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Activity

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Abstract

Title of Thesis: Angiogenin Inhibits Human Immunodeficiency Virus type 1 (HIV-1) Replication in Peripheral Blood Mononuclear Cells by a Complex Mechanism that Includes an Intracellular Component

Ping-Hsin Lin, Master of Science, 2012

Thesis Directed By: Alfredo Garzino-Demo, Ph.D.
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Angiogenin (ANG, also known as ribonuclease 5) is a 14.1kDa polypeptide secreted by T lymphocytes and epithelial cells. Other than its well-characterized function of promoting development of vasculature, ANG also possesses anti- Human Immunodeficiency Virus-1 (HIV-1) properties. Previous findings show that ANG inhibits X4 tropic HIV-1 replication in peripheral blood mononuclear cells (PBMCs). Our studies show that ANG also inhibits R5 tropic virus, the most commonly transmitted HIV-1 strain. Inhibition was detected treating cells prior to or after infection by p24 ELISA, and is not mediated by CD4, CXCR4, or CCR5 as determined by flow cytometry assays. Furthermore, we demonstrated that ANG binds to tRNA-Lys, which serves as primer for HIV-1 reverse transcription. These results indicate that ANG inhibits HIV-1 replication by a post-entry mechanism, which might be exploited to develop new antiretroviral strategies.

Angiogenin Inhibits Human Immunodeficiency Virus type 1 (HIV-1) Replication in Peripheral Blood Mononuclear Cells by a Complex Mechanism that Includes an Intracellular Component

By
Ping-Hsin Lin

Thesis submitted to the faculty of the Graduate School of the
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Master of Science
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List of Abbreviations

ANG	Angiogenin
AIDS	Acquired immunodeficiency disease syndrome
AMLV	Amphotropic murine leukemia virus
APC	Antigen-presenting cells
AZT	Azidothymidine
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DC-SIGN.	Dendritic cell (DC)-specific intercellular adhesion molecule-grabbing nonintegrin
DNA	Deoxyribonucleic acid
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FACS	Fluorescence-activated cell sorting
Gag	Group-specific antigen
gp	Glycoprotein
GPCR	G protein coupled receptor
HIV-1/2	Human Immunodeficiency Virus type 1 and 2
IL	Interleukin
kDa	Kilodalton
K_M	Michaelis constant
Lys	Lysine
MHC I/II	Major histocompatibility complex class I and II
Nef	Negative regulatory factor
nt	Nucleotide
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PHA	Phytohemagglutinin
PK	Proteinase K
Pol	Polymerase
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
Rev	Regulator of virion expression
rh	Recombind human
RNA	Ribonucleic acid

RSV	Respiratory syncytial virus
RT	Reverse transcriptase
SDF	Stromal-derived factor
Tat	Trans-activator of transcription
TBE	Tris/ Borate/ EDTA
TCID	Tissue culture infective dose
tiRNA	tRNA derived, stress induced small RNAs
tRNA	Transfer RNA
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

Chapter1: Background and hypothesis

Introduction

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency disease syndrome (AIDS). [1, 2] Based on the epidemiological estimates from The United Nations Program on HIV/AIDS (UNAIDS), more than 33.3 million people are infected by HIV worldwide nowadays. There is an urgent need to develop new agents for an effective therapeutic strategy for HIV infected patients, since current anti-retroviral reagents do not cure HIV infection, and drug resistance occurs with all retroviral classes of drugs due to the rapid replication rate and the inherent genetic variation of HIV. A previous study from Bedoya *et al.,* and Cocchi *et al*. showed that angiogenin (ANG) has HIV-1 inhibitory activity that likely contributes to limit HIV replication *in vivo*[3, 4]. ANG is a secreted RNase consisting of 123 amino acid residues[5], and is expressed by various cells including vascular endothelial cells, smooth muscle cells, fibroblasts, colonic columnar epithelium, lymphocytes, primary adenocarcinoma cells, and HIV's target cells, lymphocytes[6, 7].

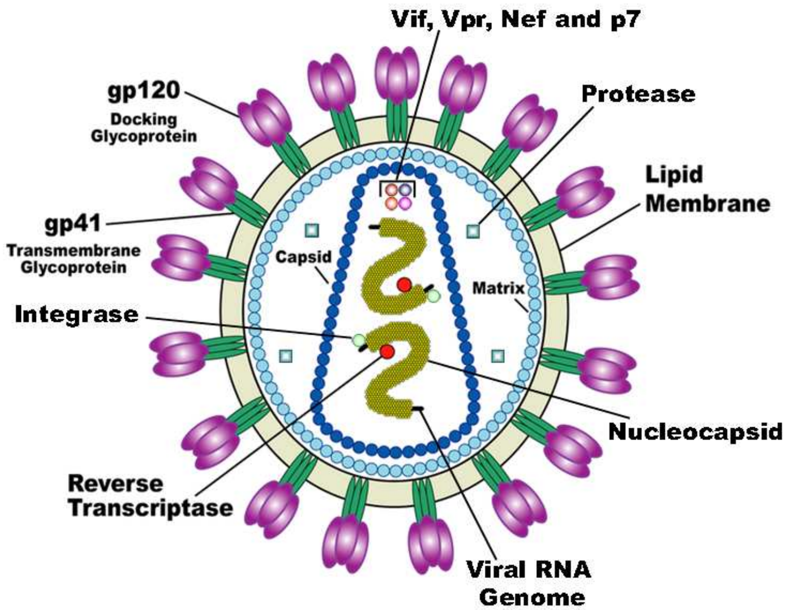
The human RNase superfamily has thirteen members (RNase1-13) including ANG (RNase 5). These human RNases recognize different RNA substrates with different functional patterns. Therefore, they perform distinct physiological functions, including angiogenesis, antitumor, and antiviral activities. The antiviral activity of an RNase, was first reported 55 years ago in a publication showed that a mammalian RNase inhibits influenza virus[8]. In addition, RNase 2 (EDN, eosinophil derived neurotoxin) and RNase 3 (ECP, eosinophil cationic protein), which are mainly secreted in the granules of eosinophils, were shown to inhibit respiratory syncytial virus (RSV) and other paramyxoviruses[9]. EDN was suggested to penetrate the viral capsid and to interact with the viral RNA genome by binding to a specific target molecule on the viral surface[10], and it also exhibits inhibitory activity against human immunodeficiency virus type 1 (HIV-1) replication in a chronically infected cell line[11]. RNase 4 and ANG; (RNase 5) which are the normal components of human plasma were originally isolated from tumor cell conditioned media[5, 12]. In addition, RNase 4 and ANG were recently identified as key component of T cell derived soluble factors that could contribute to control HIV infection *in vivo*[4]. However, ANG is considered to be an atypical human RNase as it is distinguished by its potent angiogenic activity and weak ribonuclease activity despite its structural similarity to other human RNases such as RNase 1, 2, and 4[12].

The mechanism that ANG inhibits HIV-1 replication has not been elucidated yet. The physiological features of ANG, such as receptor-mediated cell uptake[13, 14], intracellular angiogenic activity[15], and tRNA cleavage[16-19], may play an important role or provide clues on how it inhibits HIV. For example, the binding between ANG and its 172kDa putative receptor may down-regulate HIV-1 receptors, CD4, CXCR4, and CCR5. The existence of ANG in both cytoplasm and nucleus may result in inhibition of HIV-1 replication intracellularly. In addition, RNase activity of ANG may inactivate viral RNA and tRNA-Lys during the progression of HIV-1 virion replication. Based on the anti-HIV-1 activity, receptor-mediated cell signaling, and ribonuclease activity, I hypothesize that ANG inhibits both R5 and X4 tropic HIV-1 replication by a complex mechanism that involves the intracellular machinery.

Human Immunodeficiency Virus (HIV)

The first description of retroviruses was reported by Oluf Bang and Vilhelm Ellermann in 1908[20]. Retroviruses are plus-stranded RNA viruses which are able to reverse-transcribe the viral RNA genome into a DNA form called provirus by the virally-encoded enzyme reverse transcriptase. After reverse transcription, the proviral DNA is transported into the nucleus and is integrated into host chromosomes. The viral RNA and protein are then expressed by the host cell transcription and translation apparatus, eventually forming new virions that are packaged and released from host cells. There are seven genera in the retrovirus family: alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, lentivirus, and spumavirus. The structure of virion, retrovirus particle consists of an envelope about 100nm in diameter, and a diploid plus sense single stranded RNA genome each approximately 7-10 kilobases (kb) in length.

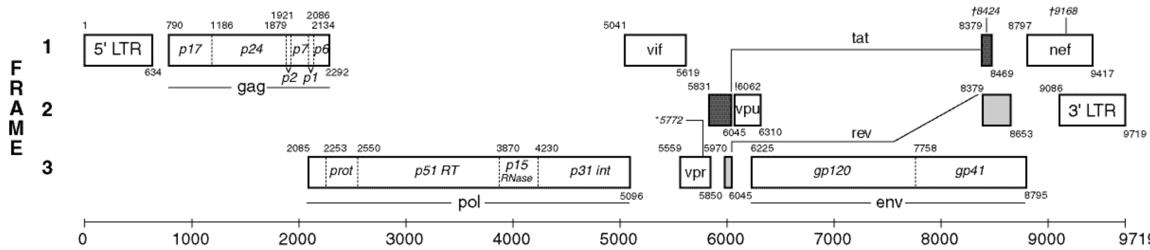
Human immunodeficiency virus (HIV) is a lentivirus which belongs to the retrovirus family[1, 2]. In a HIV particle, diploid single-stranded, positive-sense, enveloped RNA and two enzymes, reverse transcriptase and integrase, are contained in a conical viral capsid. HIV envelope is formed by viral glycoproteins, gp41 and gp120, and a lipid bilayer resulting from viral budding from the cell membrane of host cells. A schematic representation of the HIV virion is depicted in Figure 1.1.



Source: National Institute of Allergy and Infectious Diseases
Figure 1.1 Diagram of HIV virion structure.

HIV has two subtypes: HIV-1 and HIV-2. HIV-1, the predominant virus, was isolated in 1983 and subsequently identified as the causative agent of AIDS worldwide. HIV-2 was isolated in 1986 from AIDS patients in West Africa, where the most cases are found[1, 2, 21]. Both HIV-1 and HIV-2 subtypes are the causative agents of acquired immunodeficiency disease syndrome (AIDS). The strains of HIV-1 can be further classified into four groups: Group M (major), Group O (outlier), Group N (non-M, non-O), and Group P (pending the identification of further human cases) [22-26]. There are more than 90 percent of HIV-1 infections belong to HIV-1 group M which is also subdivided into clades names by letters: A, B, C, D, E, F, G, H, I, J, K. In addition, the viral strains of recombinant clades, such as these resulting from mixing of clades A and B, are classified as circulating recombinant forms (CRFs).

The genome of HIV consists of two identical copies of positive sense single stranded 9.7kb RNA molecules. The RNA has a cap at 5’ end and is polyadenylated at 3’ end, while long terminal repeats (LTRs) at the 5’ and 3’ ends play an essential role of HIV replication. The 5' end includes four regions: R, U5, Primer binding site (PBS), and L. R region is a short repeat sequence at both 3’ and 5’ ends, and it is important to ensure correct end-to-end transfer during the reverse transcription. U5 region is a short sequence between R and PBS. PBS consists of 18 bases complementary to 3’ end of tRNA primer. L region is an untranslated leader region that functions as the signal for packaging of genome RNA. The 3’ end includes three regions: polypurine tract (PPT), U3, and R. PPT is the primer for plus-strand DNA synthesis during the reverse transcription. U3 located in between PPT and R regions is a sequence including transcriptional promoter and regulatory elements involved in the recruitment of RNA polymerase II to the start site of viral RNA synthesis, and regulate the transcription of HIV proviral DNA. R is the terminal repeated sequence at 3' end. In the coding region, there are nine genes including Gag and Env for the viral structural proteins, Pol for the polymerase, Tat and Rev for regulatory proteins, and Vif, Vpu, Vpr, and Nef for the accessory proteins. A schematic representation of the HIV genome is depicted in Figure 1.2.



Source: Los Alamos National Laboratory, HIV Sequence Database.http://www.hiv.lanl.gov/.

Figure 1.2 HIV-1 genome

Gag: Group-specific antigen (Gag) is the genomic region (~1800bp) coding for Gag polyprotein, the p55 myristoylated polyprotein. The Gag protein, is processed by the viral protease (PR), into the matrix protein (MA/p17), capsid protein (CA/p24), nucleocapsid protein (NC/p7), and p6 protein. The 55kDa Gag precursor protein associates with the plasma membrane for virus assembling so it is also called assemblin.

Env: The envelope (Env) gene (~1800bp) encodes for the glycoprotein (gp) 160 which is cleaved into gp120 and gp41 by the host cell protease furin. These two glycoproteins, gp120 and gp41, interact non-covalently forming a heterodimer anchored to the viral membrane. gp120 is exposed on the surface of the viral envelope for specific binding with the CD4 receptor, while gp41 is buried in the viral envelope. However, when gp120 binds to CD4 and changes its conformation, gp41 is exposed and assists in viral fusion into host cell.

Pol: The polymerase (Pol) gene (~2900bp) encodes for three enzymes, reverse transcriptase (RT), integrase (IN), and protease protein (PR). RT (p66/55; alpha and beta subunits) is responsible to reverse-transcribe viral RNA genomes into DNA, and the function of IN (p32) is to insert the viral DNA into the host chromosomal DNA. PR (p10) is an aspartyl protease which cleaves the proteins of gag and pol.

Tat: Trans-activator of transcription (Tat) is accountable to increase the level of transcription of HIV genes via binding the trans-activation responsive region (TAR) located at the 5'-end of all nascent viral transcripts, and the host cellular factors, CycT and cdk9 which cooperate to hyperphosphorylate the carboxy-terminus domain (CTD) of RNA polymerase (RNAP) II.

Rev: Rev is a 19kDa nuclear phosphoprotein that promotes the nuclear export and stabilization of the viral mRNAs containing Rev Response Element (RRE).

Vif: Viral infectivity factor (Vif) is a 23 kDa protein that protect viral DNA mutated from the action of the cellular protein, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G), which has cytidine deaminase activity.

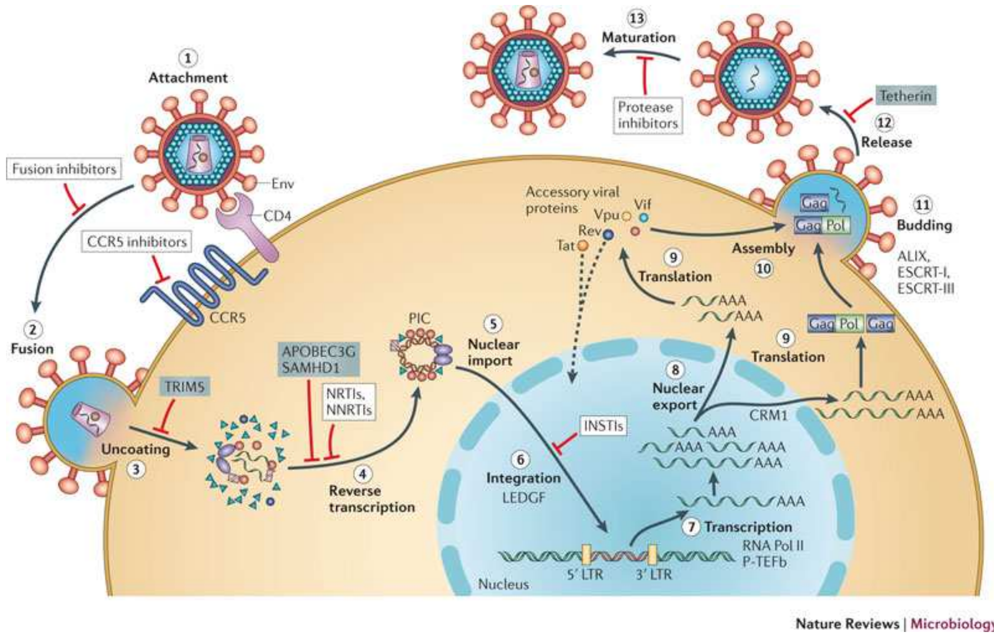
Vpr: Viral protein R (vpr) is a 14 kDa protein responsible of nuclear import of viral pre-integration complex (PIC), G2 cell cycle arrest, and induction viral replication in non-dividing cells.

Vpu: Viral Protein U (Vpu) is a 16kDa integral membrane protein which enhances HIV-1 virion release from the plasma membrane of host cells by counteracting tetherin (BST2; CD317) which is able to inhibit virion release by interaction with the viral surface envelope glycoprotein. In addition, in the endoplasmic reticulum, Vpu is able to target and degrade CD4 for enhancing virus release because CD4 could form a stable complex with Env protein.

Nef: Negative Regulatory Factor (Nef) is a 27kDa myristoylated protein that down-regulates surface expression of major histocompatibility complex-I (MHC I)[27-31] and MHC II[32] on antigen-presenting cells (APCs), and CD4 and CD28 on helper T cells.

HIV Replication Cycle

HIV infects various cell types, including memory $CD4^+$ T cells, macrophages and dendritic cells (DCs) [33-47]. HIV enters target cells by a complex interaction between its surface glycoprotein gp120 and the receptor CD4 on the host cell. The interaction of gp120-CD4 induces conformational changes that promote gp120 binding to select chemokine receptors. Chemokine receptors are seven transmembrane domain proteins that use G proteins for signal transduction so that they belong to the G protein coupled receptor (GPCR) family. Based on binding with different chemokines, chemokine receptors are classified into CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors. HIV-1 strains are divided into R5, X4 and R5X4 tropic based on their usage of two primary co-receptors, CCR5 and CXCR4[48-55], which are necessary for HIV-1 entry into host cells. The entry of HIV into cells can be blocked by co-receptors mutation. A series of seminal studies showed that individual homozygous for a 32 base pair deletion in their CCR-5 present is highly resistant to HIV infection. The interaction of gp120 with CD4 receptor and chemokine co-receptor leads gp120-gp41 to undergo a conformational change exposing hydrophobic regions that embed in the membrane of the host cell. Subsequently a conformation change of gp41 which brings the viral and target cell membrane close and which enables membrane fusion, introducing the viral capsid into the host cell. In the cytoplasm of the host cell, viral single-strand RNA is reverse transcribed to double-stranded DNA by the viral reverse transcriptase. Importantly, the tRNA-Lys3 from host cell serves as a specific primer binding to the primer binding site (PBS) on the viral RNA genome for reverse transcriptase. After reverse transcription, the viral DNA is transported to the nucleus and then integrated into a host chromosome by a viral enzyme called integrase. Following integration, the integrated DNA, also known as provirus, is then transcribed into viral mRNA. Viral RNA exists in different species. Un-spliced mRNA is essential for producing of structure protein, Gag and Pol, and it is also encapsidated in progeny virus. Singly spliced RNA encodes the Env, Vif, Vpr, and Vpu protein, while doubly-spliced RNA is translated into the regulatory proteins, Tat and Rev, and the protein Nef. Rev is involved in the shuttling of un-spliced and singly-spliced viral RNA from nucleus to cytoplasm. To assemble new viral particles, viral genome and viral proteins reach close to plasma membrane. Then the HIV virions are packaged and bud out from the cytoplasm. (Fig 1.3)[56]



Source: Engelman A *et.al*., Nat Rev Microbiol. 2012 Mar 16; 10(4):279-90

Figure 1.3 Schematic overview of the HIV-1 replication cycle.
Those host proteins that have a role in the replication cycle and are discussed in the text are indicated. The infection begins when the envelope (Env) glycoprotein spikes engage the receptor CD4 and the membrane-spanning co-receptor CC-chemokine receptor 5 (CCR5) (step 1), leading to fusion of the viral and cellular membranes and entry of the viral particle into the cell (step 2). Partial core shell uncoating (step 3) facilitates reverse transcription (step 4), which in turn yields the pre-integration complex (PIC). Following import into the cell nucleus (step 5), PIC-associated integrase orchestrates the formation of the integrated provirus, aided by the host chromatin-binding protein lens epithelium-derived growth factor (LEDGF) (step 6). Proviral transcription (step 7), mediated by host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), yields viral mRNAs of different sizes, the larger of which require energy-dependent export to leave the nucleus via host protein CRM1 (step 8). mRNAs serve as templates for protein production (step 9), and genome-length RNA is incorporated into viral particles with protein components (step 10). Viral-particle budding (step 11) and release (step 12) from the cell is mediated by ESCRT (endosomal sorting complex required for transport) complexes and ALIX and is accompanied or soon followed by protease-mediated maturation (step 13) to create an infectious viral particle. Each step in the HIV-1 life cycle is a potential target for antiviral intervention[165]; the sites of action of clinical inhibitors (white boxes) and cellular restriction factors (blue boxes) are indicated. INSTI, integrase strand transfer inhibitor; LTR, long terminal repeat; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor[56].

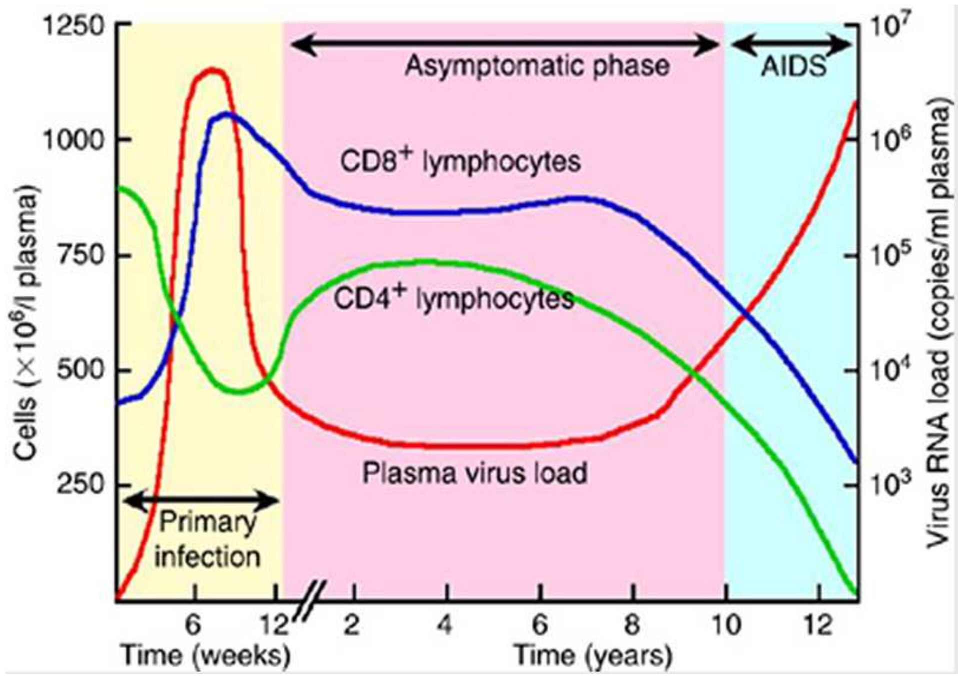
HIV Pathogenesis

The HIV infection can be separated in three phases: acute phase (2-4weeks), asymptomatic phase (8-10years), and AIDS phase (3-5 years) (Fig 1.4). The initial or acute phase of HIV infection is typically associated with rapid $CD4^+$ T cell decrease, high viral replication in lymphoid tissue, and high blood viremia. After reaching peak viremia, HIV specific antibodies become detectable in human blood, a stage known as seroconversion. HIV viral load then decreases to reach a steady state level, namely HIV set point[57]. The level of HIV set point is associated with the rate of disease progression to AIDS. A higher viral load of the HIV set point predicts faster progression to AIDS, while a lower viral load set point predicts slower progression to AIDS and longer clinical latency[58, 59]. In the HIV acute phase, HIV infects target cells mediated by CD4, and CXCR4 and/or CCR5. HIV-1 variants that use CCR5 are called R5 tropic strain, those that use CXCR4 are called X4 tropic strain, and those that can use both are designated X4R5 or dual tropic. Although both R5 and X4 viruses are present in various body fluids, R5 virus appears to be predominant in primary transmission of infection, while X4 virus variants become detectable at later stages.

In the acute phase, R5 viruses are captured from the exposed mucosal tissue and associated with processes on dendritic cells (DC) by binding its gp120 envelope glycoprotein to Dendritic Cell-SpecificIntercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)[60]. HIV is then co-internalized into lymph nodes with DC and infects $CD4^+$ T cells[61] (fig1.5). About two weeks after the initial HIV infection, acute replication of HIV in lymphoid tissues results in high level of viremia and dissemination[62, 63]. HIV replication significant damages the $CD4^+$ T cell compartment, causes gut mucosal barrier degradation, resulting in microbial translocation, immune system hyper-activation and eventually leading to immune failure. In the gut associated lymphoid tissue (GALT), the absence of lymphoid cells aggregating in terminal ileum is observed because of loss of $CD4^+$ T cells[64-66]. Patients will display some relatively unspecific clinical symptoms, such as fever, adenopathy, and leucopenia as HIV spreads beyond lymphoid tissue. Eventually, HIV replication is transiently controlled by the immune system, reducing HIV viral load. After approximately two months, the interplay between virus and immune system reaches its balance, so that the viral load of HIV reaches its set point [57].

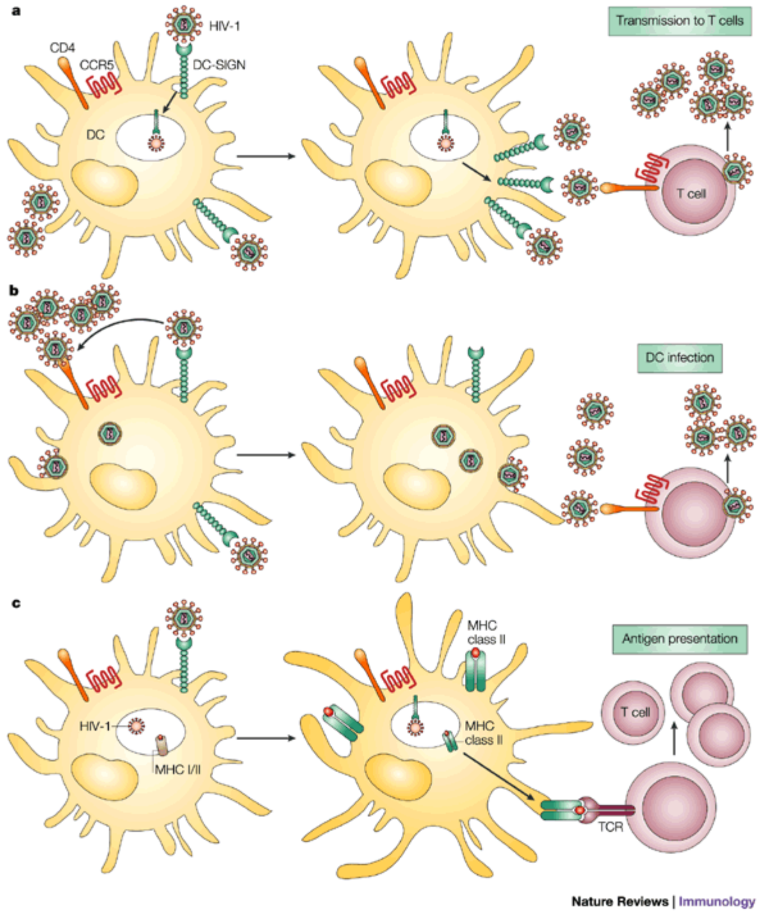
After the viral set point is reached, HIV-infected individuals start going through clinical latency or asymptomatic phase. However, during the asymptomatic phase, although immune system temporarily represses viral replication, HIV keep increasing the damages to the immune system over time, and meanwhile, $CD4^+$ T cells are still slowly depleted. If HIV infection is not treated, the clinical latency/asymptomatic phase will last 8-10 years on average, during which the immune function decline gradually.

After the latency phase, HIV infection is defined as AIDS when the number of CD4 cells falls below 200 cells per cubic millimeter of blood (<200 cells/mm3), or if AIDS-defining symptoms, such as opportunistic infections and/or certain type of cancers occur. Damage to host's immune system by HIV causes the failure of immune system and emergence of fatal opportunistic infections and tumors[67, 68]. Subjects with AIDS who do not receive treatment typically survive about 3-5 years on average. A schematic representation of the clinical progression of HIV depicting $CD4^+$ and $CD8^+$ T-cell counts and plasma viral load in peripheral blood during the acute phase, asymptomatic phase, and AIDS phase is shown in Fig1.4[61].



Source: Munier *et al*., Immunol Cell Biol 2007 Jan; 85(1):6-15.

Figure 1.4 Schematic of typical course of HIV-1 infection showing changes in CD4 and CD8 T-cell counts in peripheral blood and plasma virus load[69]



Source: Kooyk Y *et al*., Nat Rev Immunol. 2003 Sep; 3(9):697-709.

Figure 1.5 HIV-1 subverts intracellular processing by dendritic cells through DC-SIGN.

(a) DC-SIGN (dendritic cell (DC)-specific intercellular adhesion molecule-grabbing nonintegrin) is expressed by immature DCs in mucosal tissues and lymph nodes, and by DC precursors in the blood. HIV-1 is captured by DC-SIGN that is expressed by DC precursors in the blood after infection or by immature DCs at mucosal entry sites during sexual transmission. DC-SIGN-bound HIV-1 enters the cell, but escapes internalization into lysosomal compartments and recycles back to the cell surface. By hiding intracellularly in DCs, HIV-1 is protected during migration to the lymphoid tissues. On arrival at lymphoid tissues, DCs transmit HIV-1 to CD4+ T cells in *trans*, resulting in productive HIV-1 infection of CD4+ T cells. (b) High concentrations of HIV-1 allow viral infection of DCs that results in the production of HIV-1 by DCs, which subsequently infect T cells. Sequestration of HIV-1 by DC-SIGN can allow *cis*-infection of DCs by presenting the infectious virus to CD4 and co-receptors to allow efficient infection of DCs. (c) C-type lectins function as antigen receptors to internalize antigen into lysosomes to enhance antigen presentation by MHC class I and II molecules. It remains to be determined whether capture of HIV-1 by C-type lectins results in the activation of DCs and presentation of viral antigen by MHC molecules. CCR5, CC chemokine receptor 5; TCR, T-cell receptor[61].

HIV Reverse Transcription

Reverse Transcription

Reverse transcription which is one of the early steps of HIV-1 replication converts viral RNA genome into proviral DNA form which is transported to the nucleus where it integrates into the host cell genome.

Reverse transcription from viral RNA genome into DNA is one of the early steps of HIV-1 replication. The generated DNA proviral form is then transported to the nucleus where it integrates into the host cell genome. The process of reverse transcription occurs in several steps. (Fig.1.6) The viral reverse transcriptase (RT) and protease protein (PR) encoded by polymerase (pol) gene play important roles in HIV-1 reverse transcription. The tRNA-Lys3 binding to the primer binding site (PBS) on the HIV-1 RNA serves as the specific primer for HIV-1 reverse transcription and RT starts reverse transcript RNA to a single strand of complementary DNA (ssDNA), which initially the region of PBS, LTR and RU5. RNase H then degrades the RNA in the RNA/DNA duplex which leads the dissociation of PBS, LTR, and RU5 from viral RNA, so the R element on the ss cDNA is exposed. Since the R element is also presented on the 3' end of the viral RNA, the complex tRNA-Lys3, RT, and ssDNA undergoes a first "jump", hybridizing on the 3' R element of HIV-1 RNA. RT restarts reverse transcription on HIV-1 RNA generating ssDNA, and RNase H activity degrades the RNA in the RNA-DNA duplex, except for a short sequence called polypurine tract which is used by RT as a primer to start polymerizing the second strand of cDNA, including U3, LTR, U5, and PBS. RNase H eventually removes the remaining RNA, including polypurine tract and primer tRNA-Lys3. This causes the two complementary PBS elements of the 3' and 5' ends of the

DNA to recombine, forming a DNA loop and eventually allowing a second "jump" of HIV reverse transcription. Finally, RT has DNA-dependent DNA polymerase activity that copies the sense cDNA strand into an antisense DNA to form a double-stranded viral DNA, and then dsDNA loop breaks, forming linear dsDNA provirus with two LTR at the 5' and 3' ends. After reverse transcription, the dsDNA is transported into the nucleus where the dsDNA is integrated by the viral integrase into chromosomal DNA of the host cell.

tRNA-Lys3

The primer transfer RNA lysine (tRNA-Lys3) with anticodon UUU plays a crucial and essential role in the initial reverse transcription of HIV. tRNA-Lys3 folds into a classical two-dimensional cloverleaf structure, with acceptor, D, anticodon and T C arms (Figure 1.7).[70] The acceptor stem on the 3' end of tRNA-Lys3 conjugates with the PBS on the viral RNA. In addition, nuclear magnetic resonance (NMR) studies shows that the U rich anticodon loop of tRNA-Lys3 specifically binds with the A rich loop, 164-AAA-166, near the PBS, determining not only the selective use of tRNA-Lys3 as primer for HIV-1 RT, but the stabilization of tRNA U rich loop and viral RNA A rich loop in the vRNA/tRNA complex[71]. (Fig 1.8)

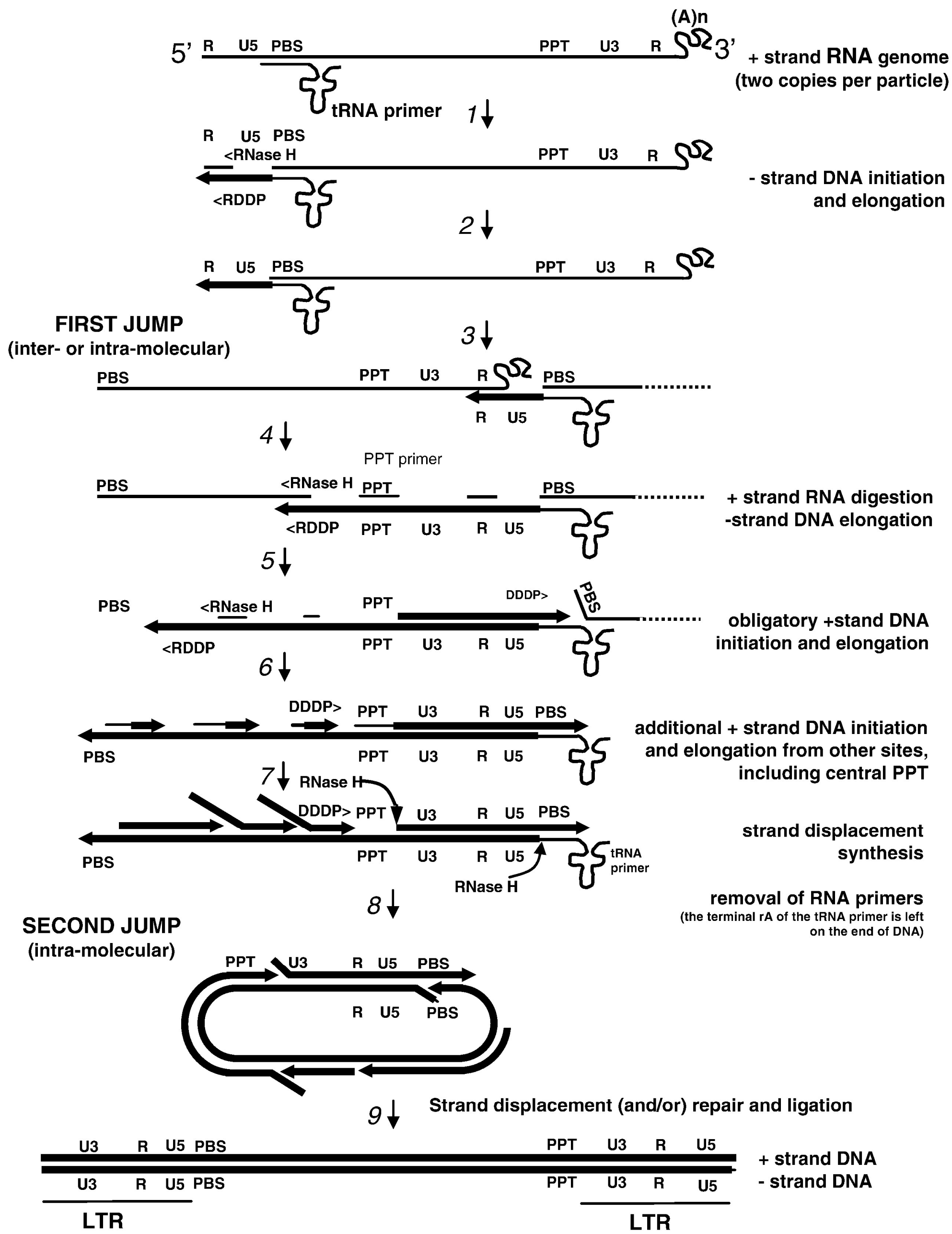


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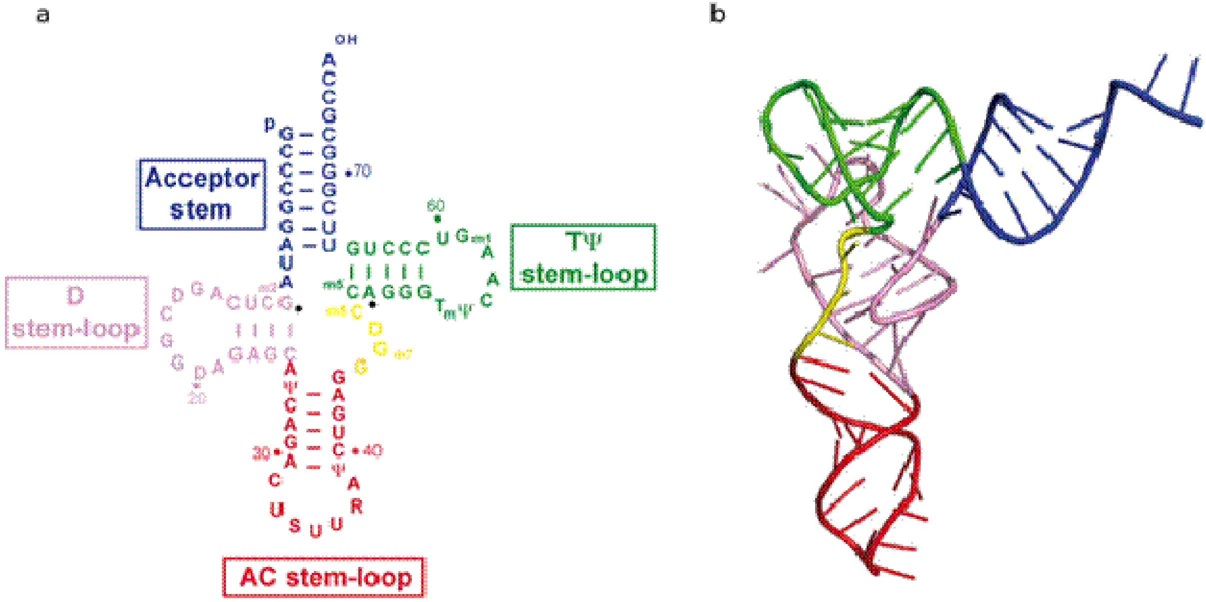
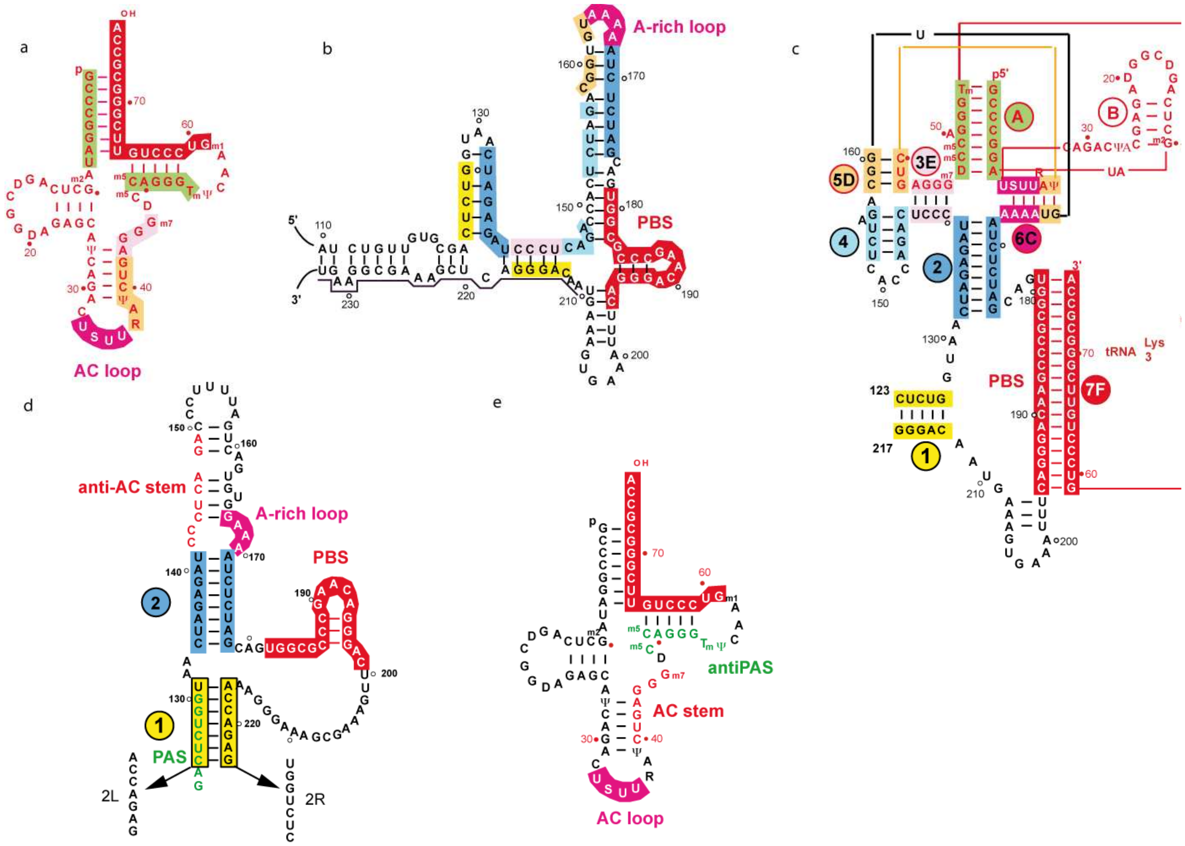
Figure 1.6 Reverse transcription of the HIV-1 genome

Each retroviral particle contains two copies of the RNA genome. Minus-strand DNA synthesis starts near the 5' end of the plus-stand RNA genome using a host tRNAlys3 that anneals at the primer-binding site (PBS) as a primer. *Step 1:* Synthesis proceeds to the 5' end of the RNA genome through the U5 region, ending at the R region at the 5'end, forming the minus-strand strong stop DNA. *Step 2:* DNA synthesis is accompanied by RNase H digestion of the RNA portion of the RNA/DNA hybrid product, thus exposing the single-stranded DNA product. *Step 3:* This exposure facilitates hybridization with the R region at the 3' end of the same, or the second, RNA genome, a strand-transfer reaction known as the first jump. *Step 4:* When minus-strand elongation passes a polypurine-rich region called the polypurine tract (PPT) region, a unique plus-strand RNA primer is formed by RNase H cleavage at its borders. Plus-strand synthesis then continues back to the U5 region using the minus-strand DNA as a template. *Step5:* Meanwhile, minus-strand synthesis continues through the genome using the plus-strand RNA as a template, and removing the RNA template in its wake *via* RNase H activity. *Step 6:* The RNase H digestion products formed are presumed to provide additional primers for plus-strand synthesis at a number of internal locations along the minus-strand DNA. *Step 7:* PPT-initiated plus-strand DNA synthesis stops after copying the annealed portion of the tRNA to generate the plus-strand DNA form of the PBS, forming the plus-strand strong stop product. The tRNA is then removed by the RNase H activity of RT. *Step 8:* This may facilitate annealing to the PBS complement on the minus-strand DNA, providing the complementarity for the second jump. DNA synthesis then continues. *Step 9:* Strand displacement synthesis by RT to the PBS and PPT ends, and/or repair and ligation of a circular intermediate, produces a linear duplex with long terminal repeats (LTRs) at both ends.[70]



Source: Isel C *et al.*, Viruses. 2010 January; 2(1): 213–243.

Figure 1.7 Secondary (a) and tertiary (b) structures of tRNA-Lys3[71]



Source: Isel C *et al.*, Viruses. 2010 January; 2(1): 213–243.

Figure 1.8 Secondary structure of the RNA partners of the HIV-1 initiation complex of reverse transcription and of the binary primer/template complexes, in the case of the HIV-1 MAL (representative of a subtype A PBS domain) and subtype B isolates. The regions undergoing intra- or intermolecular rearrangements upon formation of the primer/template complex are highlighted in various colors. Boxes or sequences of the same color represent areas that are base-paired in the binary complex. (a) The human tRNA-Lys3. (b) The PBS sub-domain in the free form of the HIV-1 MAL vRNA. (c) The HIV-1 MAL vRNA/tRNA3Lys complex. (d) The PBS sub-domain in the free form of the HIV-1 NL-4.3 (subtype B) isolate. The PAS and mutations 2L and 2R are indicated. (e) Localization of the anti-PAS region of tRNA-Lys3.[71]

Angiogenin

Angiogenin (ANG, RNase5, angiogenin-1) is a protein with angiogenic and ribonucleolytic activity. ANG was originally isolated from the culture medium of HT-29 human colon adenocarcinoma cells, for the study of its angiogenic activity. ANG is a single chain 14.1kDa polypeptide containing 123 amino acids with three intra-chain disulfide bonds link: Cys^{26}-Cys^{81}, Cys^{39}-Cys^{92} and Cys^{57}-Cys^{107} [72]. ANG is expressed in normal human tissues and fluids including plasma, and it is secreted by various cells including vascular endothelial cells, smooth muscle cells, fibroblasts, colonic columnar epithelium, lymphocytes, and primary adenocarcinoma cells as well as select tumor cell lines[6, 7].

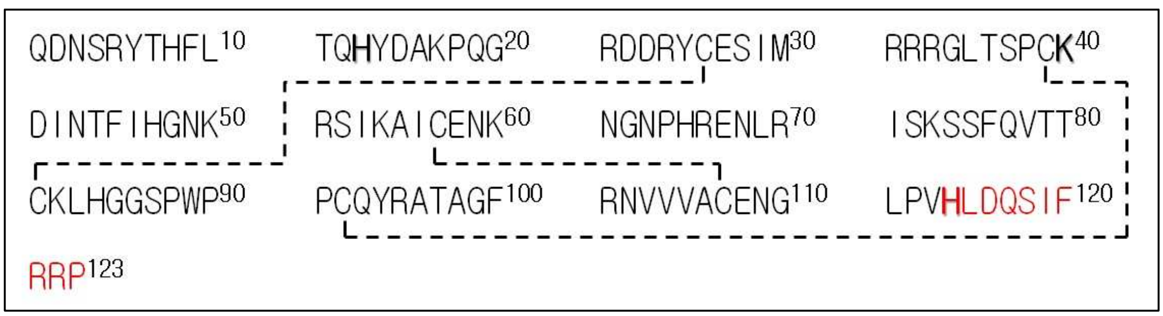


Figure 1.9 Amino acid sequence of human angiogenin.[72]
The active site residues His^{13}, Lys^{40}, and His^{114} are shown in bold. Three intra-chain disulfide bonds link: Cys^{26}-Cys^{81}, Cys^{39}-Cys^{92} and Cys^{57}-Cys^{107} are connected in dotted line [72, 73].

Angiogenin Molecular Structure and Domains

Several studies on ANG have addressed how its biological activities, angiogenesis and ribonuclease, are associated to its protein structure. Chemical modification or mutagenesis studies have identified and investigated ANG binding, cell adhesion, and actin-binding domains, as well as enzyme active site, and RNase inhibitor binding site, elucidating their contribution to angiogenic, enzymatic, or both activities of ANG . The binding site for putative receptor contains two segments, residues 60-68 and Asn-109[74], and they both are associated with angiogenic activity of ANG. Moreover, Arg-66 mutation represses angiogenic potency of ANG, showing that it is an essential site within the 60-68 region[75]. The, Arg^{66}-Glu-Asn^{68} region is responsible for epithelial cell adhesion, and Arg^{31}-Arg-Arg^{33}, Arg-^{66} and Arg^{70} are known for supporting HT-29 cell adhesion. ANG binding domain also involves the 60-68 region, which binds to a dissociable ANG binding protein on endothelial cells. The dissociable ANG binding protein was characterized as 42 kDa actin[14, 76]. The ANG ribonuclease active site is composed of three catalytic sites, His-13, Lys-40 and His 114, which are involved in the phosphodiester bond cleavage, as site-directed mutagenesis of the three catalytic sites decreases ANG ribonucleolytic and angiogenic activity[77]. In addition, the C-terminal (The red box in Fig1.9; Fig 1.10), 116-123, region plays a key role in enzymatic activity of ANG. The studies of testing ANG RNase activity on RNA, CpA, UpA, and tRNA, from Demetres *et al*. showed mutant the residues, D116H, Q117G, I119A, and F120A, increase the enzymatic activity, and the deletion mutant des(121-123) decrease the ribonuclease activity[78]. Human angiogenin binds human placental RNase inhibitor (RI) with high affinity ($Ki<0.7$ fM). The binding of RI influences 24 residues of ANG with catalytic site, Lys-40[79].

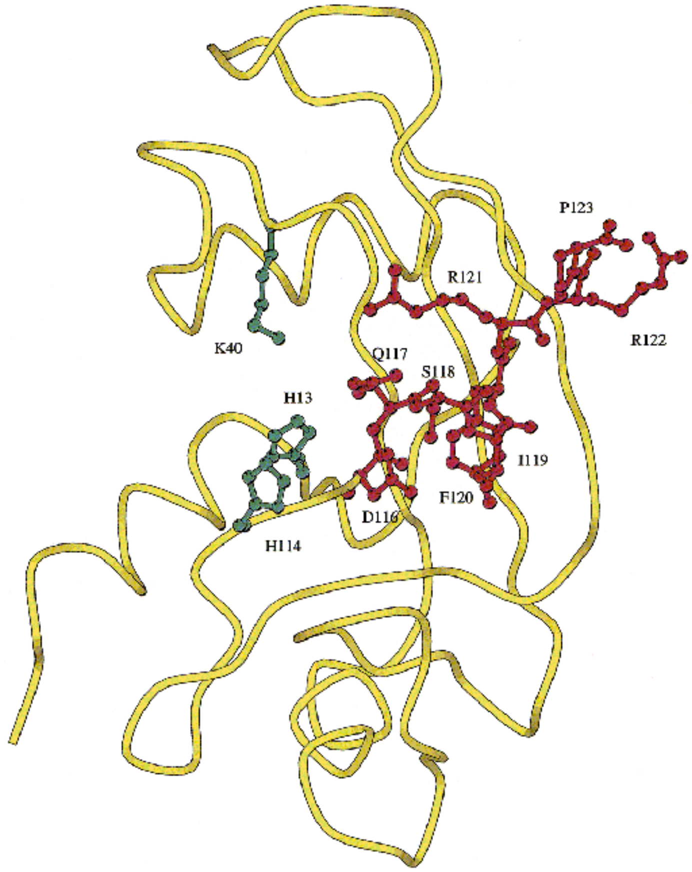


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Figure 1.10 Representation of the Ang structure showing the C-terminal segment (residues 116−123) in red and the side chains of the three catalytic residues (His13, Lys40, and His114) in cyan.

Angiogenin Receptor and Binding Protein

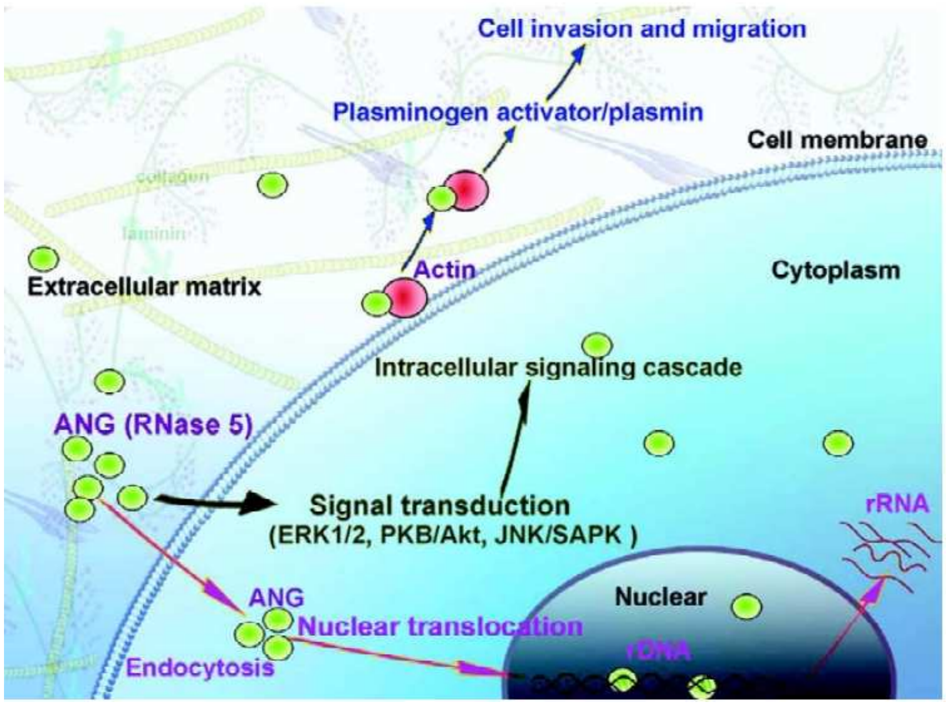
Published studies reported that ANG binds a 172kDa receptor and a 42kDa binding protein, identified as actin, on the surface of endothelial cells. The 172kDa surface receptor specifically binding to ANG is found on endothelial cells from different human tissues including human umbilical artery and vein, and human foreskin dermal tissue[13]. Previous studies speculate that ANG induces angiogenesis through a receptor-mediated signal transduction pathway, such as extracellular signal-related kinase1/2 (ERK1/2) as well as protein kinase B/Akt , and phosphorylation of stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), which are associated with cell proliferation[80-82]. The expression of 172-kDa receptor was reportedly induced by ANG, and dependent on cell density ($<2\times10^4$ cells/cm^2)[13]. Nevertheless, the nature of the receptor is still elusive, so it is named ANG putative receptor[13, 83]. A 42-kDa ANG-binding protein (ANG-BP) was also characterized as a cell surface component of the endothelial cells, and it was expressed from endothelial cells upon treatment with heparin, heparan sulfate, or ANG[14]. ANG-BP is identified as a member of the muscle-type actin family also based on amino acid analysis, tryptic peptide mapping, and partial amino acid sequencing[76]. The addition of both actin and anti-actin antibodies inhibit ANG-induced angiogenesis in the chick embryo chorioallantoic membrane (CAM) indicating that binding of ANG to the endothelial cell surface actin is an essential step in neovascularization[76].

Angiogenesis

Angiogenesis is the process of growing new blood vessels and plays an essential role in normal physiological processes[5] such as vascular structure development and wound healing[84, 85]. ANG mediates four aspects of angiogenesis including ribonuclease activity, basement membrane degradation, signal transduction, and nuclear translocation[15].

Under sub-confluent conditions, ANG specifically binds to cell surface actin resulting in the stimulation of the cell-associated protease system and cell-invasion by activating tissue-type plasminogen activator (tPA) which is a serine protease responsible for the degradation of basement membrane and extracellular matrix by catalyzing the conversion of plasminogen to plasmin. Therefore, it allows endothelial cells to migrate to the space of the degraded tissues (Fig.1.11)[76]. Endothelial cells invading and migrating into the basement membrane cause a low density of local endothelial cells. Low density of cells then induces the expression of a 172kDa receptor which is specific binding with ANG. When ANG binds to the high-affinity 172kDa receptor on subconfluent endothelial cells[86-88], it triggers several intracellular signal transductions, extracellular signal-related kinase1/2 (ERK1/2) as well as protein kinase B/Akt , and phosphorylation of stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), which are associated with cell proliferation[80-82]. When the cell number and density are elevated, the expression of the ANG receptor will decrease and stop cell division[88]. In addition, ANG translocates into cell nucleus and enhances rRNA transcription have been also reported essential for angiogenesis[89, 90]. However, ANG's nuclear translocation also is cell density dependent, and necessary for angiogenesis[91].

Some factors enhance or inhibit ANG mediated angiogenesis. Copper, *in vitro*, modulates angiogenesis by enhancing ANG binding to endothelial cells[86, 92]. Angiogenic activities of ANG can be abolished by treating with reagents that modify the residues His, Lys, Arg, or the three disulphide bonds of ANG. Placental Ribonuclease inhibitor (RI) binds tightly to ANG, potently inhibiting its activity[93, 94].



Source: Gao X *et al*., Acta Biochim Biophys Sin (Shanghai). 2008 Jul; 40(7):619-24.

Figure 1.11 Mechanism of action of angiogenin in the process of angiogenesis
Angiogenin, also known as RNase 5, induces angiogenesis mainly through four pathways: its ribonuclease activity, basement membrane degradation, signaling transduction, and nuclear translocation.

Angiogenin RNase activity

In addition to angiogenesis, ANG (RNase5) has RNase activity. Based on the comparison of amino acid sequences, ANG has 35% identity with pancreatic RNase[95,96]. However, after testing ANG on pancreatic RNase substrates, such as poly(C), poly(U), and yeast RNA, no activity was detected, suggesting that these two RNases have different specificities[12]. Although ANG has significantly weaker RNase activity than pancreatic RNase, it has been reported that ANG has specific RNA catalytic activity on 18S, 28S rRNA and tRNA[12, 95, 97]. A series of recent publications has reported that tRNA-specific ANG RNase activity is associated with protein repression under adverse condition[16-19]. The function of the tRNA fragments resulting from ANG cleavage is related to findings from the microRNA (miRNA) research field. A research group who tried to determine the liver-specific miRNA from fetal liver observed that about 41% miRNA are cleaved from tRNA[98]. Moreover, other research groups also found that these tRNA fragments are from mature tRNA cleaved at the anti-codon loop, and named these tRNA fragments tRNA halves, or tRNA derived, stress induced small RNAs (tiRNA)[16, 17]. Then ANG was proved to be the ribonuclease responsible for tRNA cleavage[16]. Both *in vitro* and *in vivo*, expression of ANG is positively correlated with the production of tRNA halves[16]. In addition, the tRNA cleavage activity of ANG was induced by various stresses including heat shock, hypothermia, hypoxia, and radiation[16]. Around the same time, the Anderson group reported the phenomenon the silencing of protein translation induced by stress[99], followed by addional studies that identified that the stress-induced silencing of protein translation is mediated by tiRNAs produced by a specific cleavage at the anticodon loop[16, 17]. In mammalian cells, studies found that the function of repression of protein translation may be to conserve anabolic energy for the repair of stress-induced damage when cell are in stress condition[99]. In addition, based on the results from Anderson *et al*, after the tRNA is split into two fragments, only the 5' end is able to inhibit protein translation.[19] In a study on whether tiRNA formation is related with RNase activity, Emara *et al*. reported that two other members of the human family of RNASES, RNaseA and RNase4, failed to produce any tiRNA. Since ANG is enzymatically less active than RNase A and RNase4 and can produce tiRNA indicates that their ribonucleolytic activity is not specific for tiRNA production [18] According to these studies, the RNase activity of ANG may play a role in not only tRNA cleavage, but also in the regulation of cellular transcription in stress condition.

Angiogenin Anti-HIV Activity

Recently two publications from Gene Shearer's and Robert Gallo's laboratories show that ANG inhibits HIV-1 *in vitro*. The Ng *et al* group showed that bovine milk contains a number of proteins known to inhibit HIV including Angiogenin-1, which has 64% sequence identity with human ANG[100, 101]. An *in vitro* study of ANG and related RNases showed inhibition of X4 tropic HIV-1 in PBMCs when cells were pre-treated or post-treated with ANG (3.55μM) and when the virus was incubated with ANG[3]. Although the mechanism(s) used by ANG to block HIV-1 replication is not completely understood, the results from Bedoya *et al.* suggested several modalities for ANG mediated HIV inhibition, such as receptors blocking and early reverse transcription inhibition[3]. The HIV-1 inhibition detected when PBMCs were incubated with ANG after HIV-1 infection may be relevant to the therapeutic potential of ANG. The study published by Cocchi *et a.l*[4] also showed dose-dependent inhibition of X4 tropic HIV-1 by ANG. However, Cocchi *et al*. used lower doses of ANG (16nM, 80nM, and 400nM[4]), which are within physiological concentrations in serum (ANG 33.17±7.48 nM[102]) in their experiments, suggesting that ANG levels in human serum are adequate for effective anti-HIV activity. Nevertheless, while both previous studies demonstrated ANG-mediated HIV-1 replication of X4 tropic isolates, they did not show whether R5 tropic HIV-1 virus is inhibited. Therefore, understanding the range of activity in different HIV isolates and the mechanism(s) of inhibition mediated by ANG may lead to the development of novel HIV therapeutics.

Specific Aims

ANG has *in vitro* anti-HIV-1 activity, reaching approximately 80% of HIV-1 inhibition[3, 4] Since ANG is produced by several cell types relevant to HIV infection, including $CD4^+$ and $CD8^+$ T lymphocytes[4, 7], it is thought to play a role in controlling infection. However, the mechanism of this activity has not been investigated in detail.

Therefore, determining the mechanism of ANG anti-HIV activity is important to better understand its role in HIV pathogenesis, and it might yield novel HIV therapeutics. The studies on the anti HIV activity of ANG from Bedoya *et al* and Cocchi *et al* focused on X4 tropic virus[3, 4]. However, R5 tropic virus is the predominant viral strain involved in primary HIV infection worldwide; therefore, it is important to characterize the activity of ANG on R5 tropic HIV. ANG is a serum protein secreted by various cells including HIV target cells, lymphocytes including CD4+ T cells.[7] In addition, ANG has angiogenic and ribonucleolytic activities[5, 1516-19, 95, 97-99]**.** Two cell surface binding sites including a 172 kDa putative receptor[13] and a 42kDa ANG binding protein[13, 83] are associated with ANG-mediated angiogenesis. Based on the anti-HIV-1 activity, receptor-mediated cell signaling and ribonuclease activity, I hypothesize that ANG inhibits both R5 and X4 tropic HIV-1 replication by a complex mechanism that includes an intracellular mechanism. The goal of my research focus on three specific aims:

Aim1: To determine if ANG inhibits X4 and R5 tropic HIV-1 replication by an intracellular mechanism.

Aim2: To determine if ANG down-regulates HIV-1 receptor and/or co-receptor expression

Aim3: To determine if ANG cleaves tRNA-Lys

Chapter 2: Materials and Methods

Isolation and culture of primary cells and cell lines

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy human subjects by Lymphoprep (Axis-Shield). For stimulating PBMCs, the isolated PBMCs were cultured with the culture medium, RPMI with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S), and 1%Gln, with 10 ng/ml Interleukin-2 (IL-2) and 2.5 μg/ml phytohemagglutinin (PHA) at 37°C in a 5% CO_2 humid atmosphere for 48 hours. Activated PBMCs were maintained in complete RPMI-1640 media with 10 ng/ml IL-2 at a density of less than $2x10^6$ cells/ml.

Virus production

HIV_{IIIB} (X4 tropic) and HIV_{BaL} (R5 tropic) both we used were prepared from acutely infected PM1 cells grown in complete RPMI-1640 media. However, because of the adaptive evolution of HIV_{BaL} caused by long term culturing in PM1 cell line, the HIV BaL (R5 tropic) were only used the first passage cultured from PM1 in RPMI-1640 media/human AB serum.

Angiogenin antiviral assay

Protocol of ANG pre-treatment: Recombinant human angiogenin (rhANG) (Calbiochem, cat# 175600) was incubated at the indicated concentration with activated PBMCs which were stimulated by 10 ng/ml IL-2 and 2.5 μg/ml phytohemagglutinin (PHA) prior to infection for 2 h at 37°C in 5% CO_2, and then cells were be washed twice by PBS buffer. PBMCs were infected 2 h with stock viruses, 2μl of 10^4 TCID50 HIV-1_{IIIB} or 0.2μl of 10^6 $TCID_{50}$ HIV-1_{BaL}, per 10^5 cells in total volume of 200μl. The infected

PBMCs were then washed two times with PBS buffer, and resuspended in 100µl of complete RPMI 1640, RPMI 1640 media with 5% fetal bovine serum (FBS), 1% Penicillin-Streptomycin(P/S), and 1% L-Glutamine (L-Gln), with 10 ng/ml IL-2 and rhANG at the indicated concentration.

Protocol of ANG post-treatment: Activated PBMCs were infected for 2 h with 2µl of 10^4 $TCID_{50}$ of stock viruses, HIV-1IIIb or HIV-1Bal, per 105 cells in total volume of 200µl, and washed twice with PBS buffer. The HIV-1 infected PBMCs were cultured with rhANG at the indicated concentration in total volume of 100µl in triplicate in flat-bottom 96-well plate.

The HIV-1-infected cultures from both two protocols were incubated for 4, 6, 8, and 10 days and the supernatants 20µl per well were harvested to determine p24 antigen levels were detected by ELISA with the use of a commercially available kit (PerkinElmer Life and Analytical Sciences) on each time point, and also replace 20µl complete RPMI 1640 culture media with 10 ng/ml IL-2 and rhANG at the indicated concentration

Infectivity assays – p24

Two procedures used in our experiments include the ANG treatment prior and after (pre-treatment) and the ANG treatment only after (post-treatment) HIV infection in PBMCs. For pre-treatment, stimulated PBMCs (10^5 cells/well) in 96-well culture plate were treated with ANG for 2 hours at 37°C. After wash with phosphate-buffered saline (PBS) twice, ANG-treated PBMCs were infected with 10^4 $TCID_{50}$ of HIV_{BaL} or HIV_{IIIB} for 2 hours, and then washed two times with PBS. Finally, the infected PBMCs were placed complete media with the appropriate treatment. Viral infectivity was monitored by p24 enzyme-linked immunoabsorbent assay (ELISA) using a commercially available kit (PerkinElmer Life and Analytical Sciences). For post-treatment, activated PBMCs (10^5 cells/well) were directly infected with 10^4 $TCID_{50}$ of HIV_{BaL} or HIV_{IIIB} for 2 hours. After 2 hours infection, PBMCs were washed with PBS two times, and complete media was added with the appropriate treatment and the rest of the experiment was carried on as that in pre-treatment.

Detecting CD4, CXCR4, and CCR5 by flow cytometry assay

Cells were surface stained with fluorochrome-conjugated antibodies and isotype-matched anti-IgG controls for 30 minutes, and then washed and fixed with 2% paraformaldehyde and washed again. After staining with antibodies, cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) with a minimum of 10,000 gated events. Lymphocytes were gated based upon forward and side scatter profiles. The FACS data from the gated cell population analysis were done by using FlowJo software (Tree Star, Inc.). The fluorochrome-conjugated antibodies including FITC-conjugated CD4, PE-conjugated CXCR4, APC-conjugated CCR5, were purchased from BD Biosciences.

Cell metabolism assay

Cell metabolism was determined by MTS assay (Promega). Cells (10^5 cells/well) were cultured in triplicate in 96-wells plates with media, and then the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) mixture was added and incubated at 37°C. MTS, in the presence of PMS, reduced by mitochondria in the cells produces a formazan product that has an absorbance maximum at 490-500 nm. Therefore, the spectrophotometric absorbance was read at 490 nm. The values of optical density (OD490 nm) were read for 1, 2, 3, 4 and 24 hours after the adding of MTS/PMS mixture. The amount of OD490 nm is directly proportional to the number of live cells in culture. The cell metabolism of each wells were compared by the OD ratios of ANG treated/non treated (control) PBMCs by percentage.

AMLV pseudo type virus production

293T cells ($2x10^5$/well) used for transfection with vectors and product pseudo type viruses were seeded and cultured in 6-wells plate with complete DMEM media (2ml/well) for 24 hours. After 24 hours culture, replace the fresh culture media 1ml per well. AMLV pseudotype viruses are made using pNL4-3.luc.R-E-, a non-infectious HIV-1 recombinant clone inserted with firefly luciferase reporter gene, and AMLV envelope DNAs. Transfection was performed by using FuGENE 6 Reagent (Promega). pNL4-3.luc.R-E- and AMLV DNA, to FuGENE 6 Reagent (Promega) diluted with serum-free medium were mixed in 3 to 1 ratio and cultured at 25^{o}C temperature for 15min. After 15min culture, transfection reagent and DNA complex were added to the cells in a drop-wise manner, and then cultured the transfected cells for 72 hours. AMLV pseudo type viruses in the suspension were collected after 72 hours culture. The suspension was aliquoted to small tubes and kept at -80^{o}C. The infectivity of AMLV pseudo-type virus was tested in PBMCs and detected by Steady-Glo luciferase assay system (promega)

Virus concentration

Sucrose Gradient assay

For preparation, ultra-clear centrifuge tubes (12ml) for SW41Ti rotor of ultracentrifuge (Beckman) were sterilized, and 8ml virus suspension was added into tubes, and then the pipette with 2ml 20% sucrose solution was inserted all the way to the bottom of the centrifuge tube, and gently and slowly expelling the 20% sucrose solution to underlay the virus suspension. After balancing the weights for each pair of tubes, the preparations were centrifuged for 2 hours and 15 minutes at 30,000 rpm. After 2hours of centrifugation, the tubes were removed from the buckets carefully. For drying the virus pellet attached on the bottom of the tubes, supernatant were poured off and tubes were set in inverted position on a paper towel for 10min to allow the residual liquid to drip away. The virus was resuspended with PBS buffer, and incubates at 4^{o}C for 30min. Virus in PBS was aliquoted in screw-cap microfuge, snap-frozen in crushed dry ice, and stored at -80^{o}C.

PEG 6000 assay

27ml of virus-containing cell culture supernatant was put into a 50ml tube, and mixed with 7ml 50% PEG 6000 solution, 3ml 4M NaCl and 3ml PBS solutions. The tube with virus mixture was stored at 4^{o}C on a shaker for 2hrs, and centrifuged at 7,000g for 10min at 4^{o}C. Viral pellet was resuspended in PBS by vortexing for 20-30 sec, snap-frozen in crushed dry ice, and stored at -80^{o}C.

Chapter 3: To determine if ANG inhibits X4 and R5 tropic HIV-1 replication by an intracellular mechanism

3.1 Angiogenin inhibits both X4 and R5 tropic virus in PBMCs

Introduction

Human angiogenin (ANG, RNase5), a member of the RNase A superfamily is a serum protein expressed in normal human tissues and fluids, and secreted by various human cells including smooth muscle cells, epithelial cells, fibroblast cells, and tumor cells.[5] In addition, ANG manifests angiogenic and ribonucleolytic activities[5]. It plays a role in the early stages of tumorigenesis[103-105]. For HIV inhibition, a variety of proteins from bovine including ANG-1 were tested for their inhibitory activity toward human immunodeficiency virus-1 reverse transcriptase by chemical assay in vitro[106],and bovine ANG-1 shows approximately 44% inhibition of HIV-1 reverse transcriptase[106]. However, bovine ANG-1 is different from human ANG. Previous studies showed that bovine ANG has a 71% identity with human ANG, and most of the polypeptide fragments responsible for the function between bovine ANG-1 and human ANGs are conserved. However, the bovine ANG-1 and human ANG are shown minor differences in the spatial arrangements based on the structural identification characterized by X-ray analysis and nuclear magnetic resonance [107-109]. Therefore, it cannot be excluded that these differences might be related to different biological and biochemical properties [110]. When the antiviral activity was analyzed by Bedoya *et al*. Their data showed that HIV-1 was inhibited by treatment with 3.55µM human ANG prior (ANG pre-treatment) and after (ANG post-treatment) HIV infection[3]. Recently another study published by Cocchi *et al*., reported about 50% inhibition of HIV-1 on day 5 when PBMCs were treated with 80nM ANG after HIV-1 infection [4]. However, the HIV-1 isolates used for both studies are X4 tropic[3,

[4], and the predominant transmitted strain worldwide is R5 tropic. Therefore, we studied inhibition of ANG on both X4, (IIIB), and R5 (Bal) strains of HIV-1 by treating PMBC prior and after (pre-treatment) or only after (post-treatment) infection.

Experimental Design

We evaluated antiviral activity in infectivity assays using two protocols, one with ANG added prior and after infection ("pre treatment") and one with ANG added after infection ("post treatment"), (Fig 3.1) as described in materials and methods. We included two controls by treating infected PBMCs with HIV-1 inhibitors: azidothymidine (AZT) and enfuvirtide (T20) for HIV-1_{IIIB} infection protocol; AZT and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) for HIV-1_{BaL} infection protocol. Among the antiretroviral compounds we used as controls, AZT is a reverse transcription inhibitor, and T20 inhibits HIV-1 fusion, and they inhibit both R5 and X4 isolates, while RANTES inhibits R5 virus by blocking CCR5. We used three concentrations of ANG (0.25µM, 0.5µM, and 1µM) for our experiments . We also tested the effect of each treatment on cell metabolism by using MTS assay (Promega), using the protocol described in material and methods.

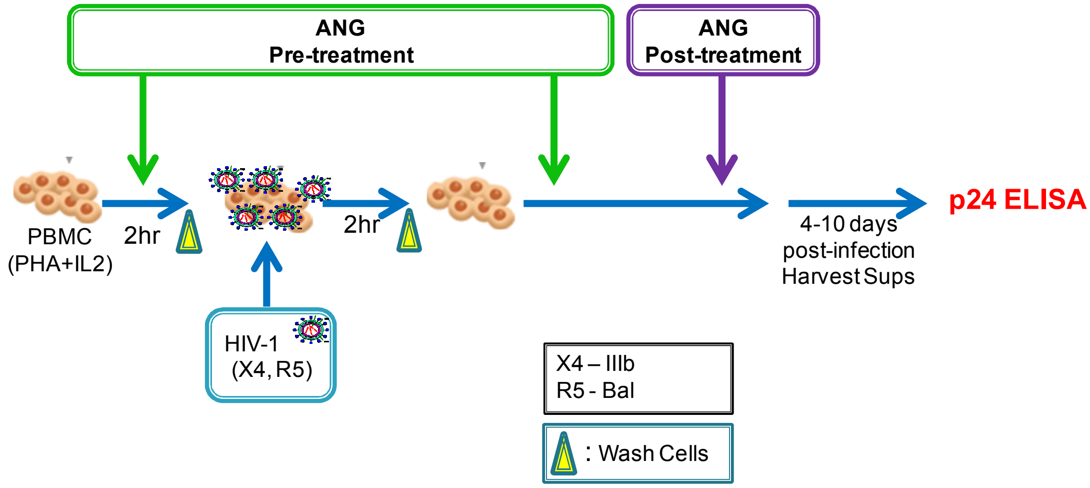


Figure 3.1 Experimental design for determining ANG HIV-1 (X4 and R5) inhibition in PBMCs using two different protocols.
ANG pre-treatment protocol: ANG added to PBMCs 2 h prior and after infection.
ANG post-treatment protocol: ANG added to PBMCs 2 h after infection. p24 antigen was measured by ELISA on 4-10 days after infection.

Results

The results show ANG inhibits the replication of both X4 and R5 tropic viruses in PBMCs with both pre treatment and post treatment protocols (Fig 3.4, 3.5). We found that both viruses could be inhibited by ANG, although we did not observe a classical dose–response curve. Differences in percent inhibition values were assessed using student's t-test, comparing control and the experimental groups, (i.e., without and with ANG), assuming that p values <0.05 are statistically significant (Fig 3.4, 3.5). There was no detectable p24 in the supernatants of infected PBMCs treated with inhibitors (AZT and T20).

We tested PBMCs metabolism by using MTS assay as indicated in materials and methods, in all groups on the last time point (Day 10). (Fig 3.4) We found no significant difference among treatment groups, indicating that ANG does not have cytotoxicity effects on PBMCs.

Discussion

The infectivity of X4 and R5 tropic HIV-1 is inhibited by ANG at all concentrations tested (0.25μM, 0.5μM, and 1μM), using both pre and post treatment protocols. Our results not only confirm the previous studies that show that X4 virus is inhibited by ANG, but also show inhibition of R5 HIV. Some of the inhibition we measured could be due to interaction of ANG with the 172kDa ANG putative cell surface receptor and 42kDa ANG binding protein revealed from previous studies. HIV-1 requires CD4 as receptor and one of two chemokine receptors, CXCR4 and CCR5 as co-receptors to enter cells. Therefore, it is possible that the binding of ANG and the cell surface receptor or binding protein may down-regulate HIV-1 receptor and/or co-receptors, and inhibit HIV-1 entry in target cells.

The HIV-1 inhibition observed in the ANG post treatment protocol may indicate that ANG influences viral replication intracellularly. We ruled out that ANG inhibits HIV-1 by directly interacting with free viral particles extracellularly since we washed out the HIV-1 virus with PBS buffer for two times before treatment. ANG has been showed to be able to enter cells by receptor-mediated endocytosis, and further translocate into the cell nucleus. Therefore, ANG may repress HIV-1 replication intracellularly. Furthermore, as ANG contains ribonuclease activity, its enzymatic activity may act to degrade intracellular RNAs that are necessary for HIV-1 replication, such as viral RNA, which is the template for reverse transcription or the genome for virion assembling, and tRNA-Lys, which is the specific primer for the reverse transcription of HIV-1.

A B

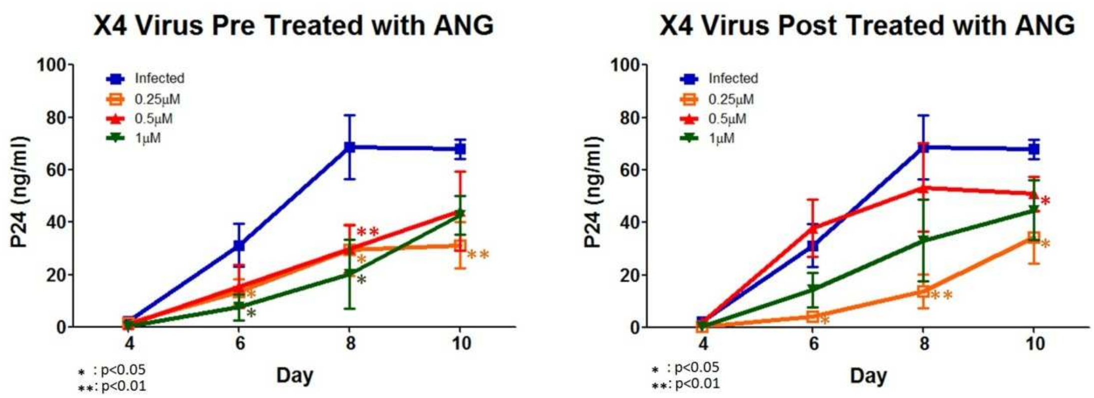


Figure 3.2 ANG treatment inhibit X4 tropic virus in PBMCs.
(A) HIV-1_{IIIB} (X4) infectivity in PHA/IL2 activated PBMCs is decreased by pre-treating with ANG. (B) HIV-1_{IIIB} (X4) infectivity in PHA/IL2 activated PBMCs is decreased by post-treating with ANG. p24 was tested by ELISA at Days 4, 6, 8, and 10. (Error bars: SD)

A B

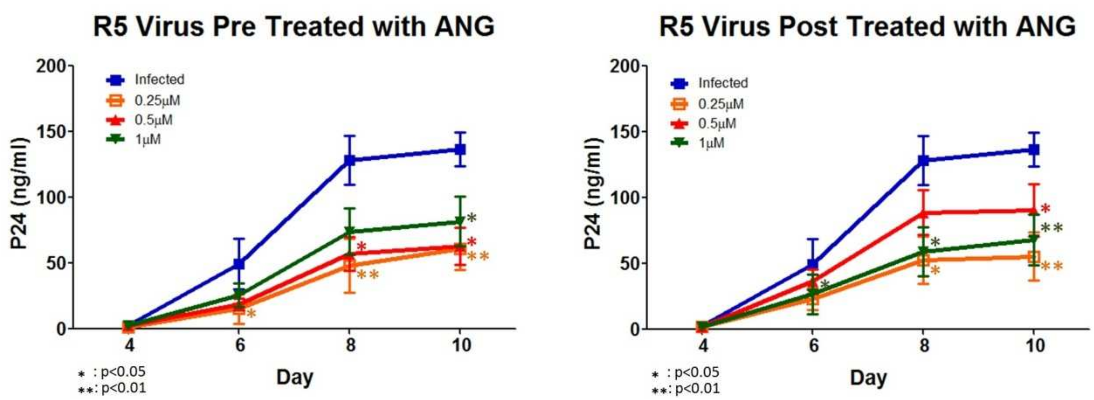
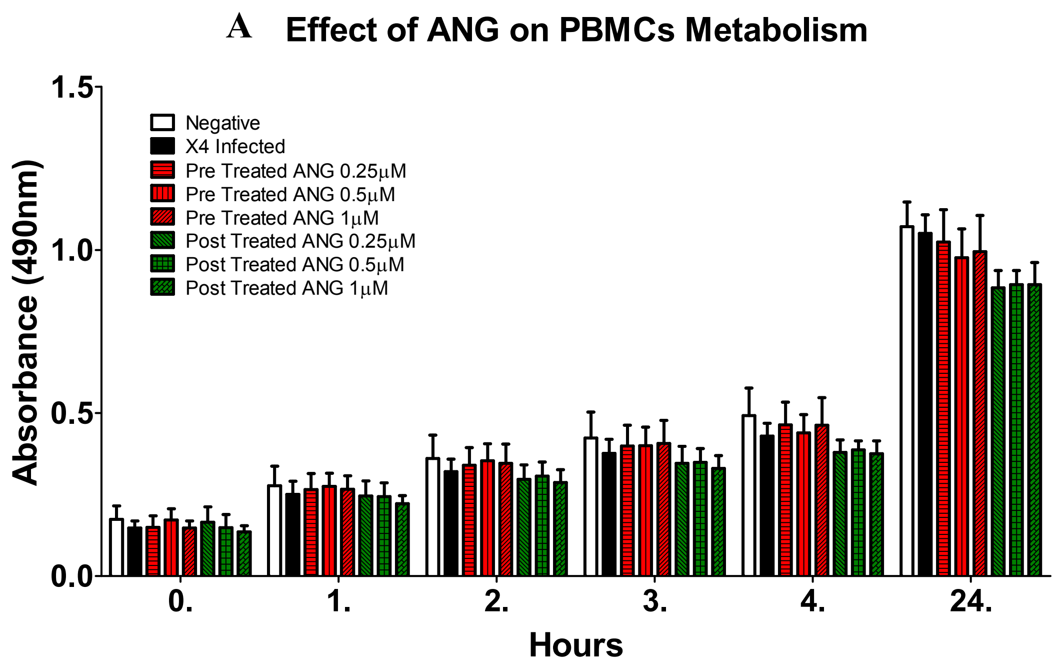


Figure 3.3 ANG treatment inhibit X5 tropic virus in PBMCs
(A) HIV-1_{BaL}(R5) infectivity in PHA/IL2 activated PBMCs is decreased by pre-treating with ANG.(B) HIV-1_{BaL} (R5) infectivity in PHA/IL2 activated PBMCs is decreased by post-treating with ANG. p24 was tested by ELISA at Days 4, 6, 8, and 10. (Error bars: SD)



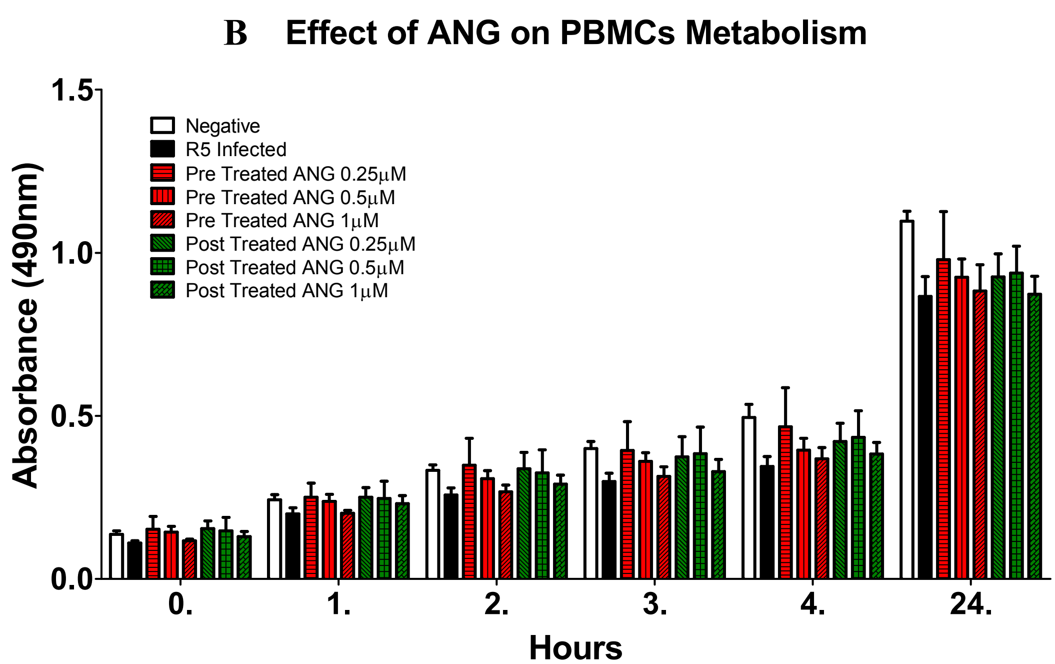


Figure 3.4 Effect of ANG on PBMCs Metabolism
(A) PBMCs Metabolism in X4 (HIV-1_{IIIB}) infection group for 10 days. (B) PBMCs Metabolism in R5 (HIV-1_{BaL}) infection group for 10 days. (Error bars: SD)

3.2 Evidence that ANG inhibits HIV-1 in PBMCs with a Intracellular Mechanism

Introduction

Based on the results of the previous experiment, we found that HIV-1 is inhibited by ANG treating prior and after HIV-1 infection in PBMCs. In particular, we noticed that the HIV-1 was inhibited when ANG was added only after infection ("post treatment"). We therefore speculated that ANG may inhibit HIV-1 replication intracellularly. In order to address this hypothesis, we infected cells with HIV-1 pseudo virus, using AMLV envelope to complement an env-defective HIV-1 genome that expresses firefly luciferase reporter gene[111-113]. The AMLV envelope of the pseudo type virus mediates viral infection in cells including $CD4^+$ T cells without binding to HIV-1 co-receptors, CXCR4 and CCR5[114]. In addition, AMLV pseudo type virus undergoes a single round of replication because the backbone used to produce it is the modified HIV-1 DNA proviral clone, pNL4-3.luc.R-E-, without *Vpr* and *Env* genes, while it contains a firefly luciferase reporter gene. Therefore, the infectivity of AMLV pseudo type virus could be detected by luciferase assay.

Experimental Design

AMLV pseudo type virus expressing firefly luciferase (luc) in place of Nef were prepared by transfecting 293-T cells, a HEK cell line have been transformed and express the SV40 large T antigen, with defective HIV provirus, pNL4-3.Luc.R-E-, and plasmids encoding *env* genes from amphotropic murine leukemia virus (AMLV). AMLV pseudo type virus was concentrated by using sucrose gradient assay before PBMC infection. Both the protocols of AMLV pseudo type virus production and viral concentration are described in material and methods. For detecting if ANG inhibit AMLV pseudo type virus, we evaluated two protocols (Fig. 3.5), one where ANG was added prior and after infection ("pre treatment") and one where ANG post was added only after infection ("post treatment"), similarly as previous HIV-1 infection experiments. We used three concentrations of ANG (0.25μM, 0.5μM, and 1μM) for AMLV pseudo type virus infection experiments. PBMCs were washed with PBS buffer prior and after AMLV pseudo type virus infecion. Finally, after 3 days incubation, the infectivity of AMLV pseudo type virus was detected by lucferase assay.

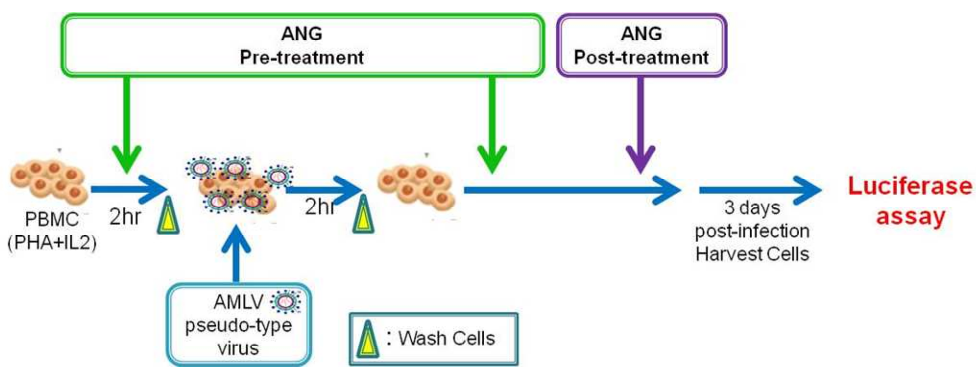


Figure 3.5 Experimental Design for determining ANG inhibits AMLV pseudo type virus.using two different protocols.
ANG pre-treatment protocol: ANG added to PBMCs 2 h prior and after infection.
ANG post-treatment protocol: ANG added to PBMCs 2 h after infection. p24 antigen was measured by ELISA 4-10 days after infection.

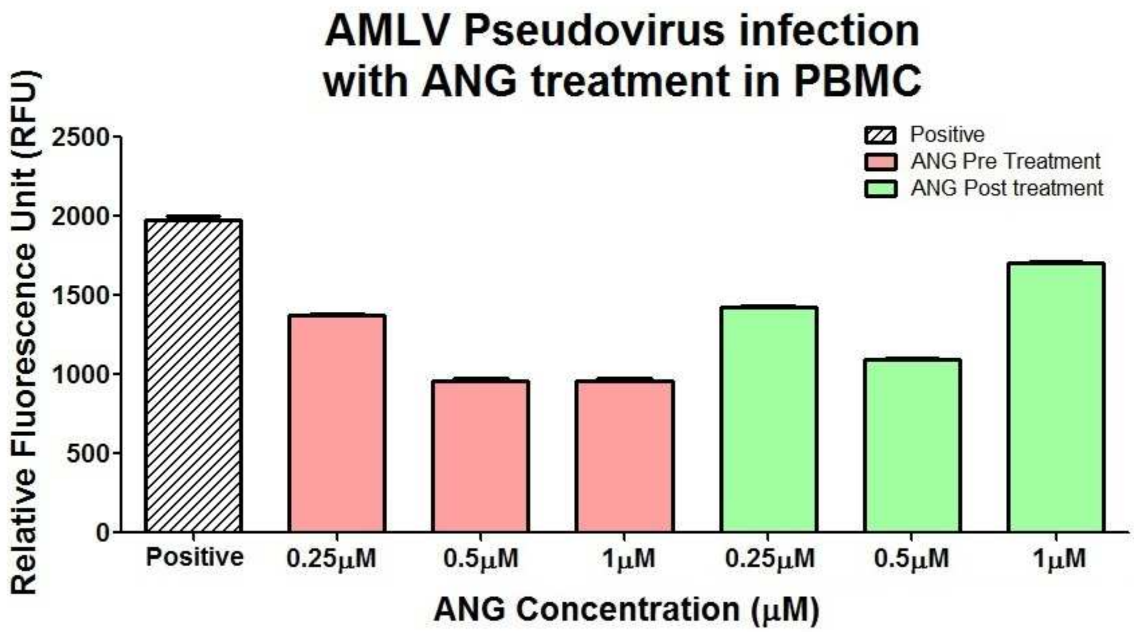
Results

The results show ANG inhibits intracellular replication of AMLV pseudo type virus in PBMCs in a dose-dependent manner when we used the pre treatment, but not with the post treatment protocol, which showed highest level of inhibitory activity with the intermediate concentration of ANG. (Fig 3.6A) After converting the data of relative fluorescence units to percentage of inhibition, we estimated the AMLV inhibition of ANG pre treatment to be: 0.25μM ANG: 28.90%; 0.5μM ANG: 49.18%; 1μM ANG: 51.23%; and that of ANG post treatment to be: 0.25μM ANG: 26.54%; 0.5μM ANG: 49.95%; 1μM ANG: 13.81%). (Fig 3.6B)

Discussion

AMLV pseudo type virus was inhibited by both pre treatment and post treatment of ANG (0.25μM, 0.5μM, and 1μM) in PBMCs, confirming the results with X4 and R5 HIV from the previous experiment. AMLV pseudo type virus enters PBMCs by using Pit2 but not CD4, CXCR4, and /or CCR5 as receptors. Moreover, since AMLV pseudo type virus has a defective HIV-1 genome, it undergoes only a single round of replication in target cells and is not able to produce new infective virions. Therefore, the inhibition of AMLV pseudo-type virus by ANG suggests that ANG affects viral replication intracellularly without interacting with HIV-1 receptor, CD4, and/or, co-receptors, CXCR4 and/or CCR5.

A



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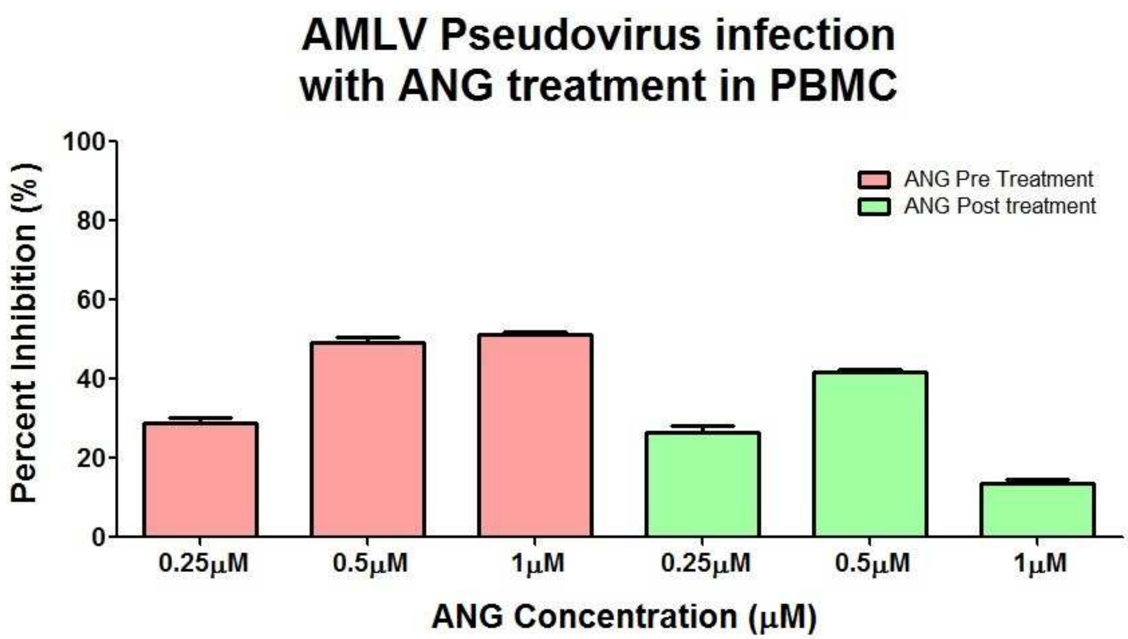


Figure 3.6 AMLV pseudotype virus infect PBMCs with ANG treatment
(A) AMLV pseudo type virus infectivity tested by luciferase assay (B) Percentage of AMLV inhibition of ANG treatment. (Error bars: SD)

Chapter 4: To determine if ANG down-regulates HIV-1 receptor and/or co-receptor expression

Introduction

From our previous studies, we concluded that treatment with ANG prior and after X4 or R5 HIV-1 infection in PBMC inhibits viral replication. Since HIV inhibition by ANG when ANG is added prior to infection in PBMCs, we investigated whether ANG could decrease expression of HIV receptors. On the HIV-1 target cells, the HIV receptor CD4, and two co-receptors, CXCR4 and CCR5[115-121] specifically bind to HIV-1 viral gp120 protein, and the bindings lead to virion fusion and HIV-1 infection. We hypothesized that ANG down-regulate HIV-1 receptor, CD4, and/or co-receptors, CXCR4 and CCR5, and we proceeded with the experiment for testing CD4, CXCR4, and CCR5 expression which expressed in the active PBMCs which have incubated with ANG by flow cytometry assay.

Experimental Design

We used flow cytometry as described in the materials and methods section to detect if ANG down-regulates expression of CD4, CXCR4, and CCR5 on the surface of PBMCs. PBMCs were isolated from healthy human donors, and stimulated with 10 ng/ml IL-2 and 2.5 μg/ml PHA for 48 hours. After wash, media (complete RPMI-1640 media with 10 ng/ml IL-2) was replaced and cells were cultured with for another 24 hours. Activated PBMCs were treated with ANG (0.25 μM, 0.5 μM, and 1 μM) for 2 hours, and washed with Fluorescence-activated cell sorting wash buffer (FACS wash buffer) for two times. The antibodies which bind with HIV-1 receptor (CD4) or co-receptors (CXCR4, or CCR5) conjugate with appropriate fluorochromes, such as fluorescein isothiocyanate (FITC), phychoerythrin (PE), or allophycocyanin (APC).The PBMCs with and without adding ANG are incubated with antibodies, and finally the cell populations with CD4, CXCR4, or CCR5 were detected and analyzed by FACSC assay. Take CD4 conjugated with PE for example. If ANG down-regulateds CD4 expression, the decasing of the population of PBMCs with PE signal would be expected.

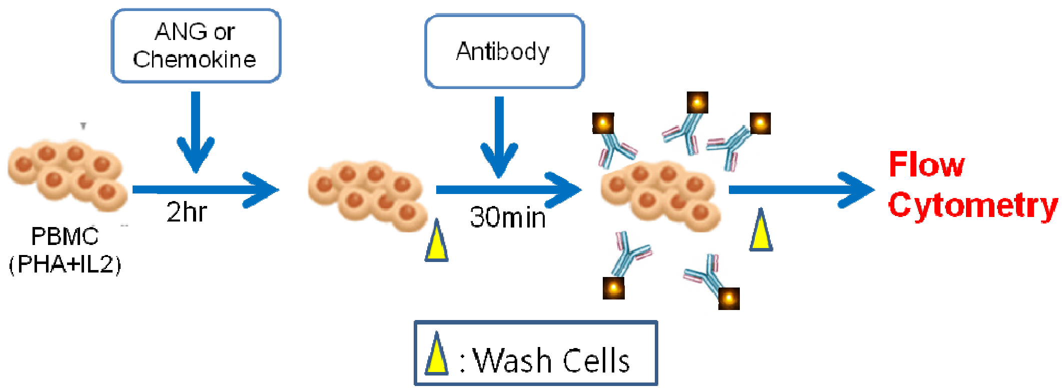


Figure 4.1 Experimental design for determining if CD4, CXCR4, and CCR5 down-regulated by ANG

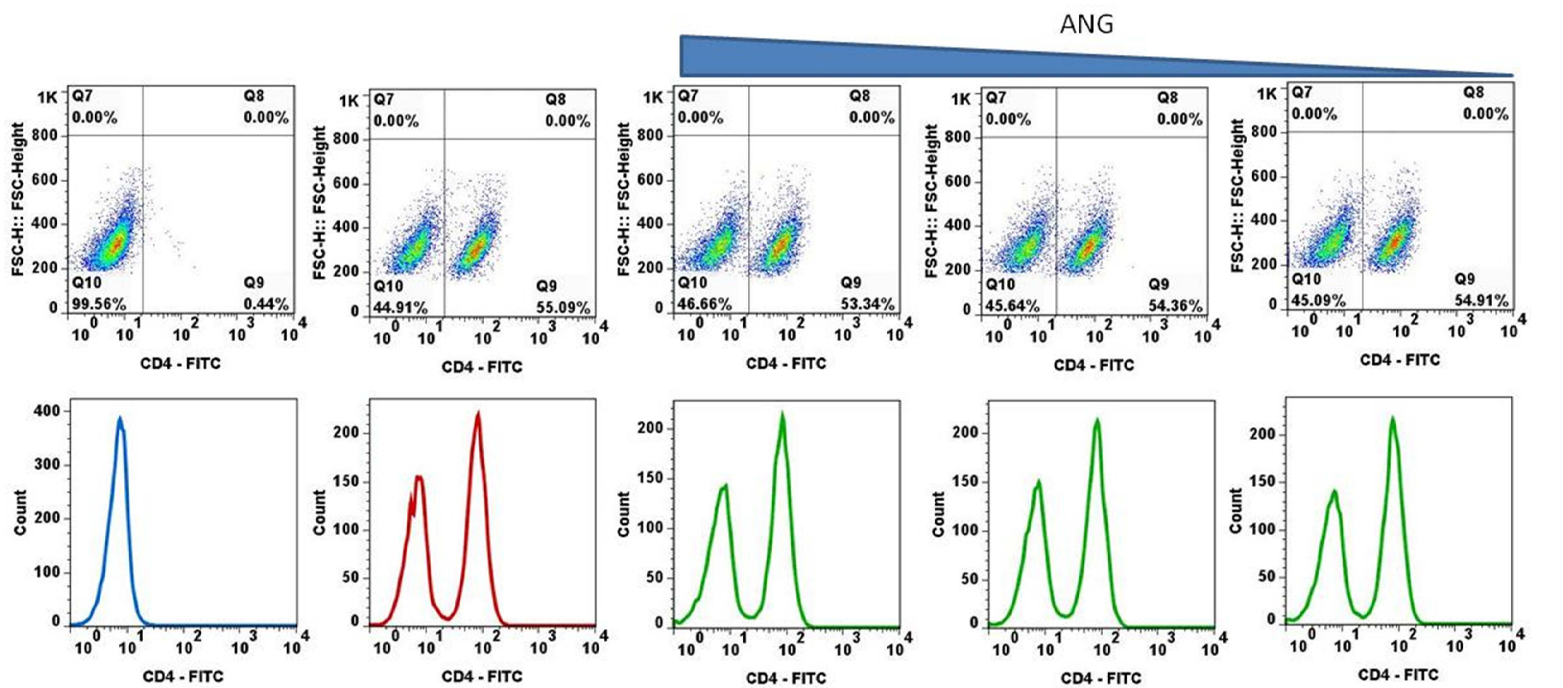
Results

Our results showed that the expression of CD4 on PBMCs was not down regulated by ANG (0.25µM, 0.5µM, 1µM) treatment. However, ANG has limited effect on CXCR4 and CCR5 expression in PBMCs. Staining of PBMCs with the negative control for CD4 (isotype antibody), is shown in blue, while the staining of PBMCs with the positive control (CD4 antibody), is shown in green in figure 4.2. Analysis of our results show CD4 expression on PBMC is not affected by ANG treatment as no shifting of the histogram curve, which is shown in blue, was observed. Figures, 4.3 and 4.4, show the expression of co-receptors, CXCR4 and CCR5. In addition to the positive and negative antibody controls, we introduced additional controls by using the chemokine stromal-derived factor-1 (SDF-1 or CXCL12) to block CXCR4 and regulated upon activation, normal T-cell expressed, and secreted (RANTES or CCL5) to block CCR5. The blue, red, orange, and green peaks, in figures 4.3 and 4.4, show negative control, positive control, inhibitor control, and ANG treatment of PBMCs respectively. ANG showed limited effect on CXCR4 and CCR5 expression as we observed ANG leads to the

MFI decreasing of CXCR4 and CCR5. However, the differences in MFI of CCR5 are difficult to assess because the signal is relatively dim.

Discussion

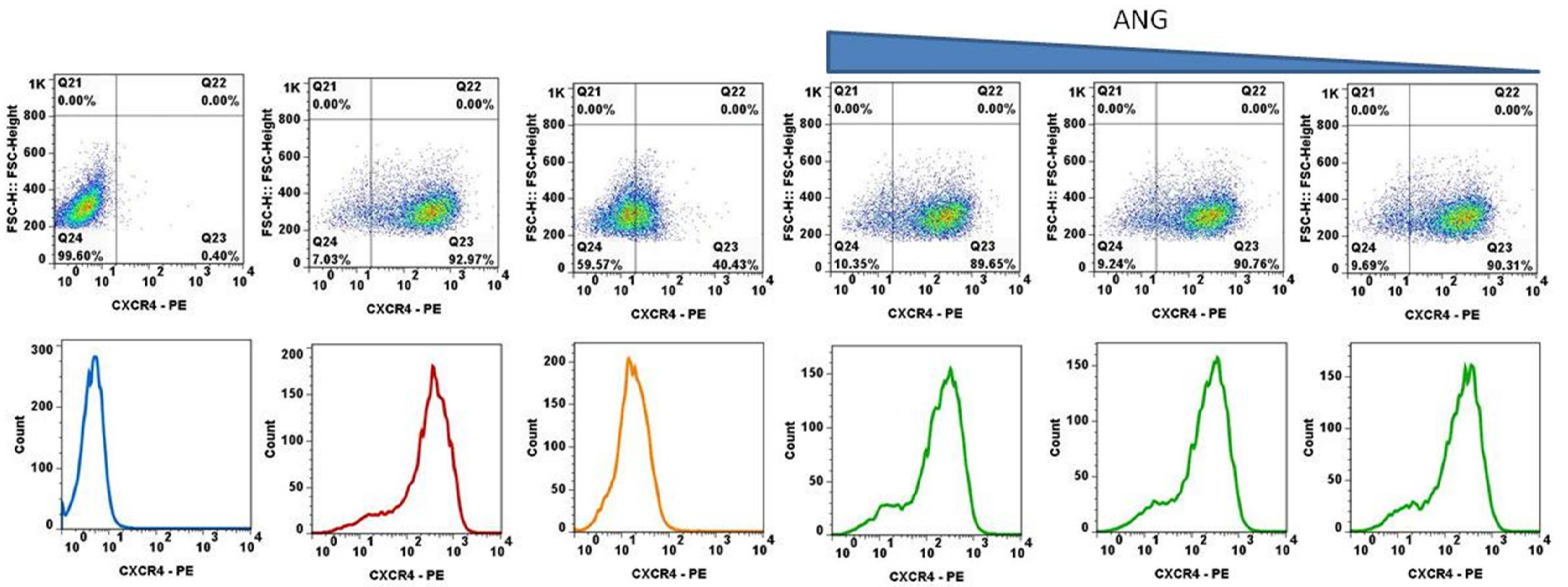
This study showed no difference of median values of fluorescence intensity of CD4 in the PBMCs with and without incubating with ANG, so it suggests that ANG does not down-regulate expression of CD4. ANG has shown limited effect on the expression of CXCR4, and CCR5 in PBMCs as we observed a decrease in median fluorescent intensity of the signal, although there was no change in the percentage of cells expressing these receptors. However, it is not clear whether the changes in MFI for CXCR4 and CCR5 are related to the antiviral activity of ANG.



Median of Fluorescence Intensity (MFI)				
7.00	47.95	41.97	42.96	46.60

Figure 4.2 CD4 expression on primary peripheral blood mononuclear cells with ANG treatment (0.25μM, 0.5 μM, and 1 μM).
The median of fluorescence intensity (MFI) of whole cell population was shown in and below the figure.

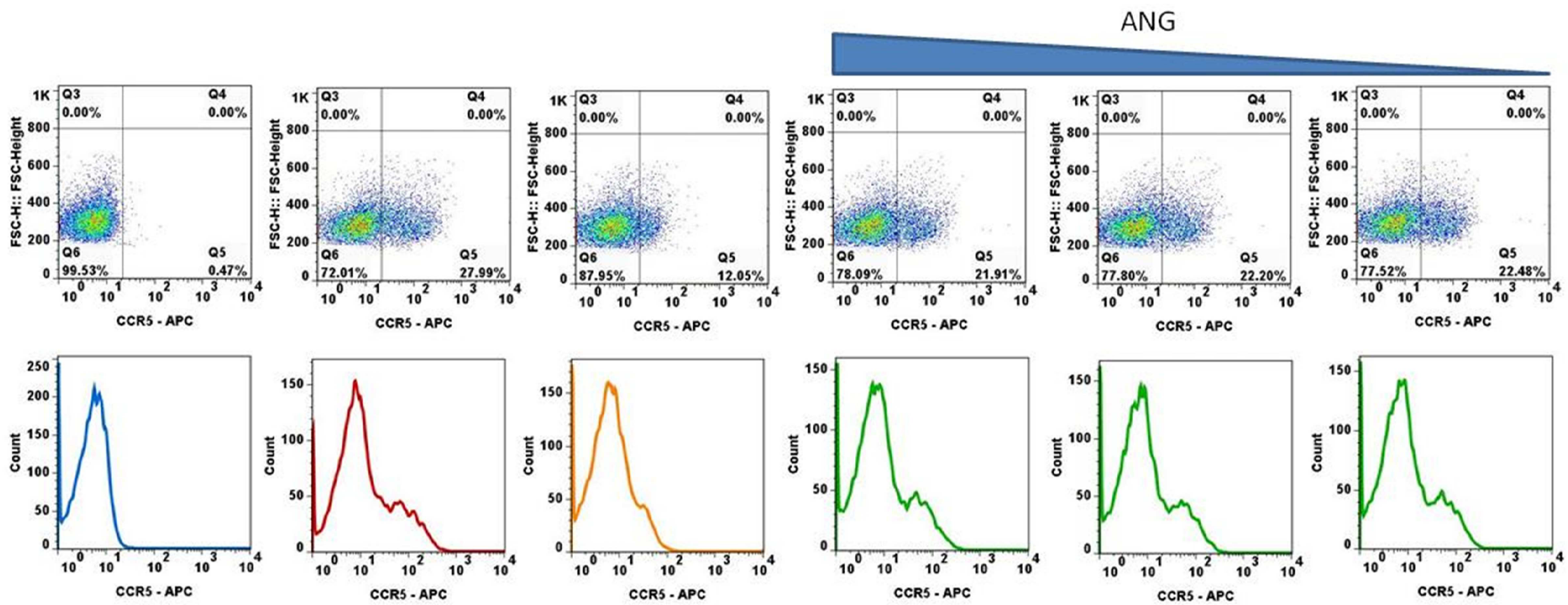
Color	Antibody
	CD4 – FITC
	IgG1 – FITC (Isotype)
	CD4 – FITC + ANG



Median of Fluorescence Intensity (MFI)					
4.55	325.51	17.28	203.36	219.72	215.77

Figure 4.3 CXCR4 expression on primary peripheral blood mononuclear cells with ANG treatment (0.25µM, 0.5 µM, and 1 µM).
The chemokine stromal-derived factor-1 (SDF-1 or CXCL12) was used to block CXCR4. The median of fluorescence intensity (MFI) of whole cell population was shown in and below the figure.

Color	Antibody
(red)	CXCR4 – PE
(blue)	IgG2 Aα – PE (Isotype)
(orange)	CXCR4 – PE+ SDF
(green)	CXCR4 – PE+ ANG



Median of Fluorescence Intensity (MFI)					
5.11	9.23	6.22	7.29	7.37	7.50

Figure 4.4 CXCR5 expression on primary peripheral blood mononuclear cells with ANG treatment (0.25µM, 0.5 µM, and 1 µM).
The regulated upon activation, normal T-cell expressed, and secreted (RANTES or CCL5) was used to block CCR5. The median of fluorescence intensity (MFI) of whole cell population was shown in and below the figure.

Color	Antibody
(red)	CCR5 – APC
(blue)	IgG2 Aα – APC (Isotype)
(orange)	CCR5 – APC+ RANTES
(green)	CCR5 – APC+ ANG

Chapter 5: To determine if ANG cleaves tRNA-Lys

Introduction

ANG is a member of the pancreatic RNase A superfamily. ANG not only possess ribonucleolytic activity, but also known for its potent angiogenic activity *in vivo*. Previous studies show that its RNase activity is relatively weak, as it is about 10^4 to 10^6-fold lower than that of bovine RNase A. However, it has been suggested that the ribonuclease activity of ANG cleaves the tRNA specifically. Some studies have shown that tRNA is cleaved by ANG in the anticodon loop when the cell is in a stress condition, resulting in two products corresponding to the 3' end and 5' end tRNA halves of tRNA.[16, 17] However, tRNAs are also important for HIV-1 replication including the processes of reverse transcription and translation. For early stage of HIV-1 reverse transcription, tRNA-Lys is primer used initiate reverse transcription that will result in proviral DNA, so that tRNA cleavage caused by ANG may lead to HIV-1 inhibition. Therefore, we hypothesize that ANG inhibits HIV-1 replication by cleaving tRNA-Lys.

Experimental Design

tRNA-Lys serving as a primer plays an important role of HIV-1 reverse transcription. As ANG has been shown its RNase activity to tRNA, HIV-1 reverse transcription could be inhibited if tRNA-Lys is cleaved by ANG. In order to identify if ANG cleave tRNA-Lys, we incubated tRNA-Lys with ANG and the results were detected by running 15% TBE-urea gel. The tRNA-Lys we use is the modified tRNA-Lys labeled with the fluorophore BODIPY-FL (BODIPY-FL-Lys-tRNA; Promega, Cat no.L5001). tRNA-Lys was incubated with and without RNase A for controls. The band of tRNA-Lys was expectedly shown on 74nt. If ANG cleaves tRNA-Lys, tiRNA (30 nt and 40 nt), or tRNA fragments (<74nt) were expectedly to see in the 15% TBE urea gel.

tRNA-Lys, in the first experiment, was treated with RNase, ANG or RNaseA, and incubated at 37°C for 3min. After the 37°C incubation, we heated the tRNA-Lys and RNase mixture at 70°C for 3min for denaturing RNA. Samples were then carefully loaded into 15% TBE-urea gel, and the gel was run at 200V. After about 2 hours gel running, gel was stained with Ethidium bromide (EtBr) with shaking at room temperature for 20min, and then destained for 20 min. Finally, the gel was exposed to ultraviolet light, and a picture was taken with an alpha innotech imager.

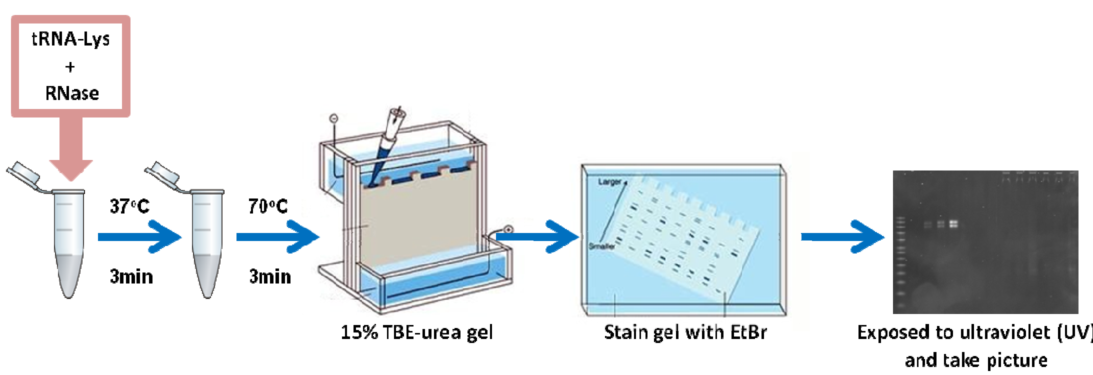


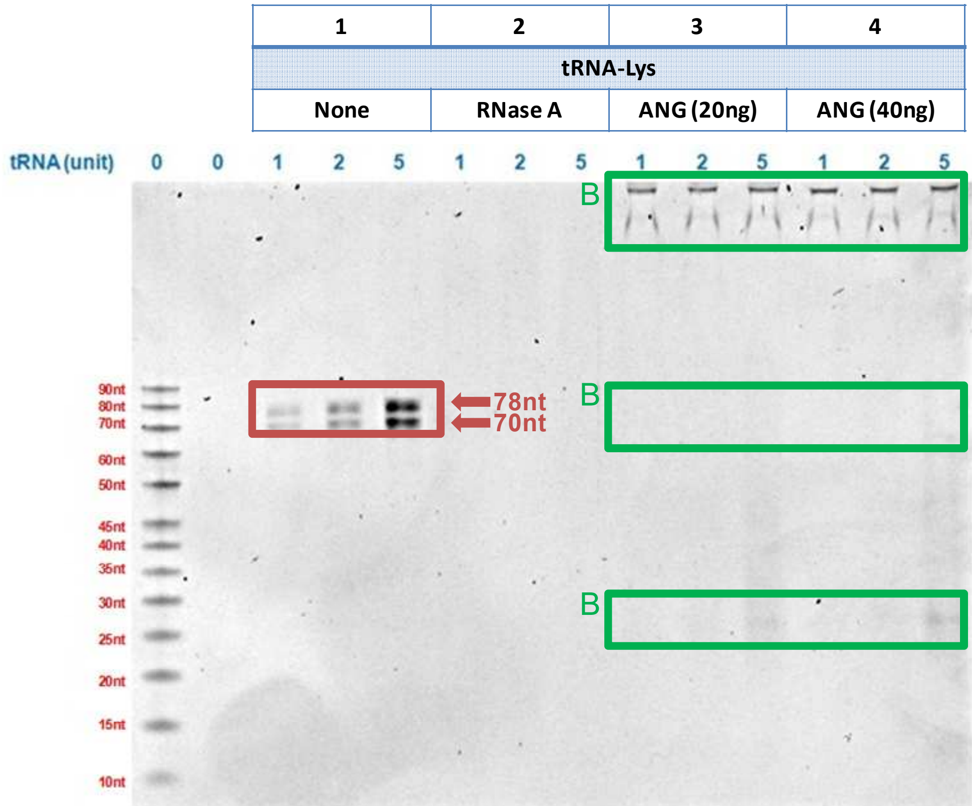
Figure 5.1 Experimental design for determining if tRNA-Lys is cleaved by ANG

We designed a second experiment to test whether bands of higher molecular weight was due ANG binding with tRNA-Lys in a complex. tRNA-Lys was incubated with 1st enzyme (ANG, RNaseA, or proteinase K (PK)) for 30min at 37^{o}C, and 2nd enzyme (ANG, RNaseA, or PK) for another 30min at 37^{o}C. Then these samples were loaded into 15% TBE-urea gel analyzed as in the previous experiment.

Results

The result of the first experiment with 15% TBE-urea gel is shown on the figure 5.2. The samples were divided in four groups, 1-4 groups, and each one made of three wells with different units of tRNA (1, 2, and 5 units). Group 1 was only loaded with tRNA-Lys. tRNA treated with RNaseA group was loaded on group 2. tRNA-Lys treated with 40ng ANG was loaded on group 3. tRNA-Lys treated with 20ng ANG was loaded in wells group 4. (Fig 5.2A) We observed two bands on the gel using tRNA-Lys without RNase treatment. We estimated the sizes of the two bands to be 70 and 78nt (expected band size=74nt) by comparison with low molecular RNA ladder. (Group 1, Fig 5.2A) Incubating tRNA with RNase A as a control resulted in no visible band on the gel (Group 2, Fig 5.2A). In the case of the treatment groups with 40ng and 20ng ANG, no bands of 70 and 78nt were detectable; however, a clear band was present in the high nucleotide size area on the top of gel, and a faint band of about 28 nt was detected. (Fig 5.2)

A



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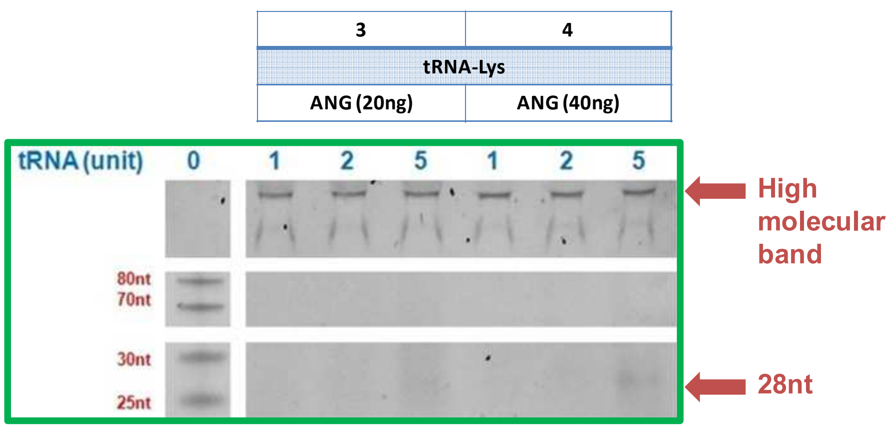


Figure 5.2 (A)Cleavage of Ethidium bromide (EtBr) staining BODIPY-FL-Lys-tRNALys by Angiogenin. In addition to the wells for molecular weight marker (M) and negative control (lane 1), four groups were analyzed including tRNA-Lys (lanes 2-4), tRNA-Lys incubated with RNase A (lanes 5-7), and tRNA-Lys incubated with 40ng ANG (lanes 8-10) and with 20ng ANG (lanes 11- 13). 1, 2, and 5 units of tRNA-Lys are used for each group. BODIPY-FL-Lys-tRNALys without RNase treatment presented two bands, 70nt and 78nt which are shown in the orange box in lanes 2-4. **(B) There is one clear band in the high nucleotide area and one faint band on 28nt. (**H: High molecular bands)

The results of the second experiment incubating tRNA-Lys with and without PK are shown in figures 5.5 and 5.6. In these two gels, the samples were set on five groups per gel **(Table 1),** each made of three wells with different units of tRNA (1, 2, and 5 units). The length of incubation (30min) was longer than the first experiment.

Group	Substrate	1st Enzyme (30min)	2nd Enzyme (30min)
I	tRNA-Lys	none	none
II	tRNA-Lys	RNase A	none
III	tRNA-Lys	ANG	none
IV	tRNA-Lys	RNase A	ANG
V	tRNA-Lys	ANG	RNase A
VI	tRNA-Lys	Proteinase K	none
VII	tRNA-Lys	Proteinase K	RNase A
VIII	tRNA-Lys	Proteinase K	ANG
IX	tRNA-Lys	RNase A	Proteinase K
X	tRNA-Lys	ANG	Proteinase K

Table 1 Groups of tRNA-Lys cleavage experiment Samples in groups A-E are not treated with PK (Fig5.3).

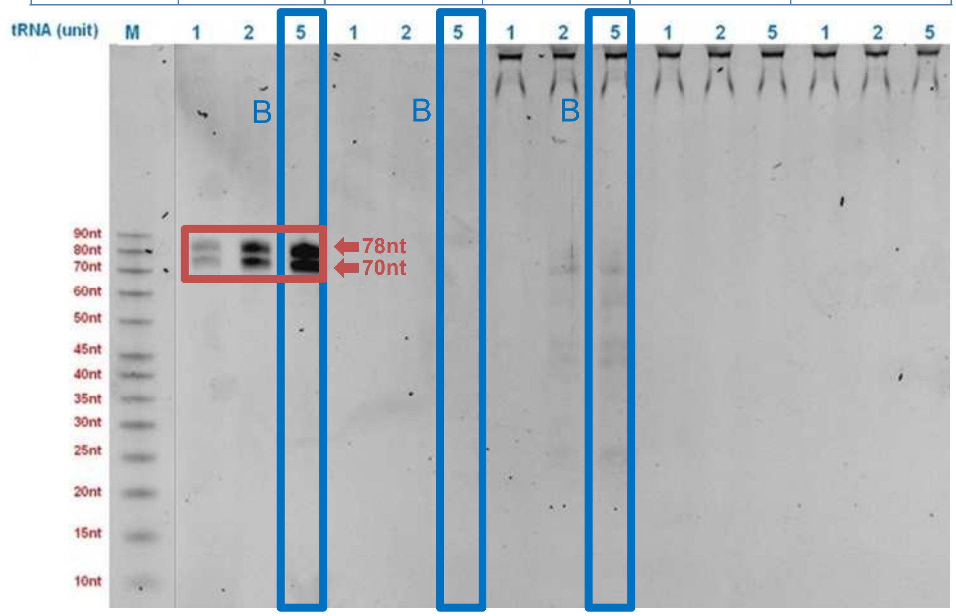
tRNA-Lys without any treatment (group I). tRNA incubated with RNaseA group (group II). tRNA-Lys incubated with 40ng ANG (group III). tRNA-Lys incubated with RNase A and then 40ng ANG (group IV). tRNA-Lys incubated with 40ng ANG and then RNase A (group V). In the experiment with PK treatment (Fig 5.4), tRNA-Lys incubated with PK group (group VI). tRNA incubated with PK and then RNaseA group (group VII). tRNA-Lys incubated with PK and then 40ng ANG (group VIII). tRNA-Lys first incubated with RNase A and then PK group (group IX). tRNA-Lys first incubated with ANG and then PK (group X).

As we observed in the previous experiment, undigested tRNA-Lys showed two bands on the gel, 70 and 78nt (expected band size=74nt) and incubating tRNA-Lys with RNase A as a control resulted in no bands (Group I and II, Fig 5.3A) Incubation of tRNA-Lys with 40ng ANG resulted in a clear band in the high nucleotide size area on the top of gel, and some faint bands on including 78, 70, 64, 48, 46, and 29 nt were detected. (Group III, Fig 5.3B)

Two bands of 70 and 78 nt were still observed when tRNA-Lys was incubated with PK (Group VI, Fig 5.4A). However, no band was observed when tRNA-Lys was incubated with PK and RNase A (goups II and VII, Fig 5.3A and 5.4A). Incubation of the tRNA-Lys with PK prior or after 40ng ANG (group VIII and X, Fig 5.4), neither the 70 and 78nt bands nor high nucleotide size bands in the top area of the gel was observed. However, in the groups VIII and X, some faint bands were detected with estimated sizes of 53, 41, and 37 nt, and 30nt (Fig 5.4B)

A

Group	I	II	III	IV	V
1st Enz	-	RNase A	ANG	RNase A	ANG
2nd Enz	-	-	-	ANG	RNase A



B

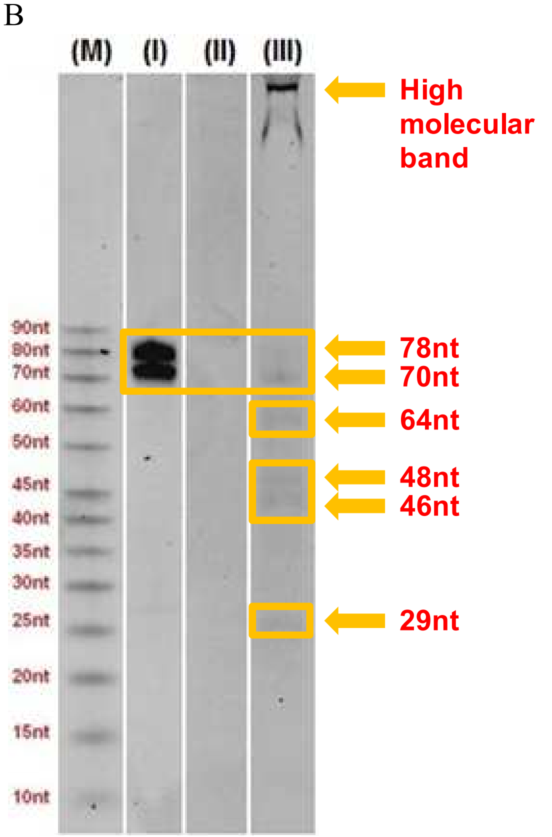
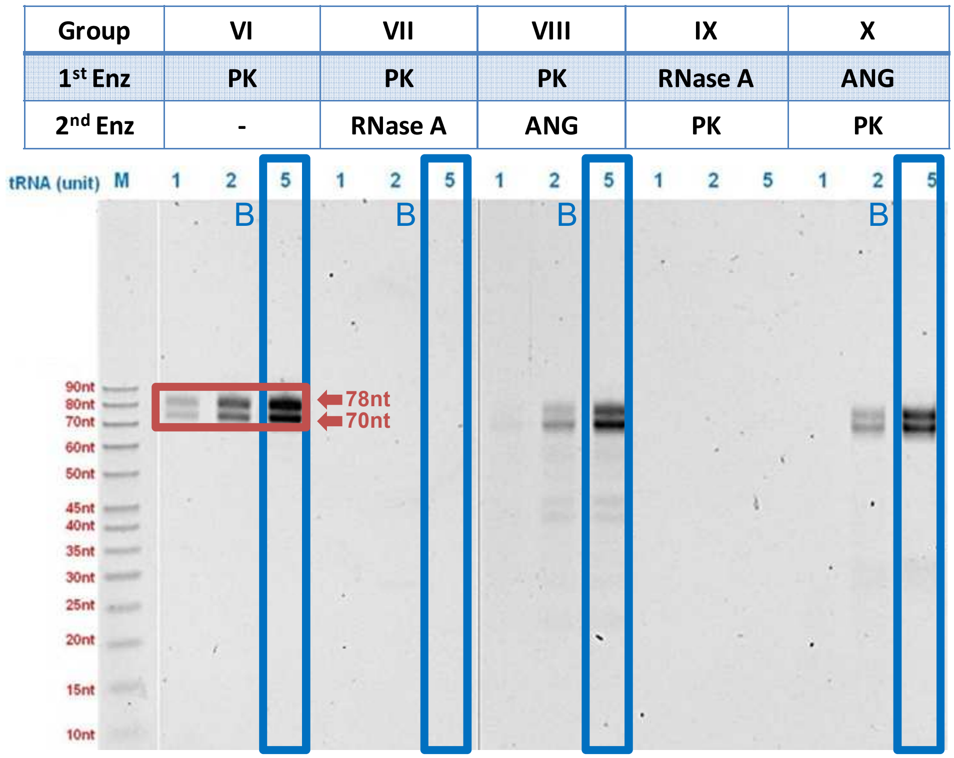


Figure 5.3 (A) tRNA-Lys incubated with RNase and ANG. In addition to marker (M) and negative control, five groups including tRNA-Lys (group I, lane1- 3), tRNA-Lys incubated with RNase A (group II, lanes 4- 6), tRNA-Lys incubated with ANG (group III, lanes 7- 9), tRNA-Lys incubated with RNaseA and then ANG (group IV, line 10, 11, 12), and tRNA-Lys incubated with ANG and then RNase A (group V, lanes 13-15). 1, 2 and 5 units of tRNA-Lys are used for each group. **(B) Compared to the lane of marker, tRNA-Lys, and tRNA-Lys treated with ANG.** We calculated the size of band based on the marker. tRNA-Lys without adding ANG (group I) shows two bands, 70 and 78nt. In the lane of tRNA-Lys incubated with ANG (group III), in additional to the strongest band of the high nucleotide size, the other weaker bands including 78, 70, 64, 48, 46, and 29 nt.

A



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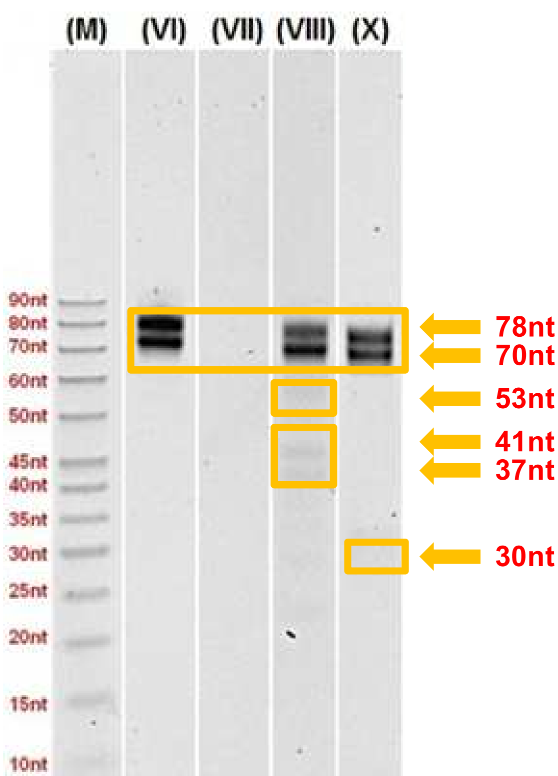

Figure 5.4 (A) tRNA-Lys incubated with RNase A, ANG, and protease K (PK). In addition to marker (M), there are five groups including tRNA-Lys (group VI), tRNA-Lys incubated with PK and then RNase A (group VII), tRNA-Lys incubated with PK and then ANG (group VIII), tRNA-Lys incubated with RNase A and then PK (group IX), and tRNA-Lys incubated with ANG and then PK (group X), and each one made of wells with different units of tRNA-Lys (1, 2, and 5 units). **(B) Compare different lanes including marker, and groups VI, VIII, and X.** Bands, 70 and 78nt, were observed on group VI, VIII, and X. None of those groups showed high molecular band. Moreover, 53, 41, and 37 nt are observed on group IX, and 30nt is observed on group X.

Discussion

The bands of estimated 70nt and 78nt that we detected in the control, untreated samples of tRNA-Lys are compatible with the expected size of tRNA-Lys (74nt). It is possible that the two bands may indicate the tRNA-Lys with and without fluorescence dye, BOPDIP-FL. For the groups of tRNA-Lys treated with ANG, the band in the high nucleotide area may be due to ANG binding with tRNA-Lys. However, the faint band detected at around 30 nt and 40 nt may indicate that a fragment of tRNA-Lys results from enzymatic cleavage. Previous studies showed ANG cleaves tRNA (70nt-90nt) in its anticodon loop to 5' end and 3' end fragments whose size is around 30 nt and 40 nt which is compatible to the size that we observed.[17] In addition, our results suggest that ANG could inhibit HIV-1 reverse transcription by cleaving the tRNA-Lys and/or by binding and sequestering it. We observed that, in absence of RNase, tRNA-Lys displayed two bands of 70nt and 78nt which disappeared after incubation with RNaseA. High molecular bands were observed when tRNA-Lys was incubated with ANG. The results suggest that high molecular band was due to ANG bound to tRNA-Lys into a high molecular weight complex while bands of 70nt and 78nt disappeared.

In order to determine if ANG cleaves or binds with tRNA-Lys, we used PK to digest ANG in the next experiment. 5 incubation groups were set up, with or without PK (Table 1.). In the control groups I and II in Fig 5.5 and F and G in Fig 5.6, tRNA-Lys was fully degraded by RNase A. The results of group I, II, and III in Fig 5.5 were comparable to results of group 1, 2, and 4 shown in Fig5.2 despite the longer incubation time used in the second experiment. Groups IV and V, where tRNA-Lys was incubated with RNase A and ANG in different order showed the high molecular bands that were observed in group

III. Therefore, the results from group I to V confirmed that the bands located on high molecular size were specific to incubation with ANG. However, the high molecular bands were not present after incubation with PK as shown in groups VIII, IX, and X. Comparing groups III and X shows that in the latter the higher molecular size complex was not observed when tRNA-Lys was incubated with PK after ANG, and the bands of 70nt and 78nt indicated that digestion did not take place. PK did not affect tRNA-Lys as expected (Group V).

In groups VIII and X, incubation of tRNA-Lys with ANG and PK in different order not only resulted in the 70nt and 78nt and no high molecular bands, but were also observed some faint bands (Fig 5.7), showing that ANG has RNase activity on tRNA-Lys. In summary, our results raise the possibility that ANG inhibit HIV-1 reverse transcription by specifically cleaving tRNA-Lys or binding with tRNA and sequester it.

Based on the Michaelis–Menten kinetic model of a single-substrate reaction, the Michaelis constant K_M indicates the dissociation constant K_D of the enzyme and substrate complex, and Kcat, the turnover number, points the number of substrate molecule each enzyme site converts to product per unit time when the enzyme is working at maximum efficiency. Therefore, as tRNA-Lys may be a substrate of ANG, our results suggest that ANG may has high affinity with tRNA-Lys with low K_M, and it may need longer time (>30min) to complete catalysis with low K_{cat}.

tRNA-Lys has been reported to interact with viral RNA in the early stage of reverse transcription. The secondary structure of viral RNA and tRNA-Lys complex showed that not only the acceptor stem binds with primer binding site of viral RNA, but the anti-codon stem loop also plays a crucial role in the stabilization. Therefore, as our studies indicated that ANG has high infinity with tRNA-Lys, it is possible that reverse transcription of HIV-1 may be inhibited by sequestering anti-codon and receptor stem of tRNA-Lys.

Chapter 6: Summary and Discussion

ANG inhibits HIV-1 replication, both X4 and R5 virus, in PBMCs

Our studies demonstrated ANG inhibits HIV-1, X4 and R5 tropic strains virus, replication in activated PBMCs. In both X4 and R5 tropic viruses infection, inhibition of HIV-1 was observed irrespective of whether PBMCs were incubated with ANG prior and after to HIV-1 infection ("pre treatment") , or only post to HIV-1 infection ("post treatment"). Both of the two previous studies from Bedoya *et al* and Cocchi *et al* showed that ANG inhibits X4 tropic HIV-1. However, our studies not only confirm that ANG inhibits X4 tropic virus, but also determine its inhibitory effect on R5 tropic virus. Notably, our study is the first to demonstrate ANG inhibiting R5 tropic virus in activated PBMCs. Our finding that demonstrates ANG inhibiting HIV-1 replication even 2 hours after infection ("post treatment") suggests that ANG is acting on this virus after the virus has entered the cells. One possible mechanism for ANG to mediate this effect is that ANG could be uptaken by cells where it could interact and/or degrade the RNA of the infecting virus and/or the newly produced viral RNA. HIV-1 Inhibition was also observed when activated PBMCs were incubated with ANG prior and after to HIV-1 infection ("pre treatment"). HIV-1 inhibition in ANG pre treatment raises the possibility that ANG can exert a direct influence on HIV-1 entry by interacting with or modulate extracellular receptors of HIV-1. In addition, it is also possible that ANG enters PBMCsvia ANG receptor in the absence of HIV-1 infection. Then after infecting PBMCs, HIV-1 enters the target cell where ANG degrades HIV-1 intracellular RNA. The inhibition of viral replication in PBMCs after 2 hour HIV-1 infection ("post treatment") points to the therapeutic potential of ANG. The time from gp120-CD4 binding to the migration of viral cDNA into the target cell nucleus has been estimated to be around 6 hours.[122] Therefore, ANG may affect the period from early HIV-1 replication to integration including interacting with and/or cleaving viral RNA or tRNA before or during reverse transcription. The tRNA specifically cleaved by ANG may be functional as tiRNA for silencing intracellular protein translation, and influence HIV-1 replication. Moreover, since tRNA-Lys serves as a primer in viral reverse transcription, it is conceivable that the degradation of tRNA-Lys by ANG result in inhibition of reverse transcription. Our results provide some mechanistic insights into the activity of ANG, which could be exploited to provide new therapeutic anti-HIV agents. In order to understand the mechanism of ANG anti-HIV-1 activity, and to determine if ANG inhibits HIV-1 replication at the level of viral entry, we tested whether the presence of ANG modulates expression of HIV-1 receptors and post-entry steps of HIV-1 replication.

ANG has limit effect on the expression of CXCR4 and/or CCR5

When we measured expression of HIV-1 receptors by flow cytometry assay, we found that ANG does not down-regulate the expression of receptor (CD4). However, ANG may have limit effect on the main co-receptors (CXCR4 and CCR5) expression. This result may raise a possibility that ANG may inhibit HIV-1 replication by down-regulating CXCR4 and/or CCR5.

ANG inhibit AMLVs

This study used AMLV pseudo-type virus (AMLVs) to determine if ANG is capable to inhibit HIV-1 intracellularly. We observed AMLVs inhibition when ANG was used prior and after infection and when it was used only after AMLV infection in activated PBMCs. This suggests that ANG may inhibit HIV-1 replication in post-entry step. Since ANG has been shown to bind to a specific receptor and to a binding protein, ANG may use one or both of them to enter PBMCs through endocytosis. During the process of HIV-1 replication in cytoplasm, ANG may inhibit HIV-1 reverse transcription or new HIV-1 assembling by targeting viral RNA or the tRNA primer.

ANG cleavage tRNA-Lys

We also found ANG may inhibit HIV-1 by cleaving tRNA-Lys which is served as a specific primer for HIV-1 reverse transcription. ANG, RNase 5, has been shown in the previous studies that it has ribonucleolytic activity, with specificity to RNA substrates including tRNA. We tested if ANG interacts with tRNA-Lys, and the results suggest ANG specifically bind to and cleave tRNA-Lys. The tight binding between ANG and tRNA-Lys that we observed raises the possibility that ANG may inhibit HIV-1 reverse transcription by binding to the primer, tRNA-Lys, and sequester it. In Michaelis-Menten kinetics, Michaelis constant (K_M) indicates dissociation constant (K_D) of enzyme-substrate complex. Therefore, low K_M enzyme may also diplay high affinity with its substrate, but requiring long incubation time to complete the enzymatic processes. After treating tRNA-Lys with ANG and using PK to digest ANG, we detected bands ranging from 29 to 48 nucleotides. Previous studies showed that ANG cleaves tRNA in the anti-codon site producing tiRNA of 33nt and 40nt, which are compatible with some of the tRNA-Lys fragments we observed. However the bands of tRNA-Lys fragments were relatively faint suggesting that either the recombinant form of ANG we used has a low activity or ANG needs longer time to fully digest tRNA-Lys.

ANG inhibit HIV-1 mediate with a intracellular mechanism

In conclusion, our studies show that ANG inhibits both X4 and R5 HIV-1 isolates, without down regulating HIV-1 receptors CD4. However, ANG may inhibit HIV-1cellular entry by its limit effect on HIV-1 co-receptors, CXCR4 and CCR5. Post-entry ANG inhibits HIV-1, and ANG inhibits single-round AMLV/HIV pseudo-type virus in PBMCs raise a possibility that ANG inhibits HIV-1 mediated with an intracellular mechanism. In addition, this study also showed ANG may inhibit HIV-1 reverse transcription by binding and/or cleaving tRNA-Lys. These results suggest that ANG inhibits HIV-1 replication mediated with a complex mechanism which including an intracellular mechanism.

Based on this studies shown ANG may inhibit HIV-1 mediated a intracellular, the continuing studies could possibly focus on determining where is the intracellular location ANG interaction with RNA, such as tRNA-Lys and viral RNA, and/or proteins, such as CD4, CXCR4, and CCR5, which are associated with HIV-1 replication. The location and interaction of ANG and other RNAs or proteins could be stained with antibody conjugated with different fluorescences and observed by conforcoal microscope. For detecting if ANG down regulate CD4, CXCR4, and/or CCR5 expression per cell, these receptors could be stained with PE and receptors could be detected by using phycoerythrin fluorescence quantitation kit (BD, Cat # 340495). In addition, ANG could also be detected if it cleaves or sequesters to viral RNA by running RNA denaturing gel. Furthermore, as previous studies suggest ANG may enhance rRNA expression by binding with nuclear DNA, ANG may also need to determine if it is able to down-regulate HIV-1

RNA transcription. In addition, it may important to determine if the tRNA fragments produced by ANG have any effect on HIV-1 protein translation.

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