5′-AGG TAG CCT TGT GTG TGG TAG ATC C-3′), the U5-specific primer (5′-AGG TAG CCT TGT GTG TGG TAG ATC C-3′), and the p-HUS-S1 probe (5′-FAM-TAG TGT GTG CCC GTC GTG TGT GTG AC-TAMRA-3′) were used. The 20 μL reaction mixture contained 2 μL of sample or standard DNA, and the final following concentrations of 1 × Premix Ex Taq (probe qPCR) (Takara), 0.2 μM sense primer, 0.2 μM antisense primer, 0.1 μM probe, and 1 × ROX Reference Dye II. PCR was performed using an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling conditions included a hot start (95 °C for 2 min), 40 cycles of denaturation (95 °C for 15 s), and an extension steps (60 °C for 30 s).

RDDP activity. HIV-1 RTase RDDP activity was measured by a DIG-labelled ELISA assay using the Reverse Transcriptase Kit (Roche, Germany), according to manufacturer’s instructions.

HIV-1_R18 recombinant RT (rRTase) expression and purification. HIV-1_R18 RTase p66 and p51 subunits were cloned into two pET-30a vectors, and recombinant p66- and p51-His subunits were over-expressed in E. coli BL21(DE3). The p66/p51-His heterodimer was purified by Ni²⁺ affinity agarose purification as previously described. The rRTase was used for the following two assays.

PPT-primed DDDP activity. HIV-1 RTase DDDP activity was measured via fluorescent resonance energy transfer (FRET) assay as previously described, with minor modifications. The DNA template (5′-AF488-AGT CCG CCC TTT TCT TTT AAA AAG TGG CTA AGA), and the DNA primer (PPT-based) (5′-TTA AAA GAA CCC CCC TTT TCT TTT AAA AAG TGG CTA AGA), and the DNA primer (PPT-based) (5′-GCC TCA ATA AAG CTT GCC TTGA-3′) were synthesized by Sangon Biotech (China). Briefly, DNA template and DNA primer were annealed at temperatures ranging from 90 °C to room temperature to form 20 μM RNA/DNA hybrid solution. The 50 μM rRTase was dissolved in reaction buffer (50 mM Tris, 80 mM KCl, 6 mM MgCl₂, 0.025% (m/v) CHAPS, 1 mM DTT, and 0.1 mM EGTA pH 7.8) at 320 ng/mL. The 50 μL enzymatic reaction, preceded by incubation for 15 min at room temperature, contained 320 ng/mL rRTase, 80 nM RNA/DNA hybrids, and the test compounds. The reaction was initiated by adding 50 μL of dXTP to a final volume of 100 μL in a black 96-well plate. The plate was then incubated at room temperature in the dark for 15 min. Finally, the plate was measured using a Flex Station 3 reader (Molecular Device, USA) with excitation at 485 nm and emission at 530 nm (cutoff = 515 nm), and the EC₅₀ was calculated.

RNase H activity. HIV-1 RNase H activity was measured by FRET assay as previously described, with minor modifications. The DNA (DD18) (5′-Dabyl-AGC TCC CAG GCT CAG ATC) and RNA (FR18) (5′-GUA CUG AGC CUG GGA GCCUC-fluorescein) fragments were synthesized by Takara (China). Briefly, DD18 and FR18 were annealed at temperatures ranging from 90 °C to room temperature to form 10 μM RNA/DNA hybrids. rRTase was dissolved in reaction buffer (the same as the one used to measure the DDDP activity) at 400 ng/mL. Fifty microiliters of a 0.4 μM RNA/DNA hybrid solution was mixed with the compound dilutions to a final concentration of 100 μM in a black 96-well plate. The reaction was initiated by adding 50 μL of rRTase solution and incubated at 37 °C for 1 h. The plate was measured using a Flex Station 3 reader, with excitation at 490 nm and emission at 525 nm (cutoff = 515 nm), and the EC₅₀ was calculated.
Viral plus-strand DNA assay. (i) Infection of C8166 by HIV-1<sub>IIIB</sub>. In a 6-well plate, 5 × 10<sup>6</sup> C8166 cells were infected by HIV-1<sub>IIIB</sub> (MOI = 0.5) with serial dilutions of SJP-L-5 (50 μM, 5 μM, and 0.5 μM) or NVP (1000 nM, 100 nM, and 10 nM) in a final volume of 8 ml RPMI-1640 media containing 10% FBS and 1 μM RAL. RAL was used to prevent viral DNA integration into host chromosomes. The plate was then incubated in a humidified CO<sub>2</sub> incubator at 37 °C for 48 h. (ii) Hirt DNA preparation. The unintegrated viral DNA was extracted following Hirt's methods<sup>30,31</sup>. (iii) Southern blot. All DNA samples were digested by <i>Pst I</i> (Fermentas, China). Following denaturation in boiling water, the DNA was immediately cooled in ice/water. DNA was then subjected to electrophoresis in 0.7% agarose gel in 0.5× TBE and analyzed by DIG-labelled chemiluminescent Southern blot according to manufacturer's instructions (DIG-high prime DNA labeling and detection starter kit II, Roche). The probes used here were a double-stranded HIV-1 DNA fragments within the RTase gene from the position 2096 to 3375 of HIV-1<sub>IIIB</sub> genome, GenBank accession number: EU541617), which hybridized to both the minus and the upstream plus strands, or a single-strand DNA fragment within the gp41 gene (from the position 6296 to 6868 of HIV-1<sub>IIIB</sub> genome), which hybridized to the downstream plus strand<sup>1</sup>. The chemiluminescent signal was recorded on X-ray film (Kodak, China). The gray value of the film was analyzed via ImageJ 1.46r software.

Selection of SJP-L-5-resistant variant viruses. The viruses resistant to SJP-L-5 were selected by adding the compound progressively to HIV-1<sub>IIIB</sub>-infected C8166 cells, according to previously described methods<sup>32,33</sup>. Briefly, HIV-1<sub>IIIB</sub>-infected C8166 cells were cultured with 0.9 μM SJP-L-5. Every ~7 days, when syncytia formation was observed to be over 80%, a doubled concentration of SJP-L-5 was added until the compound reached a concentration of 122 μM.

Genotypic resistance assays. SJP-L-5-resistant virus variants were used to analyze the phenotypic and genotypic resistance features of the compound. The RNA of the resistant virus was extracted and reverse transcribed to cDNA. RTase gene of the cDNA was amplified by PCR as previously reported<sup>32</sup>. A full-length RTase-encoding sequence of 1680 bp was cloned, and positive clones were selected. After sequencing analysis, the gene was aligned with the wild-type HIV-1<sub>IIIB</sub> strain sequence (GenBank accession number: EU541617). Mutation frequency was then calculated.

Construction of site-directed mutants (SDM) for phenotypic resistance assays. SDM were constructed based on the HIV-1 infectious clone pNL4-3. Mutagenesis was carried out by using the Fast mutagenesis system kit (TransGen, China) according to manufacturer's instructions. V108I, E138K, and Y181C were introduced into the RTase gene. These mutant plasmids were transfected into C8166 cells using TurboFect (Fermentas, China) according to manufacturer's instructions. The supernatant of each mutated virus was collected and stored at -70 °C.

Antiviral activity of SJP-L-5 against resistant viruses and site-directed mutants. Nucleoside resistant HIV-1 strains (HIV-1<sub>A018</sub> and HIV-1<sub>74V</sub>), non-nucleoside resistant HIV-1 strains (HIV-1<sub>A17</sub> and HIV-1), and RTase mutant strains (V108I, E138K, and Y181C) were used. The antiviral activity assay was performed as previously described<sup>34</sup>. Statistical analysis. The results were expressed as the mean ± standard deviation (SD). Statistical significance was determined with Student’s t-test using the SPSS 17.0 software. Differences were significant at <i>P</i> < 0.05.

References
1. Das, K. & Arnold, E. HIV-1 reverse transcriptase and antiviral drug resistance. Part 1. <i>Curr. Opin. Virol.</i> 3, 111–118 (2013).
2. Kupiec, J.-J. & Sonigo, P. Reverse Transcriptase Jumps and Gaps. <i>J. Gen. Virol.</i> 77, 1887–1901 (1996).
3. Le Grice, S. F. J. Human Immunodeficiency Virus Reverse Transcriptase: 25 Years of Research, Drug Discovery, and Promise. <i>J. Biol. Chem.</i> 287, 40850–40857 (2012).
4. Charneau, P., Alizon, M. & Clavel, F. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. <i>J. Virol.</i> 66, 2814–2820 (1992).
5. Zennou, V. et al. HIV-1 genome nuclear import is mediated by a central DNA flap. <i>Cell</i> 101, 173–185 (2000).
6. de Bethune, M. P. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989-2009).<i> Antivir. Res.</i> 85, 75–90 (2010).
7. Bai, R. et al. TMC278, a Next-Generation Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI), Active against Wild-Type and NNRTI-Resistant HIV-1. <i>Antimicrob. Agents Chemother.</i> 54, 718–727 (2010).
8. Hulme, A. E., Perez, O. & Hope, T. J. Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. <i>Proc. Natl. Acad. Sci. USA</i> 108, 9975–9980 (2011).
9. Charneau, P. & Clavel, F. A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polyuridine tract. <i>J. Virol.</i> 65, 2415–2421 (1991).
10. Huanges, O., Tjoetta, E. & Grinde, B. The Plus Strand Is Discontinuous in a Subpopulation of Unintegrated HIV-1 DNA. <i>Arch. Virol.</i> 116, 133–141 (1991).
11. Meruzzi, V. J. et al. Inhibition of HIV-1 Replication by a Nonnucleoside Reverse-Transcriptase Inhibitor. <i>Science</i> 250, 1411–1413 (1990).
12. Dueweke, T. J. et al. U-90152, a potent inhibitor of human immunodeficiency virus type 1 replication. <i>Antimicrob. Agents Chemother.</i> 37, 1127–1131 (1993).
13. Young, S. D. et al. L-743, 726 (DMP-266): a novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. <i>Antimicrob. Agents Chemother.</i> 39, 2602–2605 (1995).
14. Andries, K. et al. TMC125, a novel next-generation nonnucleoside reverse transcriptase inhibitor active against nonnucleoside reverse transcriptase inhibitor-resistant human immunodeficiency virus type 1. <i>Antimicrob. Agents Chemother.</i> 48, 4680–4686 (2004).
15. Azijn, H. et al. TMC278, a Next-Generation Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI), Active against Wild-Type and NNRTI-Resistant HIV-1. <i>Antimicrob. Agents Chemother.</i> 54, 718–727 (2010).
16. Gilboa, E., Mitra, S. W., Goff, S. & Baltimore, D. Detailed Model of Reverse Transcription and Tests of Crucial Aspects. <i>Cell</i> 18, 93–100 (1979).
17. Cauchon, E., Falgueyret, J. P., Auger, A. & Melnyk, R. A. A High-Throughput Continuous Assay for Screening and Characterization of Inhibitors of HIV Reverse-Transcriptase DNA Polymerase Activity. J. Biomol. Screen. 16, 518–524 (2011).

18. Menendez-Arias, L. Molecular basis of human immunodeficiency virus type 1 drug resistance: overview and recent developments. Antivir. Res. 98, 93–120 (2013).

19. Arts, E. J. & Hazuda, D. J. HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb. Perspect. Med. 2, a007161–a007161 (2012).

20. Kobayashi, M. et al. In Vitro Antiretroviral Properties of S/GSK1349572, a Next-Generation HIV Integrase Inhibitor. Antimicrob. Agents Chemother. 55, 813–821 (2011).

21. Velthuisen, E. J. et al. Pyridopyrimidinone inhibitors of HIV-1 RNase H. Euro. J. Med. Chem. 83, 609–616 (2014).

22. Lansdon, E. B. et al. Structural and Binding Analysis of Pyrimidinol Carboxylic Acid and N-Hydroxy Quinazolinonedione HIV-1 RNase H Inhibitors. Antimicrob. Agents Chemother. 55, 2905–2915 (2011).

23. Arhel, N. J. et al. HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore. EMBO J. 26, 3025–3037 (2007).

24. Menendez-Arias, L., Sebastian-Martín, A. & Alvarez, M. Viral reverse transcriptases. Virus Res. 234, 153–176 (2017).

25. Vingerhoets, J. et al. TMC125 displays a high genetic barrier to the development of resistance: evidence from in vitro selection experiments. J. Virol. 79, 12773–12782 (2005).

26. Clutter, D. S., Jordan, M. R., Bertagnolio, S. & Shafer, R. W. HIV-1 drug resistance and resistance testing. Infect., Genet. Evol. 46, 292–307 (2016).

27. Junghans, R. P., Le Grice, S. F. J. & Beutler, J. A. A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. Anal. Biochem. 322, 33–39 (2003).

28. Hou, E. W., Prasad, R., Beard, W. A. & Wilson, S. H. High-level expression and purification of untagged and histidine-tagged HIV-1 reverse transcriptase. Protein Expr. Purif. 34, 75–86 (2004).

29. Parniak, M. A., Min, K. L., Budihas, S. R., Le Grice, S. F. J. & Beutler, J. A. A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. Anal. Biochem. 322, 33–39 (2003).

30. Hirt, B. Selective Extraction of Polyoma DNA from Infected Mouse Cell Cultures. J. Mol. Biol. 26, 365–369 (1967).

31. Ziegler, K., Bui, T., Frisque, R. J., Grandinetti, A. & Nerurkar, V. R. A rapid polyomavirus DNA replication assay. J. Virol. Methods. 122, 123–127 (2004).

32. Zhang, X. J. et al. DB-02, a C-6-cyclohexylmethyl substituted pyrimidinone HIV-1 reverse transcriptase inhibitor with nanomolar activity, displays an improved sensitivity against K103N or Y181C than S-DABOs. PLoS ONE 8, e81489 (2013).

33. Liu, S. Y., Zhuang, D. M., Dong, R. H., Bai, L. & Li, J. Y. A pyrimidinol carboxylic acid and a pyrimidinone carboxylic acid inhibits HIV-1 reverse transcriptase and is selective for resistant virus strains. PLoS ONE 9, e105617 (2014).

Acknowledgements

This work was supported in part by grants from the National Natural Science Foundation of China (81102483, 81001462, 81422046, U1702286 and 21762048), the Key Scientific and Technological Program of China (2013ZX09103001-010 and2014ZX10005-002-006), the Yunnan Applicative and Basic Research Program (2013FB182 and 2015BC002), the CAS Institutes of Materia Medica (CASIMM0320163020), the Collaborative Innovation Center for Natural Products and Biological Drugs of Yunnan, and the Program for Changjiang Scholars and Innovative Research Team in University (IRT_17R94) and the Key Program of Natural Science of Yunnan Province to W.L. Xiao.

Author Contributions

W.L.X., and Y.T.Z. designed the study. X.J.Z., R.R.W., H.C., R.H.L., L.M.Y. and J.P.L. performed experiments. X.J.Z., R.R.W., W.L.X., and Y.T.Z. wrote the paper. H.D.S. and H.B.Z. revised the manuscript. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-20954-5.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.