


SCREENING AND CHARACTERIZATION OF ANTI-HIV SMALL MOLECULE
INHIBITORS

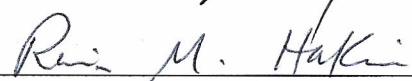
by

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A Thesis
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Committee:



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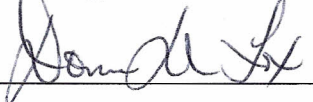
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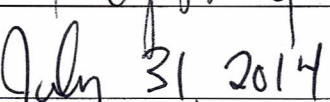
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George Mason University
Fairfax, VA

Screening and Characterization of Anti-HIV Small Molecule Inhibitors

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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Summer Semester 2014
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DEDICATION

This is dedicated to my loving parents, Shiyong Deng and Xiaohua Zou.

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I would first like to thank my supervisor Dr. Yuntao Wu heartily. He gave me invaluable guidance and persistent help in all the time of my research. I would also like to thank my committee member Dr. Jia Guo, who was always there when I had questions or met problems. My great appreciations also go to my committee member, Dr. Ramin Hakami, who gave me a lot of concerns and always encourages me.

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ABSTRACT

SCREENING AND CHARACTERIZATION OF ANTI-HIV SMALL MOLECULE INHIBITORS

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The human immunodeficiency virus (HIV) afflicts over 30 million people worldwide. The current anti-retroviral therapy has been proved effective and improved the life expectancy of HIV infected individuals. However, it still has numerous limitations, including side effects, drug interactions, poor drug absorption and viral drug resistance. Discovery and development of novel anti-HIV drugs that overcome current limitations is in great demand and still one of the major interests and focuses in the field of HIV therapy. Great efforts have been put on identifying effective anti-HIV drugs and studying the action mechanisms of the drugs on HIV in this research work. Up to forty compounds from different research institutes have been screened using a HIV Rev-dependent indicator T cell line, Rev-CEM-GFP-Luc. Eight drugs that inhibited HIV infection at non-toxic dosages have been identified through the screening. Further mechanism study has then been performed on the identified drugs and two of them

(NSC377384 and NSC44556) are selected and studied in details. The study has revealed that these two selected drugs have totally different mechanisms of action on HIV.

NSC377384 targets the late steps of HIV life cycle, and inhibits HIV replication most likely by inhibiting viral assembly and budding, causing a decrease in viral particle release. NSC44556, in contrast, targets the early steps of HIV life cycle, and likely specifically inhibits the function of HIV reverse transcriptase, leading to a decrease in viral DNA synthesis.

The discoveries in this research work will provide useful help to the development of better anti-HIV drugs overcoming some current limitations. It is also expected that this research work can contribute to the future research in better meeting the societal demand on novel anti-HIV drugs.

1. INTRODUCTION

1.1 Discovery of HIV-1 and Pathogenesis of HIV-1 infection

The development of acquired immunodeficiency syndrome (AIDS) was first clinically observed in 1981 in the United States. This term was coined to describe a condition in a group of previously healthy young males within the Los Angeles/ San Francisco area who showed a marked depletion of their immune CD4⁺ T lymphocytes, rendering them immune incompetent. As a consequence, they suffered from a number of opportunistic infections that were often fatal [1].

In 1983, two separate research groups led by Robert Gallo and Luc Montagnier independently declared that a novel retrovirus may have been infecting AIDS patients. Gallo claimed that a virus his group had isolated from the blood of individuals with AIDS was strikingly similar in shape to other human T-lymphotropic viruses (HTLVs) his group had been the first to isolate. Gallo's group called their newly isolated virus human T-cell lymphotropic virus TypeIII (HTLV-III) [2]. At the same time, Montagnier's group isolated a virus from an AIDS patient presenting with swelling of the lymph nodes of the neck and physical weakness, two classic symptoms of AIDS. Montagnier's group named their isolated virus lymphadenopathy-associated virus (LAV) [3]. As these two viruses turned out to be the same, in 1986, LAV and HTLV-III were renamed human Immunodeficiency Virus type-1 (HIV-1) [4].

HIV-1 infection targets the immune system and leads to a state of immunodeficiency. CD4⁺ T cell is the major target of HIV-1. CD4⁺ T cells play a crucial role in the immune responses by interacting with the other immune cells to fully engage in suppressing infection and inflammation [5]. The depletion of CD4⁺ T cells is one of the most fundamental events in HIV infection and leads to the subsequent loss of immune competence [6] [7].

The first hypothesis of HIV-induced CD4 T cell loss is that HIV directly infects and kills those cells [8]. However, it only partially explains the CD4 T cell pool depletion and a variety of bystander mechanisms have been described as contributing factors to CD4⁺ T cell death [9]. During HIV infection, the direct virus-mediated depletion can be attributed to cytopathic effects caused by viral replication and the cytolytic effects induced by host immune effector mechanisms, such as virus-specific cytotoxic T lymphocytes (CTLs). The indirect virus-mediated depletion results from the general immune activation induced by viral replication. This effect impairs other cellular compartments beyond CD4⁺ T cells, such as CD8⁺ T cells and B cells [10].

During the acute phase of HIV infection, virus greatly replicates and spreads from the initial site of infection to the many tissues and organs that provide the sites for replication. High levels of viremia (up to 10^7 or more copies of viral RNA per milliliter of blood) can be detected [11]. The innate immune response occurs as early as the viral RNA hits the detectable limitation (100 copies per ml), while the adaptive immune response arises as viremia approaches its peak [12].

The immune response includes both in the form of antibodies against all viral proteins, and a CD8 T-cell response against HIV-1 antigens expressed on infected cells. At the end of the acute phase, the level of viremia declines sharply, a result of both partial control by the immune system and exhaustion of activated target cells [13]. Meanwhile, a substantial drop in peripheral CD4 T cell counts and memory CD4⁺CCR5⁺ T cells also occurs [14] [15] [16].

While the immune response suppresses viral replication to some extent, it cannot stop the emergence of those viral mutants that are able to evade immune surveillance, as new variants replace the old ones [17] [18]. Continuous HIV replication results in a state of generalized immune activation persisting throughout the chronic phase [19]. The activation and proliferation of T cells that results from immune activation provides fresh targets for HIV infection. As a result, the peripheral CD4⁺ T cell count keeps dropping at a relative slow pace by the ongoing HIV replication. Although new T cells are continuously produced by the thymus to replace the ones lost, the regenerative capacity of the thymus is slowly destroyed by direct infection of its thymocytes by HIV [20] [21].

Finally, the number of CD4⁺ T cells declines to the point (below 200 cells per microliter) at which immune control of adventitious infectious agents can no longer be maintained, and opportunistic infections begin to appear, leading to AIDS [22].

1.2 HIV-1 Replication Cycle

HIV-1 only infects cells with CD4 antigen expressed on the cell surface. Entry to the cell begins through interaction of the viral surface protein complex gp120/gp41 and both CD4 receptor and a coreceptor, either CCR5 or CXCR4, on the cell surface [23] [24]. Once the viral gp120 binds to CD4 receptor and a coreceptor, a conformational change on the gp120 molecule allows gp120's shedding from the virus and reveals the gp41 fusion protein on the viral surface. The membrane fusion reaction that takes place between the lipid bilayers of the viral envelope and the host cell membrane enables the viral core to gain access to the cytoplasm [25] [26].

The viral core, including viral RNA and various enzymes, then uncoated shortly after fusion, allows for the initiation of reverse transcription. The viral single-stranded RNA genome will be converted into double-stranded DNA by the viral enzyme called reverse transcriptase [27].

The process of reverse transcription is extremely error-prone, and the resulting viral mutations may allow the virus to evade the body's immune system and also cause anti-HIV drug resistance [28]. Transcribed viral double stranded DNA is imported into the cell nucleus and then integrated into the host genome by viral integrase [29].

During viral replication, the integrated DNA provirus is transcribed into mRNA, which is then spliced into smaller pieces. These small pieces are exported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins Tat (which encourages new virus production) and Rev. As the newly produced Rev protein

accumulates in the nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they are otherwise retained until spliced [30].

At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome, it binds to the Gag protein and is packaged into new virus particles. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved into the two HIV envelope glycoproteins, gp41 and gp120 [31], which are then embedded in the host cell membrane. Viral structural proteins and genomic RNA assemble at the cell surface. The Gag (p55) and Gag-Pol (p160) polyproteins surround viral RNA form first an immature virus "bud", then the gag polyproteins is cleaved into the actual matrix, capsid and nucleocapsid proteins by the packaged viral protease. The various structural components then assemble to produce a mature HIV virion, which is then able to infect another cell [32].

1.3 Current Anti- HIV Therapy

Since its discovery in 1983 [2] [3], HIV-1 has become the most extensively studied in history. However, there is currently no cure or effective vaccine for HIV-1 infection [33]. The dysfunctional immune system caused by HIV-1 infection puts AIDS patients at a greater risk for opportunistic infections, which will consequently lead to a higher morbidity and mortality [12] [34].

Although there is no cure or effective vaccine for HIV-1 infection, anti-HIV therapy has been invented and successfully applied to control HIV replication and thus reduce AIDS-associated mortality and improve the life expectancy of HIV infected patients [35] [42]. The first effective therapy against HIV was the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine, described in 1985 as an antiviral agent inhibiting the infectivity and cytopathic effect of HIV in vitro [36] and approved by the US FDA in 1987 [37]. Subsequently several more NRTIs were developed, but all of them, even in combination, were unable to suppress the virus for long periods of time and patients still inevitably died [38].

The introduction of Highly Active Anti Retroviral Therapy (HAART) at 1996 marked a turning point in the AIDS pandemic. The HAART options are combinations consisting of at least three drugs belonging to at least two different categories, according to the mathematical predication that it is rare for HIV to mutate in more than three positions [39].

The categories of these compounds depend on the target within the HIV replication cycle they interact with. Each class of drugs attacks HIV at a different stage of

the HIV life cycle. FDA approved HIV antiretroviral compounds include entry inhibitor (or fusion inhibitor), Nucleoside reverse transcriptase inhibitors (NRTI), Non-Nucleoside reverse transcriptase inhibitors (NNRTI), Integrase inhibitors (INSTI), Protease inhibitors (PI). Typical combinations include 2 NRTIs as a "backbone" along with 1 NNRTI, PI or INSTI as a "base" [40].

The clinical results showed the HAART led to durable control of HIV replication, reducing viremia to undetectable levels. Thus, AIDS-associated mortality has greatly dropped and the life expectancy of HIV infected individuals has been improved [41] [42].

1.4 Current Problems and Research Objectives

HIV infects human immune cells and causes immune system dysfunction. So far, there is no effective vaccine for HIV infection. The current anti-retroviral therapy (HAART) is effective in controlling HIV replication and AIDS progress, but it still has limitations, including drug side effects, poor drug absorption, drug interactions, and viral drug resistance [43] [44] [45]. The HIV-affected population worldwide is over 30 million people now [World Health Organization, 2013], which places a great demand on discovery and development of novel anti-HIV drugs that overcome current limitations. Discovery and development of novel anti-HIV drugs is still one of the major interests and focuses in the field of HIV therapy. This research work aims to identify effective anti-HIV drugs from different compounds through screening and study the action mechanisms of the selected drugs on HIV. It is expected that the results from this research work can

help the development of better anti-HIV drugs overcoming current limitations and contribute to the future research in better meeting the societal demand on novel anti-HIV drugs.

2. ANTI-HIV DRUG SCREENING

A total of forty compounds from three different research institutes will be used to test the impacts on HIV replication by using a HIV indicator T cell line (described in section 2.1.2). Then several good candidates will be selected to study their action mechanism in detail, and find out on which steps they may target and inhibit HIV infection.

2.1 Materials and Methods

2.1.1 List of inhibitors to screen

Ten Chemical compounds from DTP (National Cancer Institute's Developmental Therapeutics Program): NSC377384, NSC83318, NSC50651, NSC171303, NSC44556, NSC87574, NSC273748, NSC287549, NSC156594, GR8 140873

The ten compounds were first screened and selected from a drug library of over 1000 compounds which consist of a random set of diverse structural types by a graduated PhD student, Todd Hawley, in our lab. From his previous screening results, these ten compounds showed inhibition on HIV-mediated luciferase activity in infected HIV indicator cells. In this thesis, the ten compounds will be confirmed by testing HIV-mediated GFP expression, and further selected candidates will be studied in details.

Twenty-seven ROCK inhibitors designed from TSRI (The Scripps Research Institute): SR5037, SR6443, SR6848, SR3642, SR4343, SR6925, SR7532, SR3850, SR5053, SR5834, SR6514, SR7230, SR6854, SR8046, SR6469, SR7494, SR7991, SR7492, SR6796, SR6455, SR4919, SR6513, SR6441, SR6433, SR7583, SR10677, SR10780

Rho-associated protein kinase (ROCK) is one of the upstream kinases of LIM Kinase (LIMK) and it regulates LIMK activity [46]. LIMK phosphorylates cofilin which regulate actin treadmilling. Cell lines with LIMK knockdown has been shown to decrease F-actin content and actin polarization, causing resistant to HIV infection [47] [48]. Thus ROC Kinase may also be a target to inhibit HIV infection.

Three HSP inhibitors from Dr. Ramin Hakami: HSP90 Inhibitor 17-AGG; HSP70 inhibitor KNK-437; General HSP inhibitor BAPTA-AM.

Heat shock proteins (HSP) are molecular chaperones mainly involved in protein folding and assembly [49]. These proteins are selectively expressed in cells during stress conditions in response to a range of stimuli, including heat, lymphokine and microbial/viral infections [49] [50]. Previous studies have shown that HSP are involved in HIV replication cycle and the regulation factor HSF1 has positive effects on HIV gene expression [51]. HSP may also provide a target to inhibit HIV replication.

All the inhibitors are properly dissolved in Dimethyl sulfoxide(DMSO).

2.1.2 HIV indicator T cell line

A Rev-dependent indicator T cell-line (Clone X) was used to quantify the degree of drug-mediated inhibition of HIV-1. It contains a vector, pNL-Luc-GFP-RRE-SA, which include the Rev-response element and HIV splicing sites that are efficiently used in human cells. It also contains GFP and Luciferase reading frame that are removed by cellular splicing activity in the absence of HIV Rev. In the presence of HIV Rev, singly and non-spliced transcript are delivered to the cytosol, and the reporter gene are expressed. Clone X was reconstructed from G11 cell line which has been showed responds robustly to the presence of HIV [52] [53].

2.1.3 Initiation of cell culture

There are five steps to initiate cell culture: (1) Take out one vial of cell stock (CEM-SS or CloneX) from liquid nitrogen tank, and quickly warm up cells by holding in hand or put in 37°C water bath. (2) After the cell thaw, add the cells drop by drop into 6 ml RPMI fresh medium in a 15ml tube, mix gently after each addition. (3) Collect the cells by centrifuge at 1000rpm for 5 minutes. (4) Resuspend the cell pellets with 15ml warmed RPMI plus 10% heat inactivated fetal bovine serum (FBS) medium, and plate out the cells in the T75 flask. (5) Observe cell growth and do not let cell overgrown to above 1×10^6 /ml.

2.1.4 Virus Preparation and Infection of HIV indicator T cell line

Virus stocks of the HIV-1_{NL4-3} were prepared by transfection using Lipofectamine 2000 (Invitrogen) of HEK 293 T cells with cloned proviral DNA. Supernatant was harvested between 48 to 72 hours, and filtered through a 0.45 µm nitrocellulose membrane. Virus titer (TCID₅₀) was measured by quantifying p24 release from culture supernatant using an in-house ELISA Kit. For HIV infection and treatment, cells were suspended at a concentration of 2×10^5 /100µl, and 100-200 ng(p24) of HIV-1 was added for 2 hours at 37°C. In some experiments, the infected cells were cultured in centrifuge at a speed of 600g for 2 hours at room temperature to get higher infection rate [54]. The cells were then washed twice with serum free RPMI to remove unbound virus, resuspended in RPMI plus 10% heat inactivated fetal bovine serum (FBS) and incubated at 37°C. At 48 hours post-infection, cells were analyzed for GFP expression by Flow-cytometry (FACS Calibur, BD Biosciences), which measures the degree of HIV replication. To exclude drug cytotoxicity, propidium iodide (PI) (2 µg/ml, Fluka) was added into the cell suspension prior to flow cytometry, and only viable cells (PI negative) were used for measuring GFP expression.

2.2 Results

2.2.1 Results of Screening Ten Chemical compounds from DTP

Five concentration gradients (50 μ M, 25 μ M, 5 μ M, 1 μ M, 200nm) were designed for the ten drugs at the first screening. HIV wild type NL4-3 virus were used to infect CloneX T cells (HIV indicator cell line, described in Material and Methods) for 2 hours, treated with drug or Dimethyl sulfoxide(DMSO) control 1h before infection plus add-back after infection. To get higher signal of HIV infection, the samples were cultured with spinoculation, a process whereby cell cultures are centrifuged at 600g during 2 hour infection. This process has previously been demonstrated to increase actin dynamics, facilitating increased viral infection [54]. At 48 hours post-infection, cells were collected and analyzed for GFP expression by Flow-cytometry, which measures the degree of HIV replication. Negative control was cells without HIV infection which provides a no- GFP-expression background. Infected cells were also stained with propidium iodide (P.I.) during flow cytometry to exclude drug cytotoxicity so that GFP expression was measured only in the viable cell population.

The analysis results of the ten drug's impact on HIV replication and cell viability are shown in Figure 1.1. For results of relative HIV replication in Figure 1.1.A, the black bars which below the first white bar are drug - treated samples with inhibition. In Figure 1.1.B, the black bars which below the first white bar are samples with lower cell viability than DMSO control, which means the drug concentration is toxic to cells. In our research,

those drugs which have good inhibition on HIV replication and without cytotoxicity (over 80% cell viability) will be selected as anti-HIV inhibitor candidates.

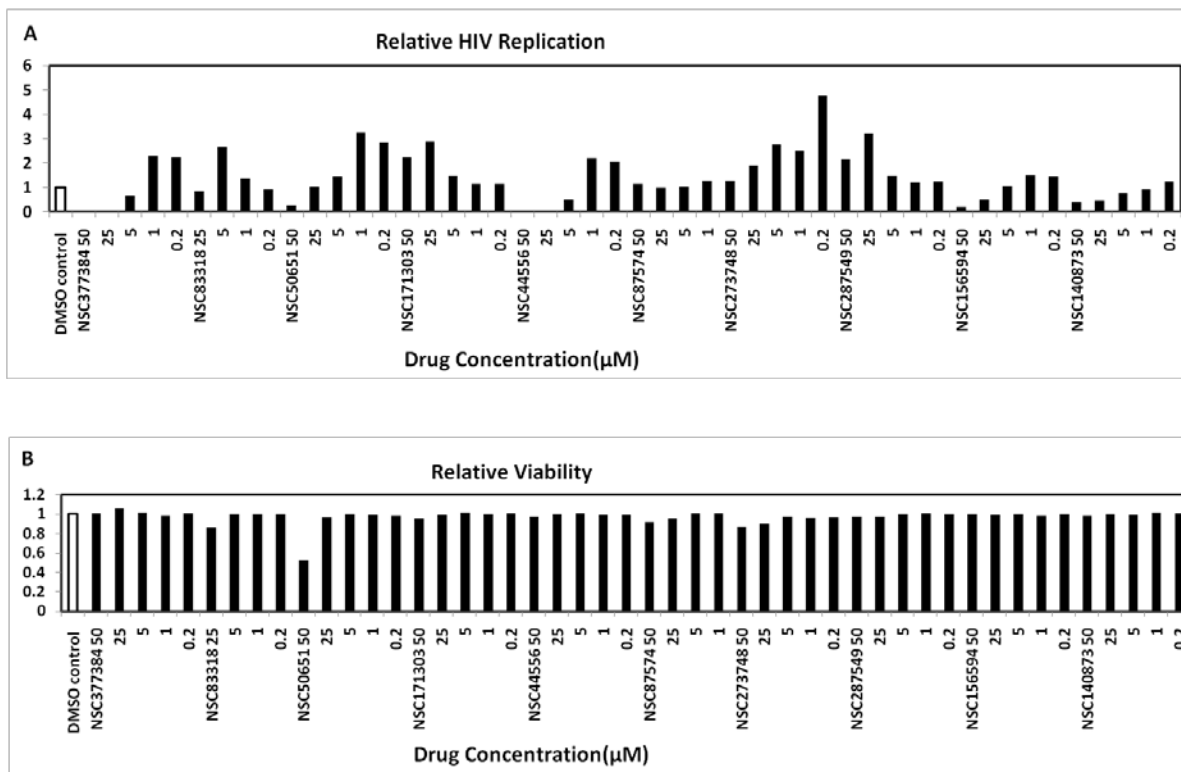


Figure 1.1. Analysis results of screening ten chemical compounds from DTP. (A) HIV- mediated GFP expression were analyzed to measure the degree of HIV replication. HIV replication level in DMSO control was set as 1 (white bar), and the relative HIV replication in each drug -treated sample are shown as black bars. **(B)** Cell viability of DMSO or drug -treated samples were measured to reflect drug cytotoxicity. Cell viability in DMSO control was set as 1 (white bar), and the relative cell viability in each drug -treated samples are shown as black bars.

(Original data can be found in Notebook Page129-133.)

Based on the analysis results of drug screening (Figure 1.1), four good inhibitor candidates which have remarkable inhibition on HIV replication without cytotoxicity are identified - NSC377384, NSC44556, NSC156594 and NSC140873. In Figure 1.2, GFP Data shows the best working dosage of each drug. NSC377384 and NSC44556 showed over 99% inhibition on HIV replication at the concentration of 25 μ M. NSC156594 and NSC140873 showed 78% and 59% inhibition respectively at the concentration of 50 μ M. Other six drugs either had no obvious inhibition on HIV or showed toxicity to cells (Figure 1.1).

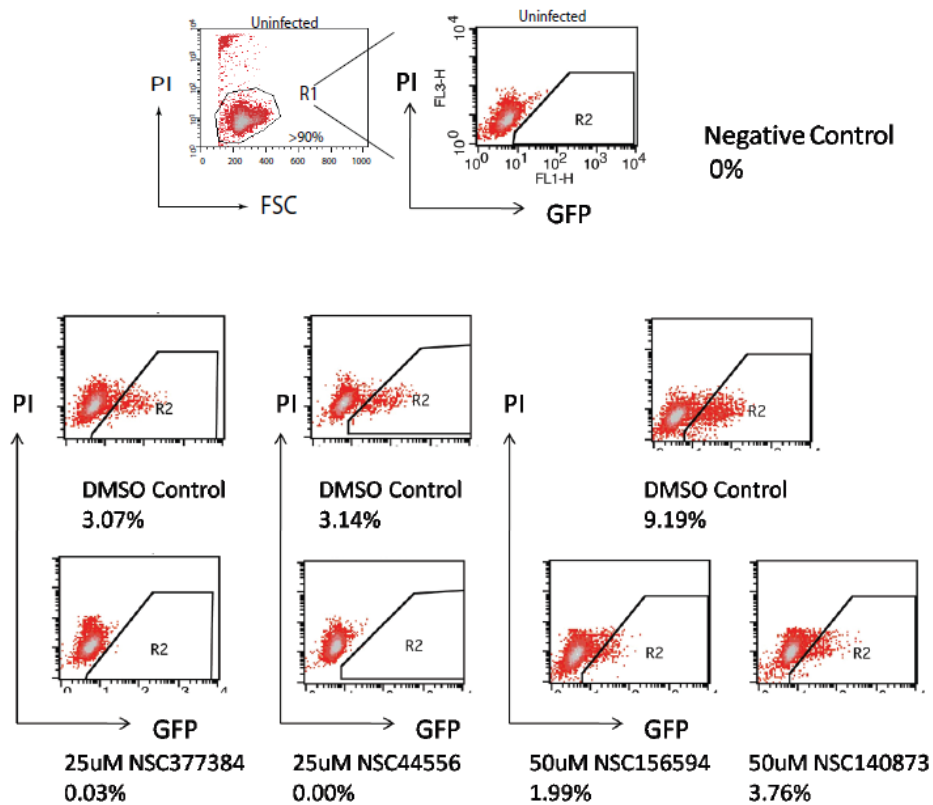


Figure1.2. Inhibition of HIV replication by NSC377384, NSC44556, NSC156594 and NSC140873. CloneX cells (HIV indicator cell line, described in Material and Methods) was pretreated with DMSO or drugs for 1 hour, and then infected with HIV-1_{NL4-3} for 2 hours in spinoculation. Cells were washed, and the drugs were added back after infection. HIV-dependent GFP expression was measured at 48 hours post infection by flow cytometry. To exclude drug cytotoxicity, propidium iodide (PI) was added into the cell suspension prior to flow cytometry, and only viable cells (R1 gate) were used for measuring GFP expression.

(Original data can be found in Notebook Page20, 24 & 28.)

2.2.2 Results of Screening Twenty-seven ROCK inhibitors from TSRI

Three concentration gradient were designed to screen ROCK inhibitors. Started from 100 μ M, did a 1:10 dilution to 1 μ M. CloneX cells (HIV indicator cell line, described in Material and Methods) was pretreated with DMSO or ROCK inhibitors for 1 hour, and then infected with HIV wild type NL4-3 virus for 2 hours at 37°C. Cells were washed, and the inhibitors were added back after infection (the first seven were not added back). After 48 hours, cells were analyzed for GFP expression by Flow-cytometry. Negative control was cells without HIV infection which provides a no- GFP- expression background. Infected cells were also stained with propidium iodide (P.I.) during flow cytometry to exclude drug cytotoxicity so that GFP expression was measured only in the viable cell population.

The analysis results of these inhibitors' impact on HIV replication and cell viability are shown in Figure 1.3 & Figure 1.4. For results of relative HIV replication, the black bars which below the first white bar are drug - treated samples with inhibition. For results of relative viability, the black bars which below the first white bar are samples with lower cell viability than DMSO control, which means the drug concentration is toxic to cells.

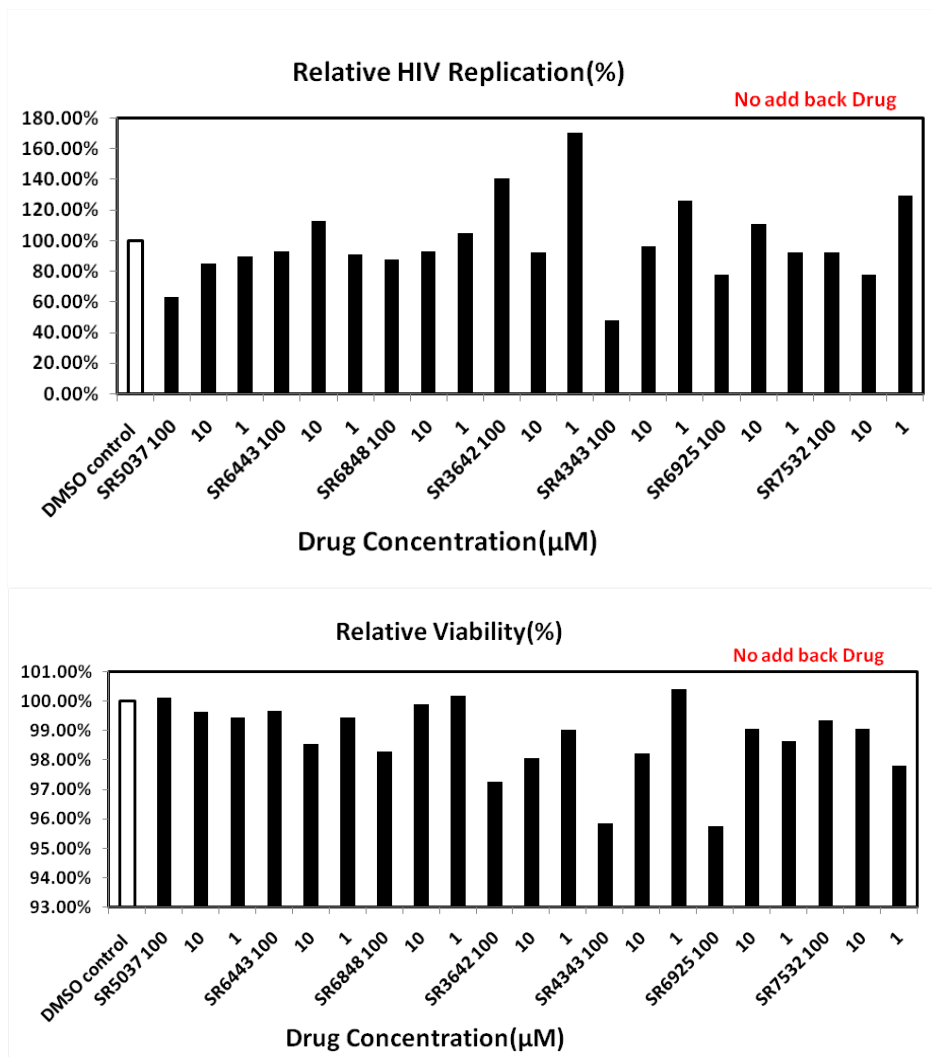


Figure 1.3. Analysis results of screening first seven ROCK inhibitors from TSRI.

HIV- mediated GFP expression were analyzed to measure the degree of HIV replication, and cell viability of DMSO or drug -treated samples were measured to reflect drug cytotoxicity. HIV replication level and cell viability in DMSO control were set as 100% (white bars), the relative HIV replication and relative cell viability in each drug -treated sample are shown as black bars. The seven inhibitor were not added back after infection. (Original data can be found in Notebook Page134-141.)

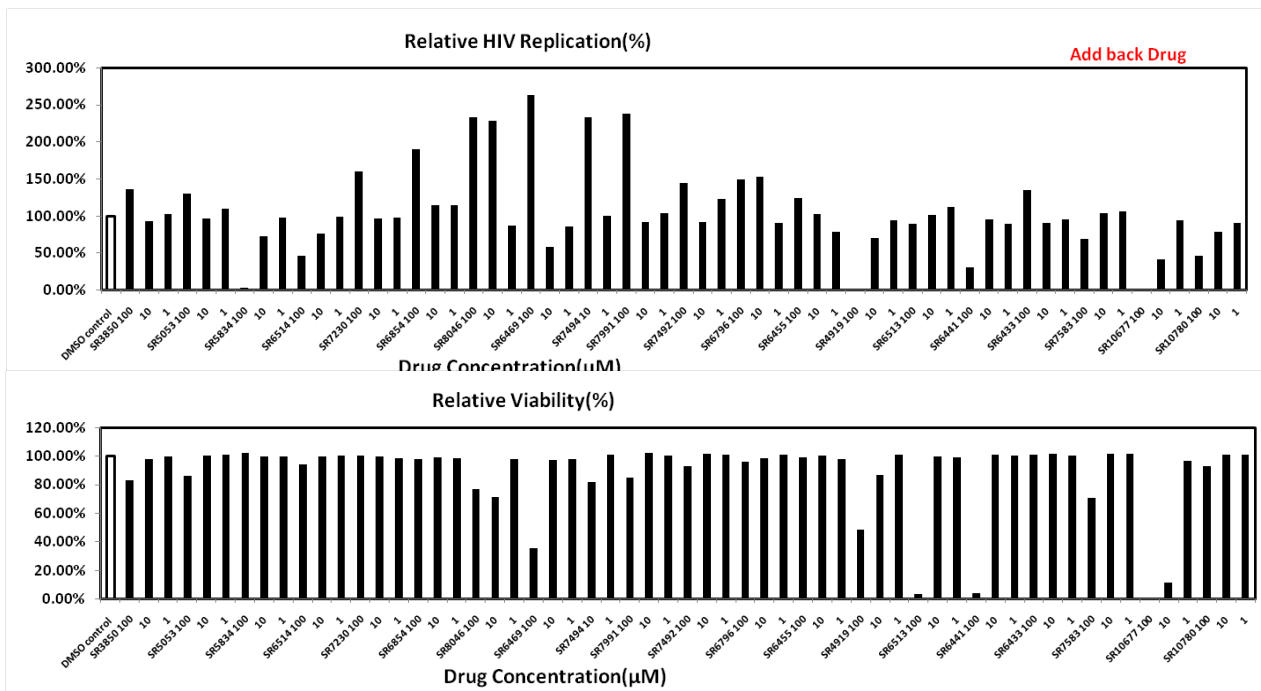


Figure 1.4. Analysis results of screening twenty ROCK inhibitors from TSRI. HIV-mediated GFP expression were analyzed to measure the degree of HIV replication, and cell viability of DMSO or drug -treated samples were measured to reflect drug cytotoxicity. HIV replication level and cell viability in DMSO control were set as 100% (white bars), the relative HIV replication and relative cell viability in each drug -treated sample are shown as black bars. The twenty inhibitor were added back after infection. (Original data can be found in Notebook Page134-141.)

According to the analysis results (Figure 1.3 & 1.4), SR5834 (100 μ M) is a good candidate without cytotoxicity (96% inhibition), SR5037 (100 μ M) and SR6514 (100 μ M) also showed inhibitions (37% and 54% respectively) but not as strong as SR5834. The data of HIV- mediated GFP expression of the three inhibitors are shown in Figure 1.5. Other ROCK inhibitors either had no obvious inhibition on HIV or showed toxicity to cells (Figure 1.3 & 1.4).

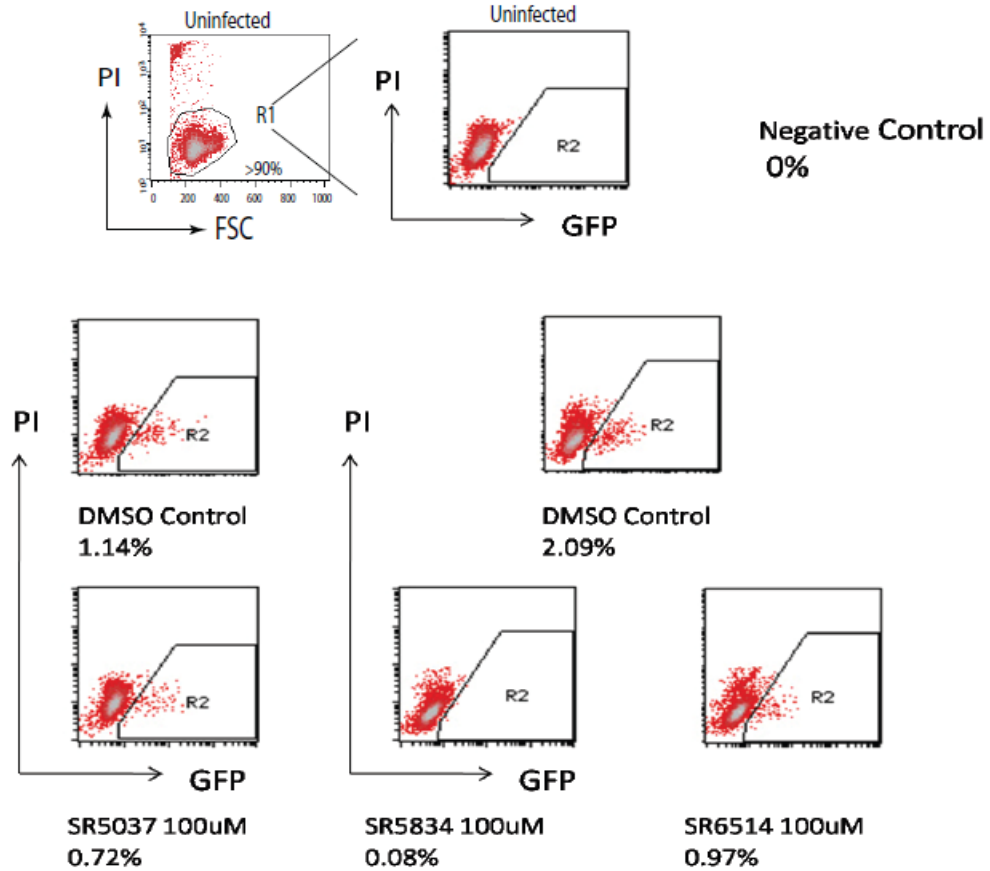


Figure 1.5. Inhibition of HIV replication by SR5037, SR5834 and SR6514. CloneX cells (HIV indicator cell line, described in Material and Methods) was pretreated with DMSO or drugs for 1 hour, and then infected with HIV-1_{NL4-3} for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. HIV-dependent GFP expression was measured at 48 hours post infection by flow cytometry. To exclude drug cytotoxicity, propidium iodide (PI) was added into the cell suspension prior to flow cytometry, and only viable cells (R1 gate) were used for measuring GFP expression.

(Original data can be found in Notebook Page142.)

2.2.3 Results of Screening Three HSP inhibitors from Dr. Ramin Hakami

Five concentration gradients were designed to test HSP inhibitors. Started from 10 μ M, did a 1:5 dilution to 16nM. Similarly, CloneX cells were infected with HIV wild type NL4-3 virus for 2 hours at 37°C, and drugs or DMSO were added 1h before infection and added-back after infection. After 48 hours, cells were analyzed for GFP expression by Flow-cytometry. Uninfected samples are cells without HIV infection which provides a no- GFP- expression background. Infected cells were also stained with propidium iodide (P.I.) during flow cytometry to exclude drug cytotoxicity so that GFP expression was measured only in the viable cell population.

The first one 17-AGG works well, with dosage-dependent inhibition from 10 μ M to 80nM (Figure 1.6.A). The second one KNK-437 doesn't show any impact on HIV infection, without any obvious difference in drug- and DMSO- treated samples (Figure 1.6.B). The third one BAPTA-AM has some toxicity at high dosage (10 μ M), and no obvious inhibition at lower dosages (Figure 1.6.C).

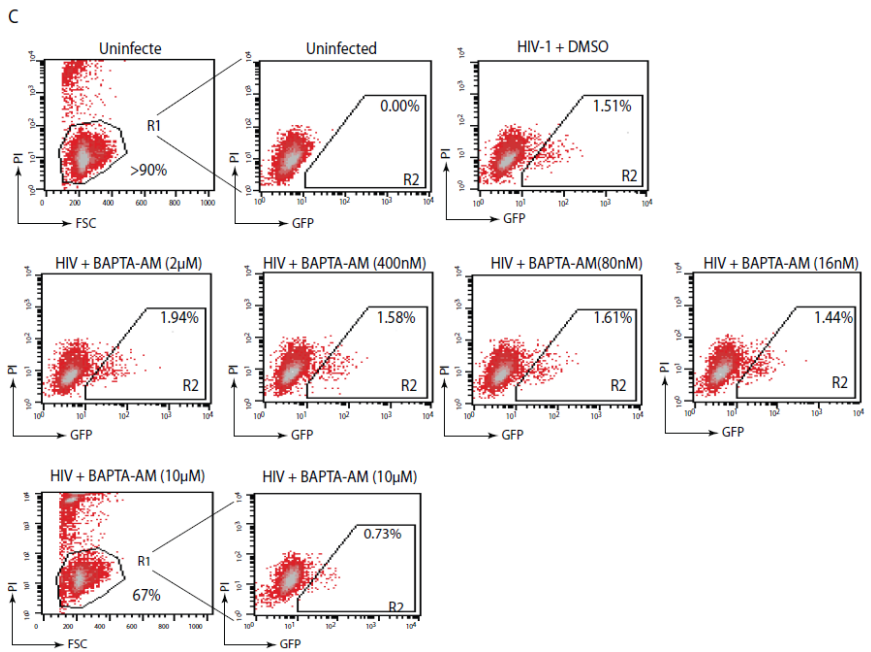
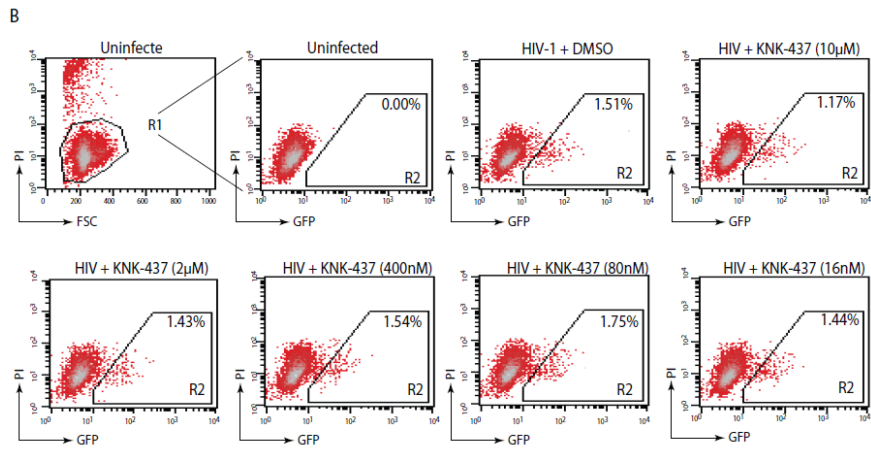
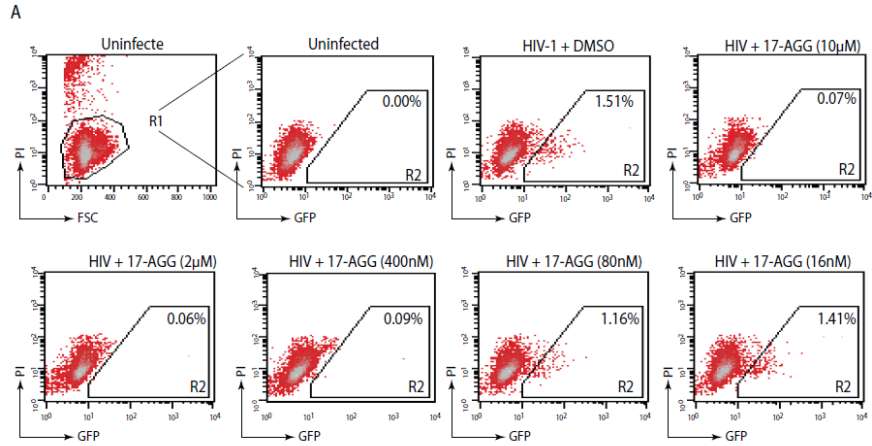


Figure 1.6. Results of screening three HSP inhibitors. CloneX cells (HIV indicator cell line, described in Material and Methods) was pretreated with DMSO or drugs for 1 hour, and then infected with HIV-1_{NL4-3} for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. HIV-dependent GFP expression was measured at 48 hours post infection by flow cytometry. To exclude drug cytotoxicity, propidium iodide (PI) was added into the cell suspension prior to flow cytometry, and only viable cells (R1 gate) were used for measuring GFP expression.

(A) 17-AGG has dosage-dependent inhibition on HIV replication from 10 μ M to 80nM.

(B) KNK-437 has no obvious impact on HIV infection. (C) BAPTA-AM has some toxicity at high dosage (10 μ M), and no obvious inhibition at lower dosages.

(All information of HSP inhibitors were given to Sarah Pierson to do further studies.)

2.3 Summary

A total of eight drugs that inhibit HIV replication at non-toxic dosages are identified. It is impossible to perform further mechanism study on all of them in this research due to the time limitation. Two of them, NSC377384 and NSC44556, are selected for this thesis work.

3. MECHANISM STUDY OF NSC377384

From the drug screening results in Chapter 2, NSC377384 has great inhibition on HIV replication (99%) at 25 μ M with spinoculation [54]. In order to find out how it inhibits HIV infection and at which step it may targets, the effects of the drug on HIV replication cycle will be tested step by step.

3.1 Materials and Methods

3.1.1 Initiation of cell culture

There are five steps to initiate cell culture: (1) Take out one vial of cell stock (CEM-SS or CloneX) from liquid nitrogen tank, and quickly warm up cells by holding in hand or put in 37°C water bath. (2) After the cell thaw, add the cells drop by drop into 6 ml RPMI fresh medium in a 15ml tube, mix gently after each addition. (3) Collect the cells by centrifuge at 1000rpm for 5 minutes. (4) Resuspend the cell pellets with 15ml warmed RPMI plus 10% heat inactivated fetal bovine serum (FBS) medium, and plate out the cells in the T75 flask. (5) Observe cell growth and do not let cell overgrown to above 1×10^6 /ml.

3.1.2 Virus Preparation and Infection of HIV indicator T cell line

Virus stocks of the HIV-1_{NL4-3} were prepared by transfection using Lipofectamine 2000 (Invitrogen) of HEK 293 T cells with cloned proviral DNA. Supernatant was harvested between 48 to 72 hours, and filtered through a 0.45 µm nitrocellulose membrane. Virus titer (TCID₅₀) was measured by quantifying p24 release from culture supernatant using an in-house ELISA Kit. For HIV infection and treatment, cells were suspended at a concentration of 2×10^5 /100µl, and 100-200 ng(p24) of HIV-1 was added for 2 hours at 37°C. In some experiments, the infected cells were cultured in centrifuge at a speed of 600g for 2 hours at room temperature to get higher infection rate [54].The cells

were then washed twice with serum free RPMI to remove unbound virus, resuspended in RPMI plus 10% heat inactivated fetal bovine serum (FBS) and incubated at 37°C. At 48 hours post-infection, cells were analyzed for GFP expression by Flow-cytometry (FACS Calibur, BD Biosciences), which measures the degree of HIV replication. To exclude drug cytotoxicity, propidium iodide (PI) (2 µg/ml, Fluka) was added into the cell suspension prior to flow cytometry, and only viable cells (PI negative) were used for measuring GFP expression.

3.1.3 Luciferase activity of infected cells by Luminescent assay

Luciferase assay kit (Cat. #E4030) was purchased from Promega. Luciferase assay reagent was prepared and stored in dark. Infected indicator cells were pelleted at 12000rpm for 5minutes, the resuspended in 1x luciferase assay lysis buffer, and added into the luciferase reading plate. Luminometer machine was primed with luciferase assay reagent and performed with reading progress according to the operation.

3.1.4 Real Time PCR for Viral DNA synthesis, 2-LTR circles synthesis and *nef* transcripts

The HIV Single cycle virus was used to infect CEM-SS cells for two hours. Drugs were added 1h before infection and added-back after infection. DNA and RNA samples in cell lysates were harvested at different time points, and purified using SV Total RNA Isolation System (Promega).

Quantitative real-time PCR analyses of viral DNA was carried out by using Bio-Rad iQ5 real-time PCR detection system as described previously [60]. The forward primer, 5'LTR-U5 (5'- AGATCCCTCAGACCCTTTTAGTCA-3'), the reverse primer, 3' gag (5'- TTCGCTTTCAAGTCCCTGTTC-3'), the probe, FAM-U5/gag (5'- (FAM) – TGTGGAAAATCTCTAGCAGTGGCGCC - (BHQ) - 3') were used for HIV total DNA analyses [56]. And the forward primer, MH536 (5'- TCCACAGATCAAGGATATCTTGTC-3'), the reverse primer MH535 (5'- AACTAGGGAACCCACTGCTTAAG-3') and the probe, MH603 (5'- 56FAMACACTACTTGAAGCACTCAAGGCAAGCTTT-36TAM-3') were used for viral 2-LTR circles [57] [58] [59]. The DNA standard used for viral total DNA quantification was pNL4-3, and the standard for 2-LTR circle was constructed by using a plasmid containing a complete 2 LTR region (pLTR-2C, cloned by amplification of infected cells with 5'-TGGGTTTTCCAGTCACACCTCAG-3' and 5'- GATTAAGTGC GAATCGTTCTAGC- 3'). Standards are ranging from 1 to 10⁶ copies of pNL4-3 or pLTR-2C mixed with DNA from uninfected cells.

For real-time PCR quantification of the *nef* transcripts, total RNA was reverse transcribed into cDNA using random decamers and M-MLV reverse transcriptase (Ambion) [55]. Nef cDNA was further quantified by real time PCR using primers 5' Nef (5'-GGCGGCGACTGGAAGAA-3'), 3' Rev (5'- AGGTGGGTTGCTTTGATAGAGAAG-3'), and the probe Nef/Rev (5'-FAM-CGGAGACAGCGACGAAGAGCTCATC-TAMRA-3').

3.1.5 HIV p24 release from drug post-treated cells after infection by ELISA

CEM-SS cells were infected with HIV single-cycle virus for 2 hours, then cells were washed with serum free RPMI medium to remove unbound virus and cultured for another 10 hours. At 12 hours-post infection, cells were collected and washed for another 3 times to remove possibly released virus, then resuspended cells in RPMI plus 10% heat inactivated fetal bovine serum (FBS) and added drug at a final working dosage.

Samples were cultured at 37°C and culture supernatants were collected between 12hours to 72 hours post-infection, pelleted and resuspended in lysis buffer for quantification of p24 by ELISA.

3.1.6 Inhibition of NSC377384 on intracellular viral protein synthesis and virion particles release by Western blot

The HIV Single cycle virus was used to infect CEM-SS cells for 2 hours. Cells were washed after 2 hours to remove extra virus and cultured for another 10 hours. At 12 hours-post infection, cells were collected and washed for another 3 times to remove possibly released virus, then resuspended cells and added NSC377384 with a final concentration at 25uM. After cultured for another 10 hours, protein samples from both cells and medium were collected at different time point for western blot. 1×10^6 cells were harvested by 1200rpm, 5min, then were lysed in NuPAGE LDS Sample Buffer (Invitrogen). The released virion particle or proteins were collected by 14000g for 2 hours at 4°C, then were resuspended in NuPAGE LDS Sample Buffer (Invitrogen). All

samples were sonicated for 4x5 seconds at 35% Amplitude with a 5 second break in between each sonication (each sample was actually sonicated 6 times because of the machine program). Samples were placed in a heating block for 10minutes at 85°C and centrifuged at 13,000 rpm for 10minutes before loading, then separated by SDS-PAGE (NuPage 4-12% Bis-Tris Gel) at 150 V for 1 hour. The separated proteins were transferred onto nitrocellulose membranes (Invitrogen) at 30 V for 2 hours on ice. The membrane was washed in TBS-T three times for 5 minutes each and blocked for 30 minutes at room temperature using 2.5% skim milk (BD Biosciences) in TBS-T. Primary antibodies targeting Total HIV (HIV immunoglobulin) and GAPDH were from NIH AIDS Reagent Program and Abcam, respectively. The membrane was incubated with 1:2000 diluted HIV immunoglobulin and 1:1000 diluted GAPDH antibody (diluted in 2.5% skim milk in TBS-T) overnight at 4°C. The membrane was washed three times for 15 minutes each and then incubated with secondary antibodies for 1 hour (1:5000 dilution of Goat anti Human Dylight 800 and 1:7500 Rabbit anti Goat antibody DyLight 680 in 2.5% BSA/TBS-T). The membrane was washed twice for 15 minutes each in TBS-T, then once for 15 minutes in PBS. Images were acquired using infrared imaging (Odyssey infrared imager, Li-cor Biosciences). IR-dye conjugated stains were imaged on an ODYSSEY imaging system (Li-Cor Biosciences).

3.2 Results

3.2.1 IC50 Assay of NSC377384

An IC50 Assay was first performed to determine the half maximal inhibitory concentration of NSC377384. Nine drug concentration gradients were used to do the test. Start from 25 μ M, did a 1:2 dilution to 100nM. CloneX cells was pretreated with DMSO or different dosage of NSC377384 for 1 hour, and then infected with HIV wild type NL4-3 virus for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. After 48 hours, cells were analyzed for luciferase expression by Luminescent assay. In the meanwhile, run each samples by Flow-cytometry to detect cell viability.

Based on the inhibition curve and the calculation formula (Figure 2.1, left), IC50 of NSC377384 is 18.4 μ M. All the drug concentrations used in the test were save to cells since the cell viability in each sample are over 90% (Figure 2.1,right).

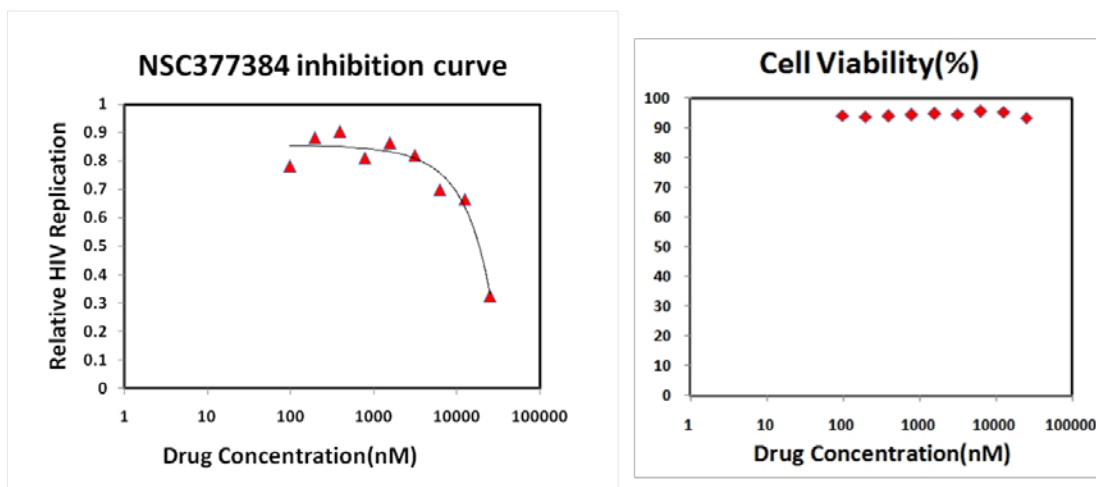


Figure 2.1. IC50 assay of NSC377384. CloneX cells was pretreated with DMSO or different dosage of NSC377384 for 1 hour, and then infected with HIV wild type NL4-3 virus for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. After 48 hours, luciferase expression were analyzed by Luminescent assay to reflect the degree of HIV replication. In the meanwhile, run each samples by Flow-cytometry to detect cell viability.

(Original data can be found in Notebook Page 126.)

3.2.2 Inhibition of NSC377384 on HIV Replication at Different Time Point

A Time point drug additional test was performed to preliminarily deduce at which stage would the drug target and inhibit HIV. Drug was added to 7 groups of CloneX at different time point independently- 12hours, 6 hours, and 1 hours before infection, the time of infection, 2 hours, 6 hours, and 12 hours after infection. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours in spinoculation [54], and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The GFP-expression percentage then was detected by Flow cytometry at 48 hours post infection.

The result showed NSC377384 inhibits HIV replication at all the time points in the experiment. Whenever the drug was added between 12 hours before infection and 12 hours after infection, the inhibition can be observed. This indicates the drug may works on the very late stage of HIV replication cycle because even when it was added at 12 hours after infection, it still had 91% inhibition (Figure 2.2).

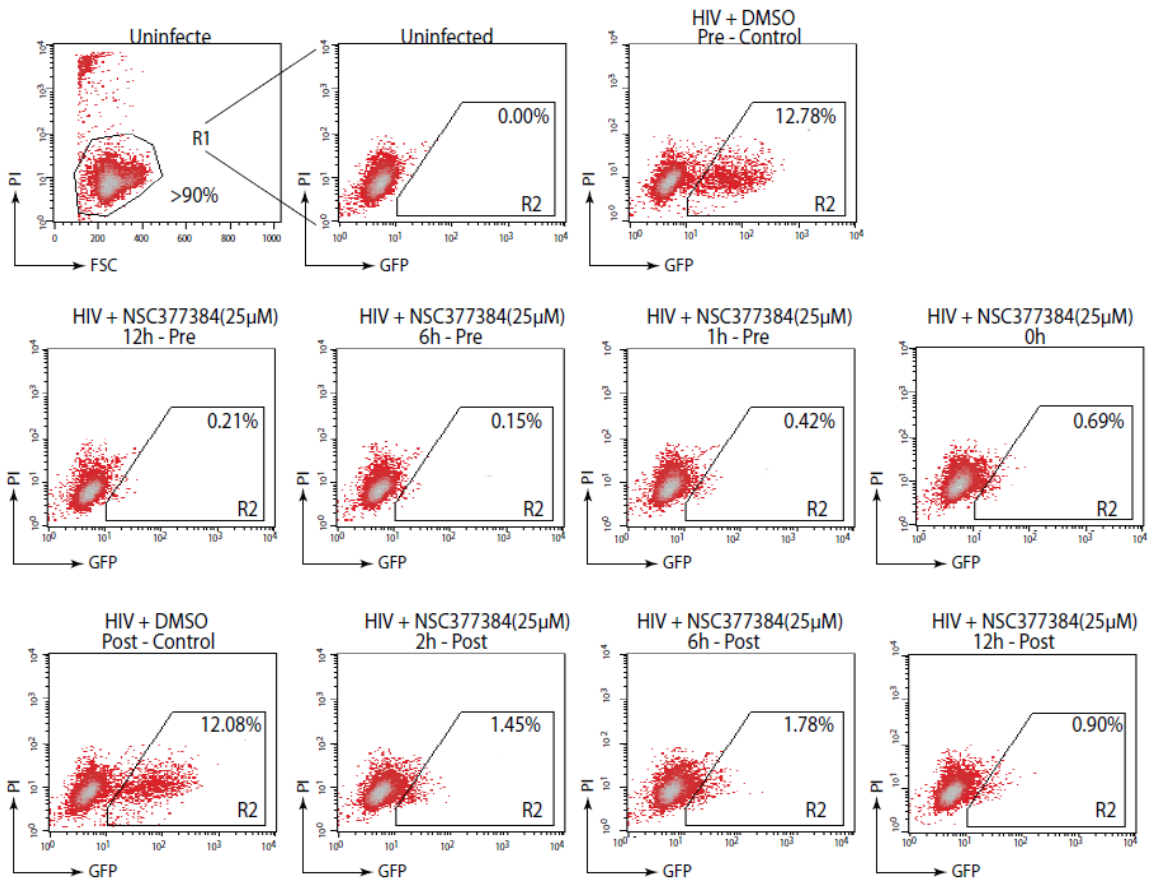


Figure 2.2. Inhibition of NSC377384 on HIV- dependent GFP expression at different time point. NSC377384 was added to 7 groups of CloneX cells at indicated time point independently. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours in spinoculation [54], and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The GFP-expression percentage was detected by Flow cytometry at 48 hours post infection.

(Original data can be found in Notebook Page35.)

Besides GFP expression, HIV-mediated luciferase activity in each cell sample was tested. The luciferase data will be more quantitative in measuring inhibition of HIV, because the luciferase activity reflects the overall viral activity. On the other hand, GFP-expression cell percentage will only reflect the relative numbers of cells infected. These two reporter systems complement each other, as luciferase data, although more quantitative, cannot easily measure the number of HIV⁺ cells, and possible drug cytotoxicity [60].

Similar results are observed in luciferase expression. The luciferase activity was decreased in both drug pre- and post- treated groups when compared to DMSO treatment controls (black bars) (Figure 2.3). In some groups, the luciferase expressions are even lower than the negative control (the first bar), suggesting that this drug may slow down the cell growth rate so we have less cells and weaker background in drug treated samples.

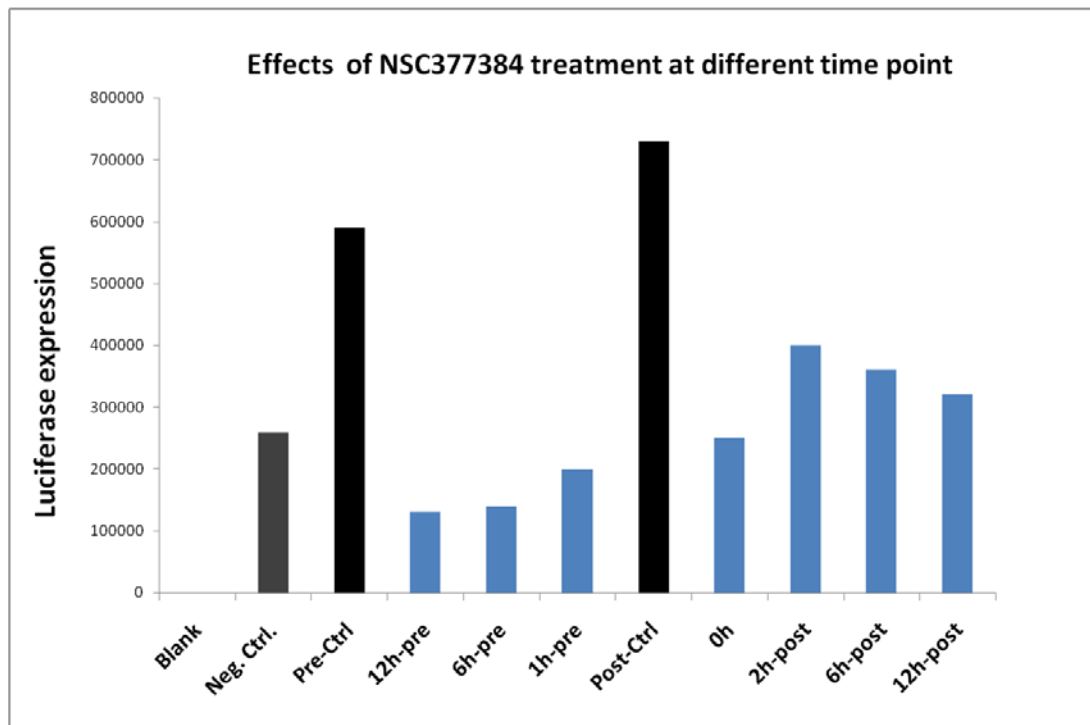


Figure 2.3. Inhibition of NSC377384 on HIV- dependent luciferase activity at different time point. NSC377384 was added to 7 groups of CloneX cells at indicated time point independently. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours at 37°C, and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The degree of luciferase expression was detected by Luminescent Assay at 48 hours post infection.

(Original data can be found in Notebook Page30-34.)

3.2.3 Effects of NSC377384 on HIV Total DNA Synthesis

The viral total DNA synthesis was then tested with drug treatment by Real-Time PCR in comparison to DMSO treatment as control. The primers and probe used for PCR were directed to HIV cDNA, and provide a measure of the degree of reverse transcription. CEM-SS cells were infected with HIV single cycle virus, and treated with drug or DMSO 1h before infection plus add-back after infection. DNA samples in cell lysates from several time points were taken for qPCR analysis.

The results shown in Figure 2.4 were derived from three independent assays by averaging the obtained DNA copy numbers. There was no inhibition in DNA synthesis between drug NSC377384- and DMSO- treated samples from 12 hours to 48 hours post-infection (Figure 2.4). At earlier time points 2 hours and 6 hours post-infection, there was some decrease of DNA synthesis, probably due to the delay of reverse transcription by drug treatment, but this was not sufficient to explain the inhibition of the drug on viral replication. Overall, the drug doesn't inhibit viral total DNA synthesis.

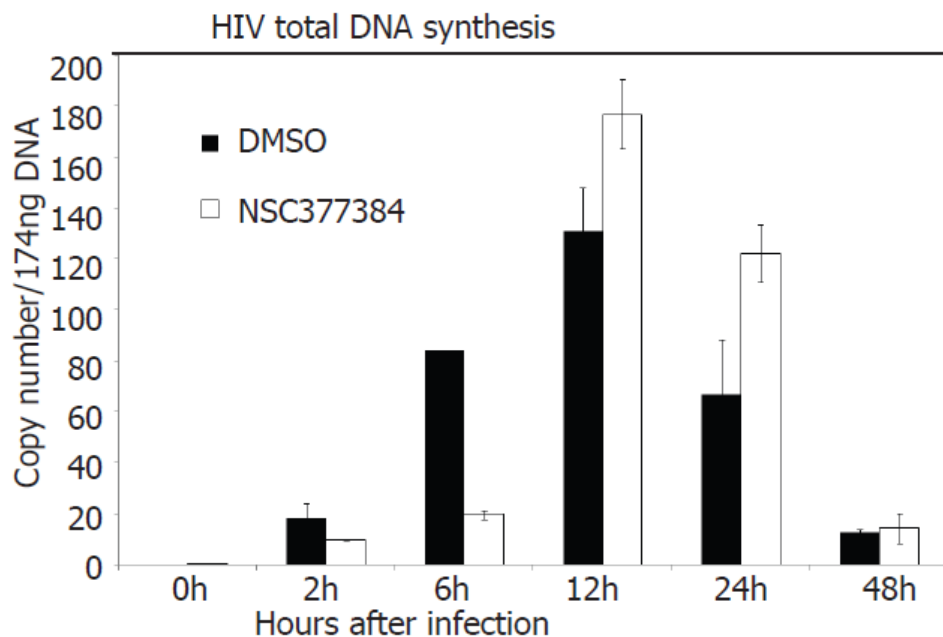


Figure 2.4. Effects of NSC377384 on HIV total DNA synthesis. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC377384 were added 1h before infection and added-back after infection. DNA samples in cell lysates were harvested at indicated time points and purified for PCR quantification of viral DNA.

(Original data can be found in Notebook Page44-46.)

3.2.4 Effects of NSC377384 on HIV2-LTR circles Synthesis

The synthesis of HIV 2-LTR circles was quantified by qPCR. 2-LTR circles are nonintegrated form of viral cDNA, which only form in the nucleus. Therefore the quantity of 2-LTR circles is directly proportional to the amount of viral DNA imported into the cell nucleus [61] [62]. No inhibition of 2-LTR synthesis in NSC377384 treated samples are observed (Figure 2.5), suggesting that the drug doesn't inhibit nucleus migration of viral DNA and 2-LTR circles forming.

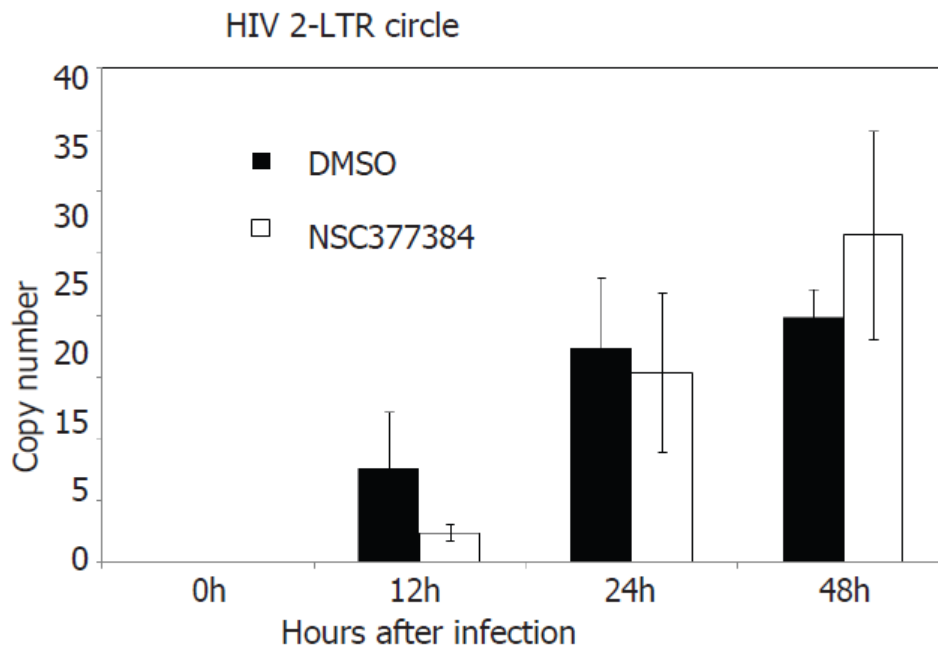


Figure 2.5. Effects of NSC377384 on HIV 2-LTR circles synthesis. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC377384 were added 1h

before infection and added-back after infection. DNA samples in cell lysates were harvested at indicated time points and purified for PCR quantification of viral 2-LTR circles.

(Original data can be found in Notebook Page 73-74.)

3.2.5 Effects of NSC377384 on HIV *nef* Transcription

Viral transcription level was tested by measuring *nef* mRNA transcripts. Extracted RNA from cell lysate was first reverse transcribed to cDNA by RT-PCR, and then the cDNA was used for qPCR of viral *nef* transcripts. *nef* transcripts were fully-spliced transcription that appear early in viral infection and can be used to quantify HIV proviral transcription [63]. The results show no inhibition of transcription at 24 hours post-infection (Figure 2.6). At 48 hours, there was a slightly decrease of *nef* transcripts but that is not statistically significant.

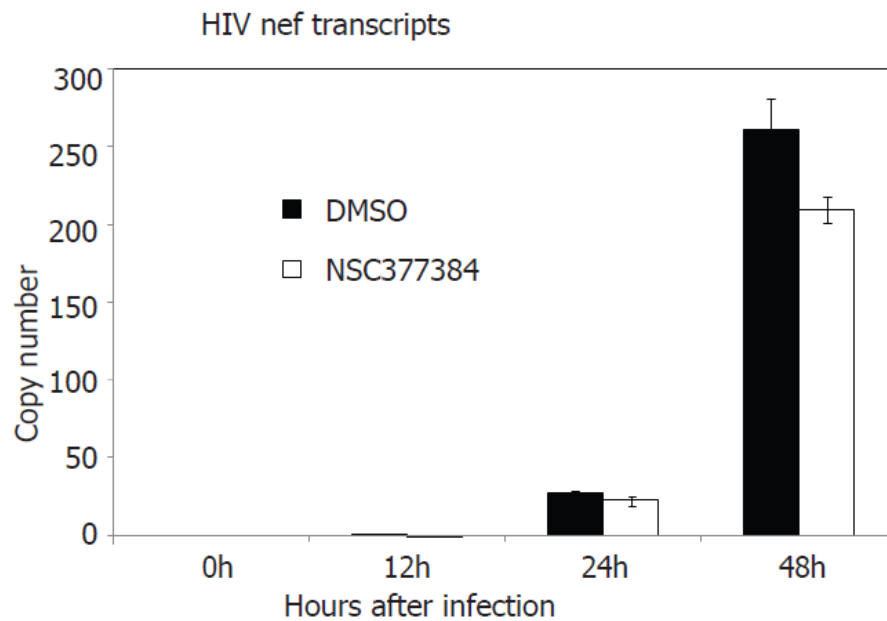


Figure 2.6. Effects of NSC377384 on HIV *nef* transcription. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC377384 were added 1h before infection and added-back after infection. RNA samples in cell lysates were harvested at indicated time points and purified using SV Total RNA Isolation System (Promega). Extracted RNA was first reverse transcribed to cDNA by RT-PCR, and then the cDNA was used for qPCR of viral *nef* transcripts.

(Original data can be found in Notebook Page 75-76.)

3.2.6 Effects of NSC377384 on HIV p24 Release

PCR results in Figures 2.4, 2.5 and 2.6 show that NSC377384 doesn't inhibit HIV total DNA synthesis, 2-LTR circular DNA synthesis or viral *nef* transcription. From the time point drug additional tests in Section 3.2.2, NSC377384 greatly inhibits HIV replication even when it was added at 12 hours after infection, suggesting that it probably targets on the late step(s) of HIV life cycle. In order to confirm this, HIV single cycle virus was used to infect CEM-SS cells and test virus release after drug post-treatment. HIV single cycle virus is only valid for one round infection and it is easy to determine which step of HIV life cycle that NSC377384 can inhibit. By testing HIV replication with drug added at 12 hours post-infection, whether NSC377384 inhibits the late step(s) of HIV infection can be determined. HIV p24 samples at different time points were harvested from culture supernatants and quantified by ELISA.

The expected result was observed: NSC377384 decreases viral p24 release even when it was added 12 hours after infection (Figure 2.7). This confirmed that this drug targets on the late step(s) of HIV life cycle.

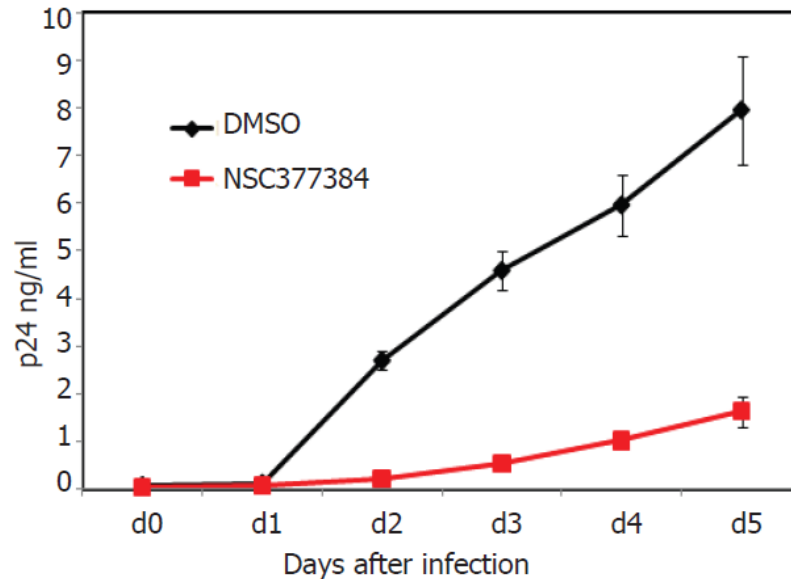


Figure 2.7 Effects of NSC377384 on HIV p24 release. CEM-SS cells were infected with HIV single-cycle virus for 2 hours, then cells were washed to remove unbound virus and cultured for another 10 hours. At 12 hours-post infection, cells were collected and washed for another 3 times to remove possibly released virus, then resuspended cells and added drug at a final working dosage. HIV p24 samples at indicated time points were harvested from culture supernatants and quantified by ELISA. Results show the release of HIV p24 from infected CEM-SS decreases when NSC377384 was added 12hours after infection.

(Original data can be found in Notebook Page 103-104.)

3.2.7 Effects of NSC377384 on Intracellular Viral Protein Synthesis

It has been confirmed that, from the above steps, NSC377384 greatly inhibits HIV replication by targeting on the late step(s) of HIV life cycle. And also, PCR results have showed NSC377384 doesn't inhibit HIV total DNA synthesis, 2-LTR circular DNA synthesis and viral nef transcription. Thus, the effects of NSC377384 on viral protein synthesis in infected cells are tested in order to find out whether it inhibits viral protein translation. Infected cells were treated with the drug at 12 hours post-infection and harvested for western blot. Human anti-HIV antisera was used to detect total HIV protein. The blots image showed a general diminished viral protein synthesis in drug treated samples compare to DMSO control. Viral p24 and a similar size as the p24 precursor, Pr55gag, were clearly detected (Figure 2.8.A). Quantifications of normalized Pr55 and p24 expression revealed around 21% (Figure 2.8.B) and 37% (Figure 2.8.C) decrease of viral protein synthesis in infected cells by drug treatment.

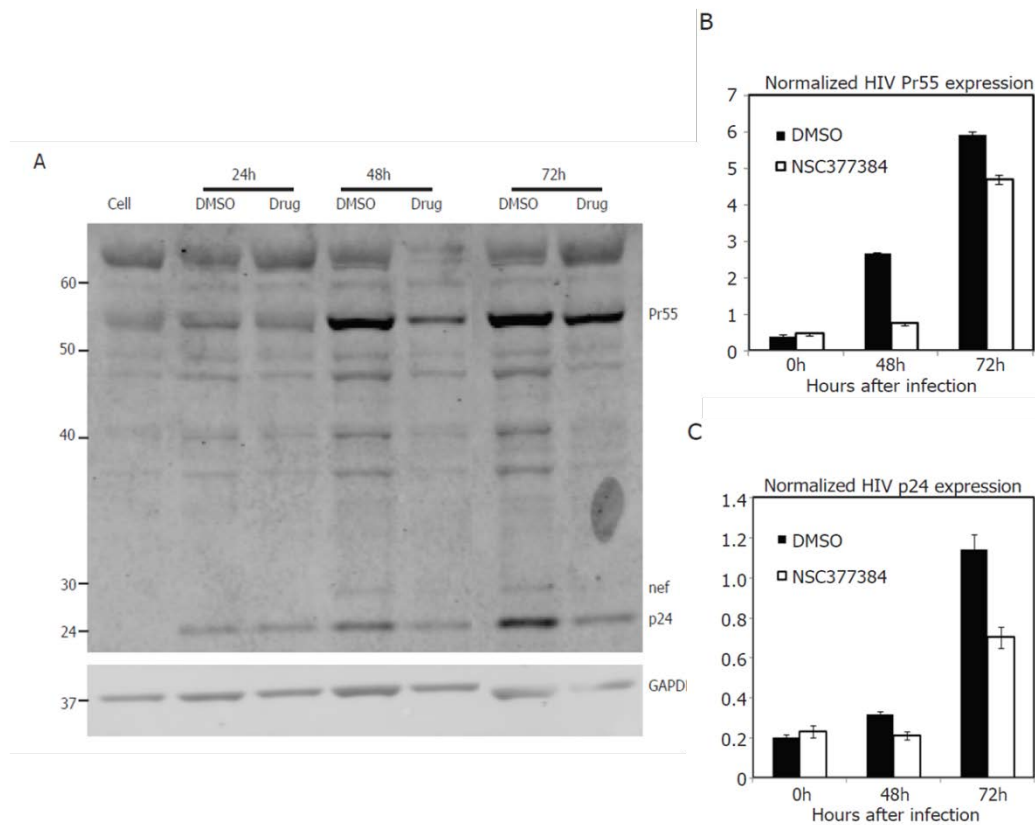


Figure 2.8 Effects of NSC377384 on intracellular viral protein synthesis. CEM-SS cells were infected with HIV single-cycle virus for 2 hours, then cells were washed to remove unbound virus and cultured for another 10 hours. At 12 hours-post infection, cells were collected and washed for another 3 times to remove possibly released virus, then resuspended cells and added NSC377384 with a final concentration at 25uM. Cell samples were harvested at indicated time point and lysed in NuPAGE LDS Sample Buffer for western blot.

(Original data can be found in Notebook Page 97.)

3.2.8 Effects of NSC377384 on Virion particle Assembly and Release

The effect of NSC377384 on virion release from infected cell are also tested. The released virion particle or proteins were collected by 14000g for 2 hours at 4°C, then were resuspended in NuPAGE LDS Sample Buffer for western blot. Human anti-HIV antisera was used to detect total HIV protein. Decreases of detected protein were observed in drug treated samples (Figure 2.9.A). Quantification of HIV proteins Pr55 and p24 revealed around 60% (Figure 2.9.B) and 86% (Figure 2.9.C) decrease of released viral protein in the culture supernatant of drug treated samples.

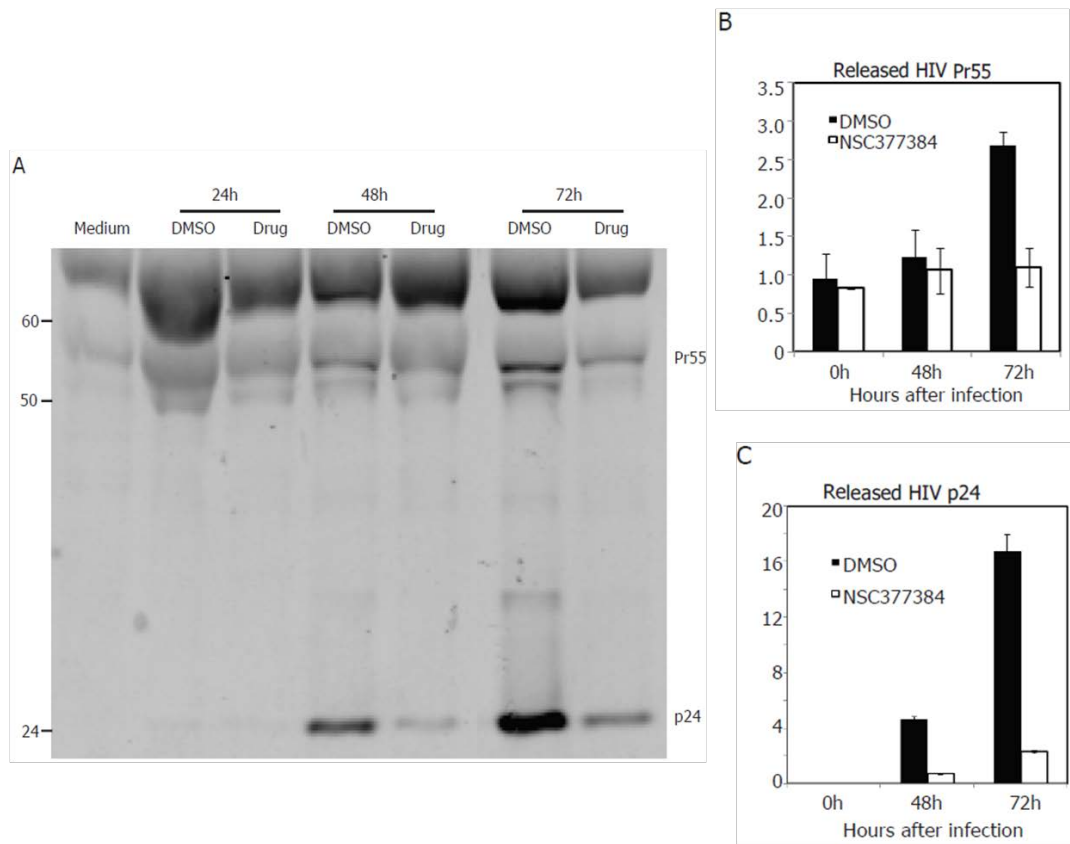


Figure 2.9 Effects of NSC377384 on virion particle assembly and release. CEM-SS cells were infected with HIV single-cycle virus for 2 hours, then cells were washed to remove unbound virus and cultured for another 10 hours. At 12 hours-post infection, cells were collected and washed for another 3 times to remove possibly released virus, then resuspended cells and added NSC377384 with a final concentration at 25uM. The released virion particle or proteins were collected by 14000g for 2 hours at 4°C, then were resuspended in NuPAGE LDS Sample Buffer for western blot. Human anti-HIV antisera was used to detect total HIV protein.

(Original data can be found in Notebook Page 97.)

3.3 Discussion

In this chapter, the impacts of HIV inhibitor NSC377384 on viral replication cycle have been tested step by step. First of all, the results of time point drug additional test showed NSC377384 inhibits HIV replication whenever it was added between 12 hours before infection and 12 hours after infection (Figure 2.2 & 2.3), indicating the drug may work on the very late stage of HIV replication cycle because even when it was added at 12 hours after infection, it still had 91% inhibition. Next qPCR results showed, the viral total DNA synthesis, which reflects the degree of HIV reverse transcription, is not inhibited by NSC377384 (Figure 2.4). Similarly, no inhibition is observed in the synthesis of HIV 2-LTR circles (Figure 2.5), which is directly proportional to the amount of viral DNA imported into the cell nucleus. NSC377384 doesn't inhibit HIV proviral transcription either (Figure 2.6), which is measured by *nef* mRNA transcripts. Also, around 21% -37% reduced viral protein expression has been observed in drug treated cells (Figure 2.8), revealing that NSC377384 has some inhibition on viral protein translation. Furthermore, around 60% -86% reduced viral protein released in the culture supernatant have been observed (Figure 2.9), revealing that NSC377384 also inhibits viral assembly or budding, causing a decrease in viral particle release. All the step-by-step test results are just consistent with the very first time point drug additional data that NSC377384 inhibits very late steps of viral replication, and no inhibitions are observed on viral DNA synthesis or transcriptions that relate early steps.

In conclusion, we found the drug NSC377384 inhibits HIV replication by slightly inhibits viral protein translation (21% -37%) and mainly inhibits viral assembly and budding (60% -86%), leading to a decrease of viral particle release.

Further tests can be performed to find the exact targets of NSC377384, determining whether it inhibits directly to HIV particles or it targets on cellular factors which are necessary for HIV replication.

4. MECHANISM STUDY OF NSC44556

4.1 Materials and Methods

4.1.1 Initiation of cell culture

There are five steps to initiate cell culture: (1) Take out one vial of cell stock (CEM-SS or CloneX) from liquid nitrogen tank, and quickly warm up cells by holding in hand or put in 37°C water bath. (2) After the cell thaw, add the cells drop by drop into 6 ml RPMI fresh medium in a 15ml tube, mix gently after each addition. (3) Collect the cells by centrifuge at 1000rpm for 5 minutes. (4) Resuspend the cell pellets with 15ml warmed RPMI plus 10% heat inactivated fetal bovine serum (FBS) medium, and plate out the cells in the T75 flask. (5) Observe cell growth and do not let cell overgrown to above 1×10^6 /ml.

4.1.2 Virus Preparation and Infection of HIV indicator T cell line

Virus stocks of the HIV-1_{NL4-3} were prepared by transfection using Lipofectamine 2000 (Invitrogen) of HEK 293 T cells with cloned proviral DNA. Supernatant was harvested between 48 to 72 hours, and filtered through a 0.45 µm nitrocellulose membrane. Virus titer (TCID₅₀) was measured by quantifying p24 release from culture

supernatant using an in-house ELISA Kit. For HIV infection and treatment, cells were suspended at a concentration of 2×10^5 /100 μ l, and 100-200 ng(p24) of HIV-1 was added for 2 hours at 37°C. In some experiments, the infected cells were cultured in centrifuge at a speed of 600g for 2 hours at room temperature to get higher infection rate [54]. The cells were then washed twice with serum free RPMI to remove unbound virus, resuspended in RPMI plus 10% heat inactivated fetal bovine serum (FBS) and incubated at 37°C. At 48 hours post-infection, cells were analyzed for GFP expression by Flow-cytometry (FACS Calibur, BD Biosciences), which measures the degree of HIV replication. To exclude drug cytotoxicity, propidium iodide (PI) (2 μ g/ml, Fluka) was added into the cell suspension prior to flow cytometry, and only viable cells (PI negative) were used for measuring GFP expression.

4.1.3 Luciferase activity of infected cells by Luminescent assay

Luciferase assay kit (Cat. #E4030) was purchased from Promega. Luciferase assay reagent was prepared and stored in dark. Infected indicator cells were pelleted at 12000rpm for 5 minutes, then resuspended in 1x luciferase assay lysis buffer, and added into the luciferase reading plate. Luminometer machine was primed with luciferase assay reagent and performed with reading progress according to the operation.

4.1.4 Real Time PCR for Viral DNA synthesis, 2-LTR circles synthesis and *nef* transcripts

The HIV Single cycle virus was used to infect CEM-SS cells for two hours. Drugs were added 1h before infection and added-back after infection. DNA and RNA samples in cell lysates were harvested at different time points, and purified using SV Total RNA Isolation System (Promega).

Quantitative real-time PCR analyses of viral DNA was carried out by using Bio-Rad iQ5 real-time PCR detection system as described previously [60]. The forward primer, 5'LTR-U5 (5'- AGATCCCTCAGACCCTTTTAGTCA-3'), the reverse primer, 3' gag (5'- TTCGCTTTC AAGTCCCTGTTC-3'), the probe, FAM-U5/gag (5'- (FAM) – TGTGGAAAATCTCTAGCAGTGGCGCC - (BHQ) - 3') were used for HIV total DNA analyses [56]. And the forward primer, MH536 (5'- TCCACAGATCAAGGATATCTTGTC-3'), the reverse primer MH535 (5'- AACTAGGGAACCCACTGCTTAAG-3') and the probe, MH603 (5'- 56FAMACACTACTTGAAGCACTCAAGGCAAGCTTT-36TAM-3') were used for viral 2-LTR circles [57] [58] [59]. The DNA standard used for viral total DNA quantification was pNL4-3, and the standard for 2-LTR circle was constructed by using a plasmid containing a complete 2 LTR region (pLTR-2C, cloned by amplification of infected cells with 5'-TGGGTTTTCCAGTCACACCTCAG-3' and 5'- GATTA ACTGCGAATCGTTCTAGC- 3'). Standards are ranging from 1 to 10⁶ copies of pNL4-3 or pLTR-2C mixed with DNA from uninfected cells.

For real-time PCR quantification of the *nef* transcripts, total RNA was reverse transcribed into cDNA using random decamers and M-MLV reverse transcriptase (Ambion) [55]. *Nef* cDNA was further quantified by real time PCR using primers 5'

Nef(5'-GGCGGCGACTGGAAGAA-3'), 3' Rev (5'-AGGTGGGTTGCTTTGATAGAGAAG-3'), and the probe Nef/Rev (5'-FAM-CGGAGACAGCGACGAAGAGCTCATC-TAMRA-3').

4.1.5 Cell-free PCR-based RT Assay

As a template, RNA isolated from transfected HeLa cells (transfected with turboGFP plasmid DNA) was used. Total RNA was extracted using SV total RNA Isolation System (Promega). Purified HIV reverse transcriptase was ordered from NIH (Catalog#3555), provided with 150ng Total RNA template, and a RT reaction mixture containing 5 μ M random decamers, 1x RT buffer, 0.5mM dNTP and 0.5U/ μ L RNase inhibitor (all from Ambion RT kit). In the meanwhile, treated the reverse transcriptase with either DMSO control or NSC44556 for 10 minutes on ice. As a positive control, a known HIV RT inhibitor- Nevirapine (NVP, NIH reagent, Catalog#4666) was used at a working dosage of 1 μ M. DMSO Controls are diluted exactly the same as each drug sample.

The RT reaction was performed at 37°C for 1 hr, then 92°C for 10min for heat inactivation. After this, cDNA was used for quantitative real-time PCR analyses of turboGFP DNA. The forward primer, turboGFP-sense (5'-CAAGATGAAGAGCACCAAAGGC -3'), the reverse primer, turboGFP-anti sense (5'-TGTTGATGGCGTGCAGGAAG - 3'), and the probe, Probe160 -TurboGFP (5'-FAM-TTCAGCCCCTACCTGCTGAGCCACG-3BHQ_1-3') were used for turboGFP DNA analyses.

In this Assay, a control group for HIV RTase was also set to determine whether the drug is specific to HIV RTase. A commercial M-MLV RT (Ambion, AM2044) was used to perform the RT reaction of control group.

4.2 Results

4.2.1 IC50 Assay of NSC44556

An IC50 Assay was first performed to determine the half maximal inhibitory concentration of NSC44556. Ten drug concentration gradients were used to do the test. Start from 50 μ M, did a 1:2 dilution to 100nM. CloneX cells was pretreated with DMSO or different dosage of NSC44556 for 1 hour, and then infected with HIV wild type NL4-3 virus for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. After 48 hours, cells were analyzed for luciferase expression by Luminescent assay. In the meanwhile, run each samples by Flow-cytometry to detect cell viability.

Based on the inhibition curve and the calculation formula (Figure 3.1, left), IC50 of NSC44556 is 5.2 μ M. All the drug concentrations used in the test were save to cells since the cell viability in each sample are over 90% (Figure 3.1,right).

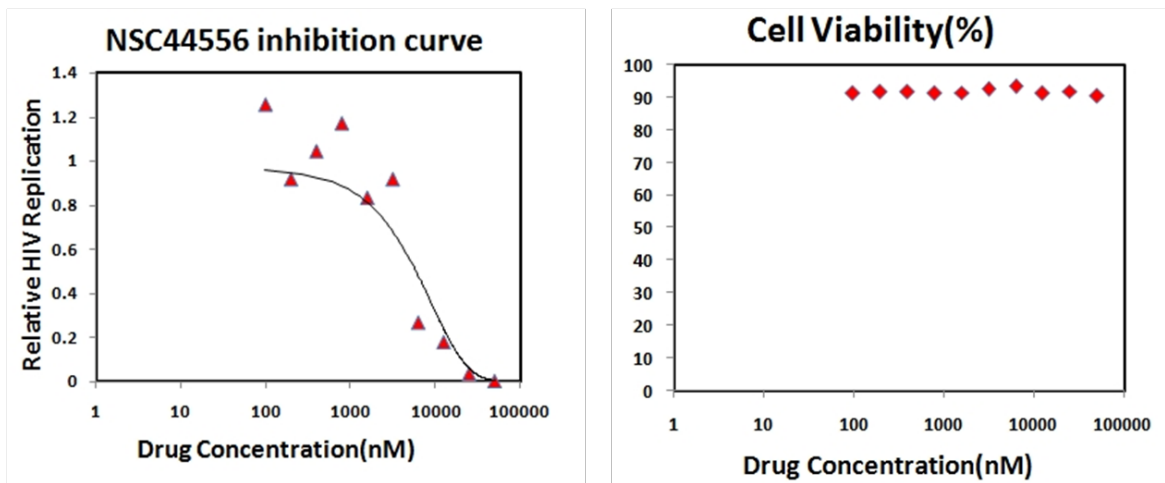


Figure 3.1. IC50 assay of NSC44556. CloneX cells was pretreated with DMSO or different dosage of NSC44556 for 1 hour, and then infected with HIV wild type NL4-3 virus for 2 hours at 37°C. Cells were washed, and the drugs were added back after infection. After 48 hours, luciferase expression were analyzed by Luminescent assay to reflect the degree of HIV replication. In the meanwhile, run each samples by Flow-cytometry to detect cell viability.

(Original data can be found in Notebook Page 127-128.)

4.2.2 Inhibition of NSC44556 on HIV replication at different time points

A Time point drug additional test was pursued to preliminarily deduce at which stage will the drug target and inhibit HIV. Drug was added to 7 groups of CloneX at different time point independently- 12hours, 6 hours, and 1 hour before infection, the time of infection, 2 hours, 6 hours, and 12 hours after infection. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours in spinoculation [54], and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The GFP-expression percentage then was detected by Flow cytometry at 48 hours post infection.

The results showed, NSC44556, works when we add it before, during, and immediately after infection. Around 99% inhibitions were observed at 12hours, 6 hours, 1 hours pre-treatment time points, as well as at the time of infection point. 93% and 73% inhibition were observed respectively at 2 hours and 6 hours post-treatment groups, and no inhibition showed at 12 hours post-treatment (Figure 3.2).

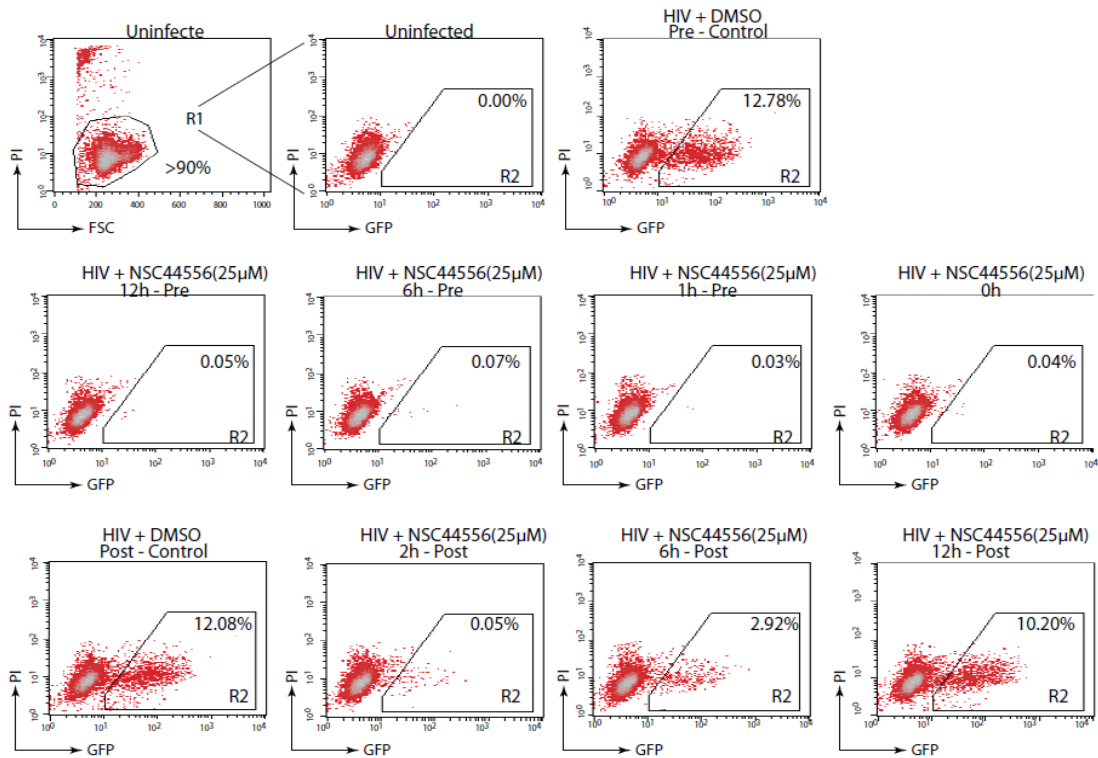


Figure 3.2. Inhibition of NSC44556 on HIV- dependent GFP expression at different time point. NSC44556 was added to 7 groups of CloneX cells at indicated time point independently. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours in spinoculation [54], and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The GFP-expression percentage was detected by Flow cytometry at 48 hours post infection.

(Original data can be found in Notebook Page35.)

Besides GFP expression, HIV-mediated luciferase activity in each cell sample was also tested. The luciferase data will be more quantitative in measuring inhibition of HIV, because the luciferase activity reflects the overall viral activity. On the other hand, GFP-expression cell percentage will only reflect the relative numbers of cells infected. These two reporter systems complement each other, as luciferase data, although more quantitative, cannot easily measure the number of HIV⁺ cells, and possible drug cytotoxicity [60].

Similar results are observed in luciferase expression. The luciferase activity was decreased from 12 hours-pretreated group to 6 hours post-treated group. And no inhibition showed at 12 hours post-treatment (Figure 3.2). In some groups, the luciferase expression are even lower than the negative control (the first bar), suggesting that this drug may also slow down the cell growth rate so we have less cells and weaker background in drug treated samples.

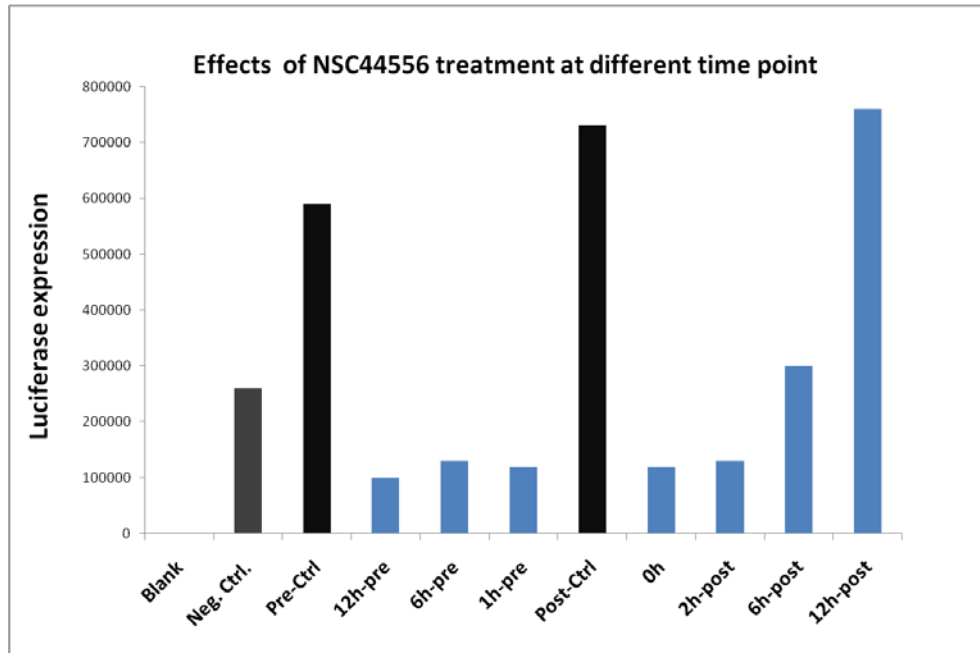


Figure 3.3. Inhibition of NSC44556 on HIV- dependent luciferase activity at different time point. NSC44556 was added to 7 groups of CloneX cells at indicated time point independently. Each groups of CloneX was infected with HIV-1_{NL4-3} for 2 hours at 37°C, and then washed with fresh RPMI medium. The pre -treated groups all had drug added back after wash. The degree of luciferase expression was detected by Luminescent Assay at 48 hours post infection.

(Original data can be found in Notebook Page30-34.)

4.2.3 Effects of NSC44556 on HIV Total DNA Synthesis

The viral total DNA synthesis was tested by Real-Time PCR with drug treatment in comparison with DMSO treatment as control. The primers and probe used for PCR were directed to HIV cDNA and measured the degree of reverse transcription. CEM-SS cells were infected with HIV single cycle virus, and treated with drug or DMSO 1h before infection plus add-back after infection. DNA samples in cell lysates from several time points were taken for qPCR analysis.

The results shown in Figure 3.4 were derived from three independent assays by averaging the obtained DNA copy numbers. Obvious inhibition of DNA synthesis showed in the drug NSC44556 treated samples, and it had roughly 70% fewer copy numbers than DMSO treated control (Figure 3.4).

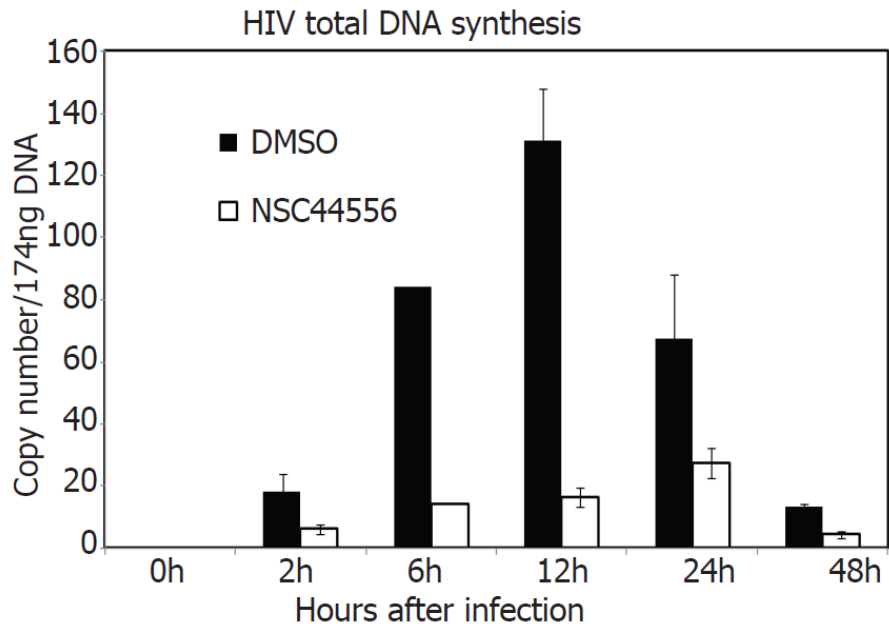


Figure 3.4. Effects of NSC44556 on HIV total DNA synthesis. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC44556 were added 1h before infection and added-back after infection. DNA samples in cell lysates were harvested at indicated time points and purified for PCR quantification of viral DNA.

(Original data can be found in Notebook Page44-46.)

4.2.4 Effects of NSC44556 on HIV 2-LTR circles Synthesis

After the study of NSC44556 on total DNA synthesis, the synthesis of HIV 2-LTR circles was quantified by qPCR. 2-LTR circles are nonintegrated form of viral cDNA which only form in the nucleus. Therefore the quantity of 2-LTR circles is directly proportional to the amount of viral DNA imported into the cell nucleus[61] [62]. The copy numbers of viral 2-LTR circles greatly decreased in NSC44556 treated samples from 6 hours to 24 hours post-infection(Figure 3.5), likely resulting from lower total DNA synthesis in Figure 3.4. There is slight enhancement at 48 hours post infection, could be due to the delay of 2-LTR degradation.

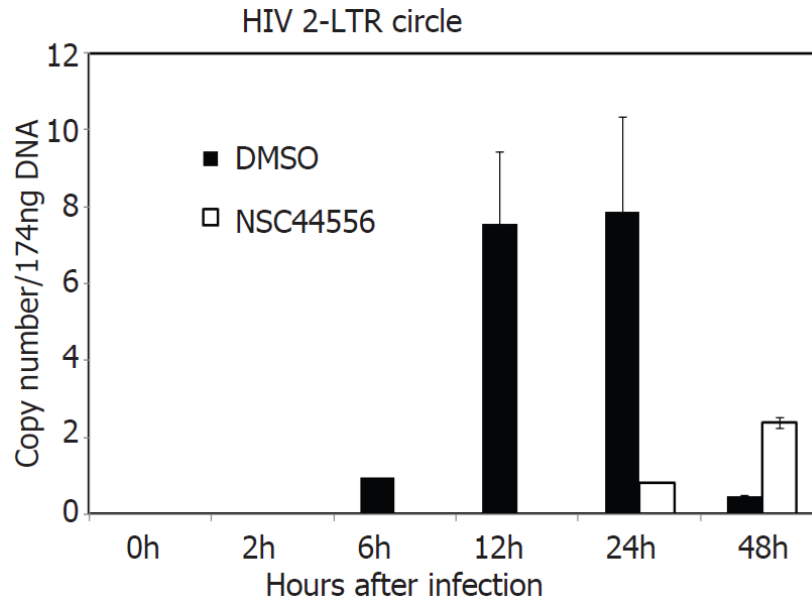


Figure 3.5. Effects of NSC44556 on HIV 2-LTR circles synthesis. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC44556 were added 1h before infection and added-back after infection. DNA samples in cell lysates were harvested at indicated time points and purified for PCR quantification of viral 2-LTR circles.

(Original data can be found in Notebook Page 49-51.)

4.2.5 Effects of NSC44556 on HIV *nef* Transcription

The viral transcription level was tested by measuring *nef* mRNA transcripts. Extracted RNA from cell lysate was first reverse transcribed to cDNA by RT-PCR, then the cDNA was used for qPCR of viral *nef* transcripts. *nef* transcripts were fully-spliced transcription that appear early in viral infection and can be used to quantify HIV proviral transcription [63]. qPCR results showed almost completely inhibited transcripts in NSC44556 treated samples (Figure 3.6). Again, this is most likely attributed to the decrease in HIV DNA synthesis and nuclear migration (Figure 3.4 & 3.5).

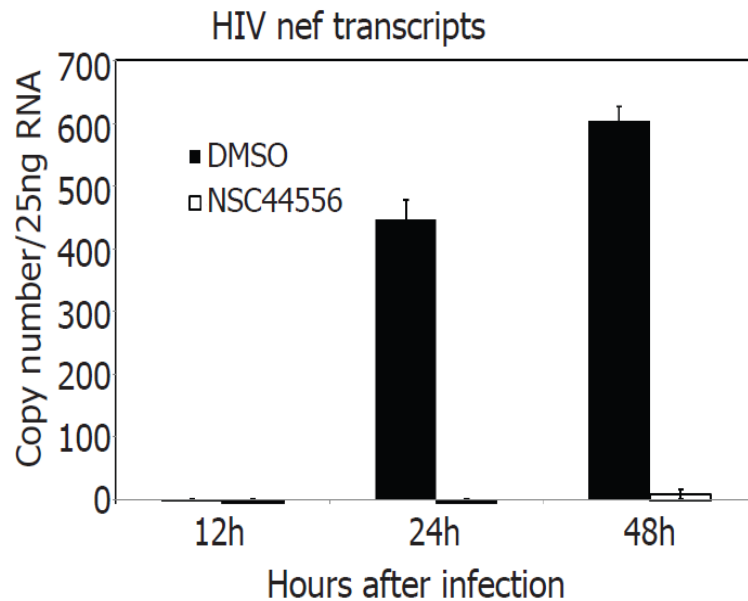


Figure 3.6. Effects of NSC44556 on HIV *nef* transcripts. CEM-SS cells were infected with single-cycle HIV(Env) virus for 2 hours, NSC44556 were added 1h before infection and added-back after infection. RNA samples in cell lysates were harvested at indicated time points and purified using SV Total RNA Isolation System (Promega). Extracted RNA was first reverse transcribed to cDNA by RT-PCR, and then the cDNA was used for qPCR of viral *nef* transcripts.

(Original data can be found in Notebook Page 58.)

4.2.6 Effects of NSC44556 on the Function of HIV Reverse Transcriptase

Based on the results of time point drug additional test (section 4.2.2), NSC44556 shows inhibition on early step(s) of HIV life cycle. It inhibits viral replication when the drug is added before, during, and immediately after infection (2 hours post infection), and it starts losing effects when added after 6 hours post-infection. The next qPCR results shows it inhibits viral total DNA synthesis (Figure 3.4), and following reduced 2-LTR circular DNA synthesis and viral *nef* transcription were also observed (Figure 3.5 & 3.6). It can be inferred that NSC44556 might inhibits viral total DNA synthesis by inhibiting the function of viral reverse transcriptase. To confirm this, a RT assay in vitro (described in section 4.2.6) was designed to determine whether it is a RT inhibitor.

Both purified M-MLV RTase and HIV RTase were treated with DMSO or inhibitors (NVP or NSC44556) and then added to RT reaction, the obtained cDNA was used for quantitative real-time PCR analyses. As a positive control, NVP is a know RT inhibitor specific to HIV RTase. By comparing DNA copy numbers in DMSO- and drug-treated samples, the effects of drugs on reverse transcriptase can be detected. From the results in Figure 3.7.A, neither NVP nor NSC44556 have impacts on the activity of M-MLV RTase, but they all inhibit the activity of HIV RTase (Figure 3.7.B), around 66% inhibition was observed in 25 μ M NSC44556 treated sample and 44% inhibition in 5 μ M NSC44556 treated sample. This indicates NSC44556 likely specifically inhibits the function of HIV reverse transcriptase.

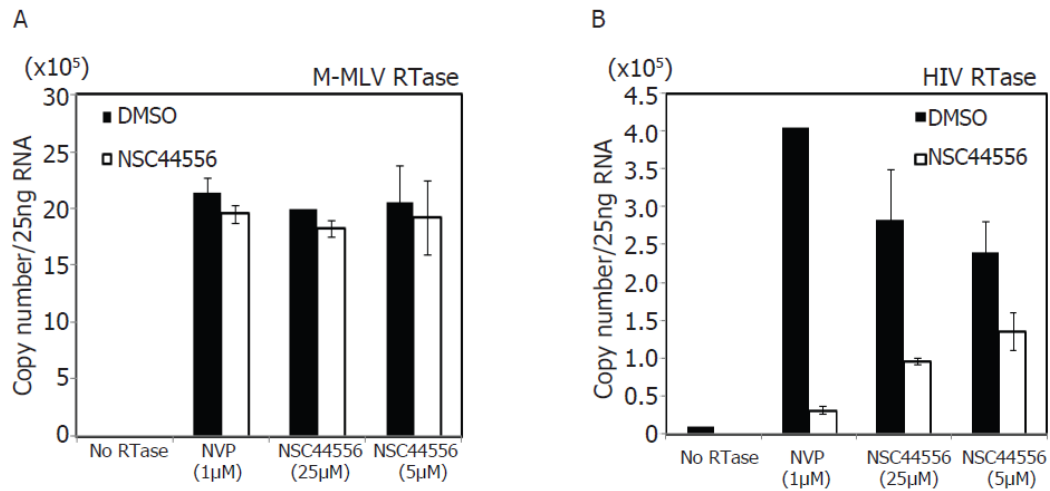


Figure 3.7. Effect of NSC44556 on the function of HIV reverse transcriptase. Total RNA isolated from transfected Hela cells (transfected with turboGFP plasmid DNA) was first reverse transcribed to cDNA by RT reaction using either M-MLV RTase or HIV RTase. M-MLV RTase and HIV RTase were pretreated with DMSO or inhibitors (NVP or NSC44556) for 10 minutes on ice. NVP is a know RT inhibitor specific to HIV RTase and was used as positive control. The obtained cDNA was used for quantitative real-time PCR analyses of turboGFP DNA.

(Original data can be found in Notebook Page 123-125.)

4.3 Discussion

From the results of time point drug additional test (Figure 3.2 & 3.3), NSC44556 inhibits viral replication when the drug is added before, during, and immediately after infection (2 hours post infection), and it starts losing effects when added after 6 hours post-infection, indicating it targets on early step(s) of HIV life cycle. The next qPCR results shows it inhibits viral total DNA synthesis (70% inhibition) (Figure 3.4), which reflect the degree of HIV reverse transcription. This consist with the results of time point drug additional test, because HIV reverse transcription is a early step after virus entering the infected cell. We also observed following reduced 2-LTR circular DNA synthesis and viral *nef* transcription (Figure 3.5 & 3.6), it is most likely resulting from lower total DNA synthesis in Figure 3.4. Based on these results, it can be inferred that NSC44556 might inhibits viral total DNA synthesis by inhibiting the function of viral reverse transcriptase. To confirm this, a RT assay in vitro (described in section 4.2.6) was designed to determine whether it is a RT inhibitor. The expected result was observed that NSC44556 inhibits the activity of HIV RTase (66% inhibition) (Figure 3.7.B). What's more, it showed on inhibition on the activity of M-MLV RTase (Figure 3.7.A), indicating it is a RTase inhibitor specific to HIV.

To conclude, NSC44556 inhibits HIV replication by inhibiting the function of HIV reverse transcriptase, leading to a decrease in viral total DNA synthesis. All results in the research are consistent with each other. NSC44556 inhibits the step of HIV reverse transcription, explained the results of time point drug additional test that it only works when the it is added before, during, and immediately after infection, once the reverse

transcription is done, and then the drug is added, it doesn't inhibit HIV replication any more.

5. CONCLUSION AND FUTURE WORK

This research work has identified eight effective anti-HIV drugs that inhibit HIV replication at non-toxic dosages, among a total of forty compounds from three different research institutes, through testing their impacts on HIV replication by using a HIV indicator T cell line (screening). For example, NSC377384 has great inhibition on HIV replication (99%) at 25 μ M. In order to find out how different drugs may inhibit HIV infection differently, two of the eight identified drugs, NSC377384 and NSC44556, are selected to study the drug action mechanism in detail. The effects of each of them on HIV replication cycle will be tested step by step to find out which steps that the drugs may target and inhibit HIV infection. The tests show that NSC377384 targets the late steps of HIV life cycle and inhibits HIV replication by mainly inhibiting viral assembly and budding, causing a decrease in viral particle release. NSC44556, in contrast, targets the early steps of HIV life cycle, and likely specifically inhibits the function of HIV reverse transcriptase, leading to a decrease in viral DNA synthesis.

The discoveries in this thesis work are helpful to the development of novel anti-HIV drugs that overcome current limitations in the field of HIV therapy.

The future work will be focused on better clarifications of the drug action mechanisms and improvement of drug effectiveness and efficiency. For NSC377384, it has been confirmed that the drug inhibits viral assembly and budding, which are new and

unique targets of anti- HIV inhibitors. Further tests can be performed to find the exact targets of NSC377384, determining whether it inhibits directly to HIV particles or it targets on cellular factors which are necessary for HIV replication. Besides, since all experiments in this research were done on T cell line, we can further confirm these results in primary blood cells.

REFERENCES

1. Nicholas Acheson. *Fundamentals of Molecular Virology*. 2nd Edition, page 355.
2. Gallo RC, et al. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science*. 1983; 220 (4599): 865-867.
3. Montagnier L, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*. 1983; 220 (4599): 868-871.
4. Robert Aldrich, Garry Wotherspoon. Who's who in gay and lesbian history. Page 154.
5. Zhu, J. and W.E. Paul. CD4 T cells: fates, functions, and faults. *Blood*, 2008. 112(5): p. 1557-69.
6. Klatzmann D, et al. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature*, 1984. 312(5996): p. 767-8.
7. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *The Journal of experimental medicine*. 2004. 200:761–70.
8. Daniel Douek. Making sense of HIV pathogenesis. *The PRN notekook*. 2005.
9. Saout CL, Lane HC, Catalfamo M. The Role of Cytokines in the Pathogenesis and treatment of HIV Infection. *Cytokine Growth Factor Rev*. 2012 Aug-Oct; 23(0): 207–214.
10. Garg H, Mohl J, Joshi A. HIV-1 induced bystander apoptosis. *Viruses*. 2012. 4 (11): 3020-43.
11. John Coffin and Ronald Swanstrom, HIV Pathogenesis: Dynamics and Genetics of Viral Populations and Infected Cells. *Cold Spring Harb Perspect Med* 2013.

12. McMichael, A.J., et al. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol*, 2010. 10(1): p. 11-23.
13. Lane HC. Pathogenesis of HIV Infection: Total CD4+ T-Cell Pool, Immune Activation, and Inflammation. *Top HIV Med*. 2010. 18(1): 2-6.
14. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *The Journal of experimental medicine*. 2004; 200:749-59.
15. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *The Journal of experimental medicine*. 2004; 200:761-70.
16. Davis CB, Dikic I, Unutmaz D, Hill CM, Arthos J, Siani MA, Thompson DA, Schlessinger J, Littman DR. Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J Exp Med*. 1997;10:1793-1798.
17. Leslie AJ, et al. HIV evolution: CTL escape mutation and reversion after transmission. *Nature Medicine*. 2004. 10: 282 - 289.
18. Judy Lieberman. Defying death - HIV mutation to evade cytotoxic T lymphocytes. *N Engl J Med*, 2002. 347(15): 1203-1204.
19. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. *J. Pathol*.2008. 214 (2): 231-41.
20. Hazenberg MD, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*, 2003. 17(13): p. 1881-8.
21. Deeks SG, et al. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood*, 2004. 104(4): p. 942-7.
22. Lackner AA, Lederman MM, Rodriguez B. HIV pathogenesis -The host. *Cold Spring HarbPerspect Med* 2011.
23. Bour S, Geleziunas R, Wainberg MA. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. *Microbiol Rev*. 1995, 59:63-93.
24. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism and disease. *Annu Rev Immunol*. 1999, 17:657-700.

25. Chan D, Kim P. HIV entry and its inhibition. *Cell*. 1998. 93 (5): 681-4.
26. Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science*. 1998. 280 (5371): 1884-8.
27. Patel PH, et al. Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase. *Biochemistry*. 1995. 34, 5351-63.
28. St Clair, M. H. et al. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science*. 1991. 253, 1557-9.
29. Freed EO. HIV-1 replication. *Somat Cell Mol Genet*, 2001. 26(1-6): p. 13-33.
30. Pollard VW, Malim MH. The HIV-1 Rev protein. *Annu. Rev. Microbiol.* 1998. 52: 491-532.
31. Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk HD, Garten W; Bosch; Angliker; Shaw; Klenk; Garten. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature*. 1992. 360 (6402): 358-61.
32. Gelderblom HR. *Fine structure of HIV and SIV*. Page 31-44.
33. Spear M, Guo J, Wu Y. Novel Anti-HIV therapeutics targeting chemokine receptors and actin regulatory pathways. *Immunological Reviews*.
34. Mario Stevenson. HIV-1 pathogenesis. *Nature Medicine*. 2003. 9 (7): 853-860.
35. Ho DD and Bieniasz PD. HIV-1 at 25. *Cell*, 2008. 133(4): p. 561-5.
36. Mitsuya H, Weinhold KJ, Furman PA, St Clair MH, Lehrman SN, Gallo RC, et al. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc Natl Acad Sci*. 1985;82:7096-100.
37. U.S Approves Drug to Prolong Lives of AIDS Patients. *New York Times*. 3/21/1987.
38. Moore RD, Chaisson RE. Natural History of Opportunistic Disease in an HIV-Infected Urban Clinical Cohort. *Ann Intern Med*. 1996. 124(7):633-642.

39. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, et al. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*. 1995. 373, 117–122.
40. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. *US Department of Health and Human Services*. 2/12/2013.
41. Ho DD and Bieniasz PD. HIV-1 at 25. *Cell*, 2008. 133(4): p. 561-5.
42. De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents*. 2009. 33(4): p. 307-20.
43. Vandamme AM, Van Vaerenbergh K, De Clercq E. Anti-human immunodeficiency virus drug combination strategies. *Antiviral Chemistry & Chemotherapy*. 1998. 9(3):187-203.
44. Kumari G, Singh RK. Anti-HIV drug development: structural features and limitations of present day drugs and future challenges in the successful HIV/AIDS treatment. *Curr Pharm Des*. 2013. 19(10):1767-83.
45. Mark A. Public Health Implications of Antiretroviral Therapy and HIV Drug Resistance. *JAMA*. 1998. 279(24): 1977-1983.
46. Ohashi K, Nagata K, Maekawa M, Ishizaki T, Narumiya S, Mizuno K. Rho-associated Kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J Biol Chem*. 2000. 275: 3577-82.
47. Vorster PJ, et al. LIM kinase 1 modulates cortical actin and CXCR4 cycling and is activated by HIV-1 to initiate viral infection. *J Biol Chem*. 2011. 286: 12554-64.
48. Xu X, Guo J, Wu Y. Involvement of LIM Kinase 1 in actin polarization in human CD4 T cells. *Communication & Integrative Biology*. 2012.
49. Brenner BG, Wainberg Z. Heat shock proteins: novel therapeutic tools for HIV-infection? *Expert. Opin. Biol. Ther*. 2001. 1:67-77.
50. Creagh EM, Sheehan D, Cotter TG. Heat shock proteins—modulators of apoptosis in tumour cells. *Leukemia*. 2000. 14:1161-1173.
51. PratimaRawat and DebashisMitra. Cellular heat shock factor 1 positively regulates human immunodeficiency virus-1 gene expression and replication by two distinct pathways. *Nucleic Acids Research*. 2011, 1–14.

52. Wu Y, Beddall MH, Marsh JW. Rev-dependent lentiviral expression vector. *Retrovirology*. 2007. 10:12.
53. Wu Y, Beddall MH, Marsh JW. Rev-dependent indicator T cell line. *Curr HIV Res*. 2007. 10:395–403.
54. Guo J, Wang W, Yu D, Wu Y. Spinoculation triggers dynamic actin and cofilin activity facilitating HIV-1 infection of transformed and resting CD4 T cells. *J Virol*. 2011;10:9824–9833.
55. Wu Y and Marsh JW. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science*. 2001. 293(5534):1503-1506.
56. Yoder A, et al. HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. *Cell*. 2008. 134(5):782-792.
57. Kelly J, et al. Human macrophages support persistent transcription from unintegrated HIV-1 DNA. *Virology*. 2008. 372:300-312.
58. Butler SL, Hansen MS, & Bushman FD. A quantitative assay for HIV DNA integration in vivo. *Nat Med*. 2001. 7(5):631-634.
59. Wu Y & Marsh JW. Early transcription from nonintegrated DNA in human immunodeficiency virus infection. *J Virol*. 2003. 77(19):10376-10382.
60. Hawley T, Spear M, Guo J and Wu Y. Inhibition of HIV replication in vitro by clinical immunosuppressants and chemotherapeutic agents. *Cell Biosci*. 2013. 3: 22.
61. Mandal D, Prasad VR. Analysis of 2-LTR circle junctions of viral DNA in infected cells. *Methods Mol Biol*. 2009. 485: 73–85.
62. Richard D Sloan and Mark A Wainberg. The role of unintegrated DNA in HIV infection. *Retrovirology*. 2011. 8:52.
63. Karn J, Stoltzfus CM. Transcriptional and Posttranscriptional Regulation of HIV-1 Gene Expression. *Cold Spring Harb Perspect Med*. 2012. 2: 1–17.

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