# MicroRNA Machinery as an Integral Component of Viral Transcription Mechanisms and Drug Efficacy

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## Dedication

To my family, friends, and co-workers who helped and supported me in my research and thesis.

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## Abstract

### MicroRNA Machinery as an Integral Component of Viral Transcription Mechanisms and Drug Efficacy

Human immunodeficiency virus is the foremost cause of acquired immunodeficiency syndrome. The current treatments do not focus on how in latent cells, the virus could still mutate. A drug resistant virus within the host will eventually lead to AIDS. Researchers must gain an appreciation of how latent cells function to keep the virus alive and away from immune response. In recent findings, the ability of HIV-1 to create its own viral microRNA from the TAR hairpin element is important for manipulating host cell mechanisms and maintaining latency. HIV-1 manipulates host cell mechanisms, such as cyclin dependent kinases and their corresponding cyclin elements, to suppress apoptotic cellular pathways and to maintain a latent infection. The therapeutic focus now turns to developing effective cdk inhibitors that eliminate viral replication, but do not affect normal cellular mechanisms. To do this, researchers need to understand the importance of how RNAi plays a role in viral activation and drug efficacy. A thorough understanding of HIV-1 microRNA pathways and latency could lead to alternative treatment options for HIV-1 infection.

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| | |
|---|---|
| AIDS | Acquired Immunodeficiency Syndrome |
| HIV-1 | Human Immunodeficiency Virus – 1 |
| HAART | Highly Active Antiretroviral Therapy |
| PIC | Pre-integration Complex |
| LTR | Long Terminal Repeat |
| TAR | Transactivation Response Element |
| RNAi | RNA interference |
| pTEFb | Positive Transcription Elongation Factor b |
| CDK | Cyclin-dependent Kinase |
| CTD | C-terminal Domain |
| PBS | Phosphate Buffered Saline |
| CAT Assay | Chloramphenicol Acetyltransferase Assay |
| ChIP | Chromatin Immunoprecipitation |

# CHAPTER I: INTRODUCTION

## A. The Global Impact of HIV-1

Human immunodeficiency virus (HIV-1) is the etiological agent of acquired immunodeficiency syndrome (AIDS). Approximately 33 million people are currently infected with HIV-1 and 3 million new cases will be identified this year alone [1]. Highly active antiretroviral therapy (HAART) is currently the most effective HIV-1 treatment and has been shown to significantly reduce AIDS-related mortality. HAART is an effective multi-drug therapy that improves immune response to HIV-1 and most importantly lowers viral loads. Therefore, patients present with significantly less symptoms of complete HIV-1 infections, including AIDS. One of the most well known drugs composing HAART therapy is a nucleoside reverse transcriptase inhibitor, 3'-azido-3'deoxythymidine (zidovudine/AZT). Since reverse transcriptase is integral for retroviral infection, AZT seems to be the key to preventing further HIV-1 infection. However, these drugs target specific viral proteins. This makes them less effective if small nucleotide polymorphisms (SNPs) occur. Since latently infected cells still produce small amounts of virus, there is a chance that one of the new virus variants have a SNP that allow some resistance to the drugs. The threat of a SNP-derived resistance to

therapies is possible since the viral reverse transcriptase has a high rate of mutation. If the HAART therapy becomes ineffective or a patient decided to halt treatment, the little amounts of virus in the latently infected cells would have the ability to infect healthy cells and lead towards AIDS.

Current therapies, including HAART, for HIV-1 are ineffective at eliminating the virus from the system and drug-resistant virus production is a possibility. Therefore, research should focus on developing treatments that target the viral life cycle to eliminate production. HIV-1 only encodes for nine genes and creating a drug that targets a region should seem relatively simple. However, HIV-1 is significantly more complex and incorporates the use of cellular mechanisms for its own benefit. Mapping out these viral-cellular interactions enables researches to examine various biomarkers. Understanding the virus-host relationship is integral in developing any effective future therapeutic.

## B. The Biology of HIV-1

### 1. Basics of HIV-1 Infection.

HIV-1 is a retrovirus (lentivirus subfamily) that primary infects the human immune system cells ($CD4^+$ T cells, macrophages, dendritic cells). Retroviruses have an icosahedral shape, but are enveloped by a lipid bilayer from the host plasma membrane. This family of virus only has a virion diameter of 80-130nm, specifically 120nm for HIV-1 [2]. HIV-1 infection begins with a short acute phase followed by a long chronic phase. During the acute phase, there is a spike in the viral load and plateau in the chronic phase. $CD4^+$ T cell population is significantly decreased in the chronic phase. The body becomes very susceptible to various opportunistic infections and eventually will progress

to AIDS. The treatments are currently sufficient to lower new viral infection and keep $CD4^+$ T cells at a level that will prevent progression to AIDS. These anti-retroviral drugs are the only approach keeping HIV-1 infected patients alive.

**2. The HIV-1 genome and life cycle.**

Retroviruses have a genome size between 3.5-9kb. These are the smallest of RNA viruses in terms of genome size. Retroviruses contain (+) single-stranded RNA separated into two copies. The genetic material is surrounded by a lipid membrane that have a gp41 (transmembrane glycoprotein) and gp120 (docking glycoprotein) to recognize and enter cells. Within the lipid membrane is viral capsid composed of thousands of p24 viral protein. The capsid surrounds the two copies of the viral (+)-ssRNA. This type of virus contains its own virion polymerase, reverse transcriptase. This reverse transcriptase uses the viral RNA to make DNA that integrates into the host genome.

The HIV-1 genome consists of nine genes that are expressed from a single promoter located in the viral long terminal repeat (LTR) [3, 4] (Figure 1). Three conserved lentiviral genes are *gag, pol,* and *env*; which are in charge of making the structural proteins necessary for new viral production. The other six genes, *tat, rev, nef, vpr, vpu,* and *vif* are HIV-1 regulatory genes that determine infectivity of the virus. A few of the more important and most understood viral genes are discussed in terms of their part in infecting target cells and propagating HIV-1 within the host.

HIV-1 *env* plays a critical role in the initial virion-host cell interaction. This gene codes for the proteins responsible for cell-to-cell interactions, gp160. The gp160

precursor polypeptide is cleaved into two non-covalently associated subunits, gp120 and gp41 [5, 6]. The viral gp120 subunit is responsible for binding to CD4 and chemokine host cell receptors. Once gp120 determines the tropism of binding, the viral gp41 subunit directs the fusion between the viral particle and host cell membrane [5].

Once the virus enters the host cell, the HIV-1 genome intends to incorporate into the cellular DNA. However, host cell mechanisms attempt to eliminate the virus and its production as soon as possible. The HIV-1 *nef* gene (negative factor) codes for a protein very important to viral pathogenesis. This protein alters cellular signal transduction pathways by downregulating vesicle trafficking and presence of cell-surface receptors. This downregulation allows the infected cells to avoid the immune system responders, allowing the virus to reproduce and eventually infect more cells [7-11]. Viral *vpr* also aids in allowing more time for the virus to replicate by arresting cells in G2 and interacting with cdk2 [12-14]. The viral RNA is then able to freely transcribe into viral cDNA using the *pol*, reverse transcriptase.

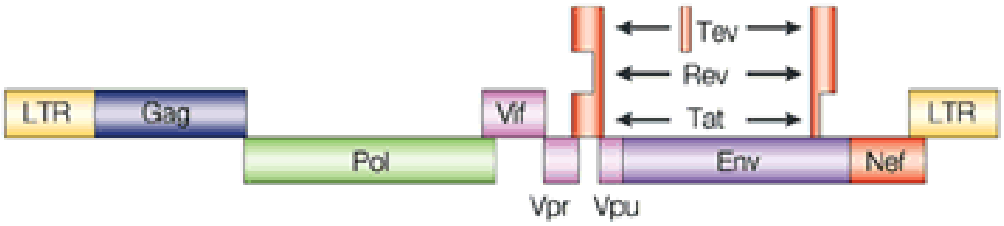
The HIV-1 *gag* protein drives the localization of the viral genome into the cell nucleus. *Gag* is translated first into a precursor Pr55Gag protein. This polyprotein does not actually cleave until after HIV-1 buds from the cellular membrane. Shortly after budding, HIV-1 protease cleaves the Pr55Gag protein to the mature *gag* proteins: p6, p7 nucleocapsid, p24 capsid, and p17 matrix [15]. The *vpr* protein then associates with p6 to aid in nuclear import. The mature HIV-1 *gag* proteins, viral integrase and nucleic acids are encompassed into a preintegration complex. Upon infection, this preintegration complex associates with some cellular proteins and cytoskeleton for shuttling to the nuclear membrane [16]. The viral-host complex is able to penetrate the nuclear

membrane and release the HIV-1 genome safely into the nucleus [17]. The viral genome is then able to integrate with the host cell DNA. From there, many viral factors such as *vpr* and *tat,* stimulate viral transcription. More specifically, *vpr* and *tat* associate with cyclin T1, CBP/p300, and Sp1 to activate the HIV-1 LTR [13, 18, 19-22]. The effects of *tat* are vast and will be discussed in a future section. After many transcripts are transcribed, the new viral RNA needs to be exported from the nucleus. *Rev* contains a nuclear export signal that allows the viral unspliced RNA to avoid the nuclear retention signal and leave the nucleus [151]. When the viral RNA leaves the nucleus, new virions are created and released to infect other host cells.

HIV-1 relies on its complex interactions with host cell mechanisms in order to survive and reproduce. The viral life cycle includes binding of the virion to the host cell, fusion of the virus to the cell membrane, assembly of the preintegration complex, reverse transcription of the viral RNA, HIV-1 cDNA transformation, viral gene expression and maturation, viral assembly, and budding of a new HIV-1 virion [23-29]. After a host becomes exposed to HIV-1, the virions bind to a specific target host cell via the interaction of viral gp120 with the cellular membrane CD4 surface receptor. This interaction with the recruitment of other cellular co-receptors leads to a conformational change in the gp120. Viral gp41 is now exposed to bind to the host cell membrane and allow the virion contents to enter the cell. Upon entering the cell, the HIV-1 RNA is transcribed to pro-viral DNA via the viral reverse transcriptase. This pro-viral DNA is combined into the preintegration complex and shuttled to the host cell nucleus. Once the viral DNA incorporates into host cell genome, the HIV-1 LTR is able to mimic a polymerase II promoter and initiate transcription using the host cell machinery. At first,

*tat* and *nef* genes are translated. *Tat* protein then binds to the transactivation response element (TAR) in order to phosphorylate cellular transcription factors to increase viral transcription via a positive feedback cycle [30-33]. Once many transcripts of the viral genome are created, new virions form and bud off from the host cell membrane to target new host cells.

**Figure 1: Organization of viral genes on the HIV-1 genome.** All nine viral proteins (*gag, pol, vif, vpr, tat, rev, env, vpu, and nef)* are indicated, in addition to the long terminal repeats (LTRs) found at both the C-terminal and N-terminal end. The 5' LTR contains the TAR hairpin element that can be part of the abortive transcripts marked by latency. During active infection, the PolII is able to transcribe the entire HIV-1 genome. This includes the *Tev* element, a fusion of parts of the *tat*, *env*, and *rev* genes, only present in a few isolates of HIV-1. The functions of the viral proteins are discussed in further detail in the text.


Tev
Rev
Tat
LTR
Gag
Vif
LTR
Pol
Env
Nef
Vpr
Vpu

**3. Tat as the viral transactivator.**

Viral Tat (transactivator of transcription) protein is arguably the most important protein of the HIV-1 genome. Tat has also been shown to be released by HIV-1 infected cells into the serum and become absorbed into uninfected T-cells (due to Tat having a protein transfer domain) [34]. The Tat that is present in the serum acts as a toxin that induces cell death in these uninfected T-cells [150]. Tat exhibits some apoptotic effects; however, the most important effect of tat is on viral transcription. It is the main transcriptional activator of the HIV-1 LTR and also induces some cellular genes to help the virus reproduce and survive [31-33, 35-37]. Tat binds to the viral TAR element, an RNA stem-loop structure important for elongation. This Tat-TAR complex then recruits both viral and cellular components to initiate and elongate the viral promoter. For example, tat recruits the pTEFb complex. The activated components of this complex, cdk9 and cyclin T1 then hyper-phosphorylate the large subunit of the RNA polymerase II C-terminal domain (CTD) to activate transcription elongation [38]. In terms of affecting viral factors, tat stabilizes the TFIID/TFIIA complex on the HIV-1 TATA box and then recruits TBP/TFIID transcription factor [39-45]. Tat also can change chromatin structure around the HIV-1 LTR. It can shift the nucleosomes so that the activated RNA polymerase II and CBP/p300 can initiate and elongate the viral transcript [46-51]. All of the functions of tat have yet to be discovered; however, the post-translational modifications on tat, such as acetylated lysine 28 and 50, ubiquitinated lysine 71, and methylated arginine residues, could play important roles in how tat is able to employ host cell mechanisms to significantly activate viral transcription [52-54].

**4. Viral manipulations of cellular cyclin dependent kinases.**

The host cell cycle is dependent on the activity of cyclin-dependent kinases (cdks) and their cyclin subunits. The cdk/cyclin complexes aid in the advancement of eukaryotic cells through the G1/S and G2/M cell cycle checkpoints. For the G1/S checkpoint, the cdk2/cyclin E complex phosphorylates the retinoblastoma (Rb) protein [55]. When this G1/S checkpoint is passed, integral proliferative genes are transcribed in order to significantly increase HIV-1 genome replication. The activity of the cdk2/cyclin E complex can be tied to viral tat activity. Tat recruits the cdk2/cyclin E complex to phosphorylate the RNA polymerase II CTD [56-59]. The cdk2/cyclin E is essential for the phosphorylation of the CTD and therefore, tat-activated viral transcription [60-62]. In latently infected cells that have decreased p21/waf1, cdk2/cyclin E showed increased activity to initiate viral transcription and even phosphorylate tat in a positive feedback mechanism *in vitro* [61, 63]. To test the importance of the cdk2/cyclin E complex, researchers knocked down cdk2 with siRNA and found that tat-induced HIV-1 transcription was significantly downregulated [64]. Therefore, the cdk2/cyclin E complex and tat-dependent transcription seem like viable therapeutic targets to prevent increase viral transcription, especially since cdk2 is not essential for eukaryotic proliferation [65].

**C. MicroRNA Machinery and Gene Silencing**

RNA interference (RNAi) is a regulatory mechanism conserved in higher eukaryotes, such as primates and mice. The basis of RNAi is that a small RNA molecule guides a protein effecter complex to a complementary or mostly complementary

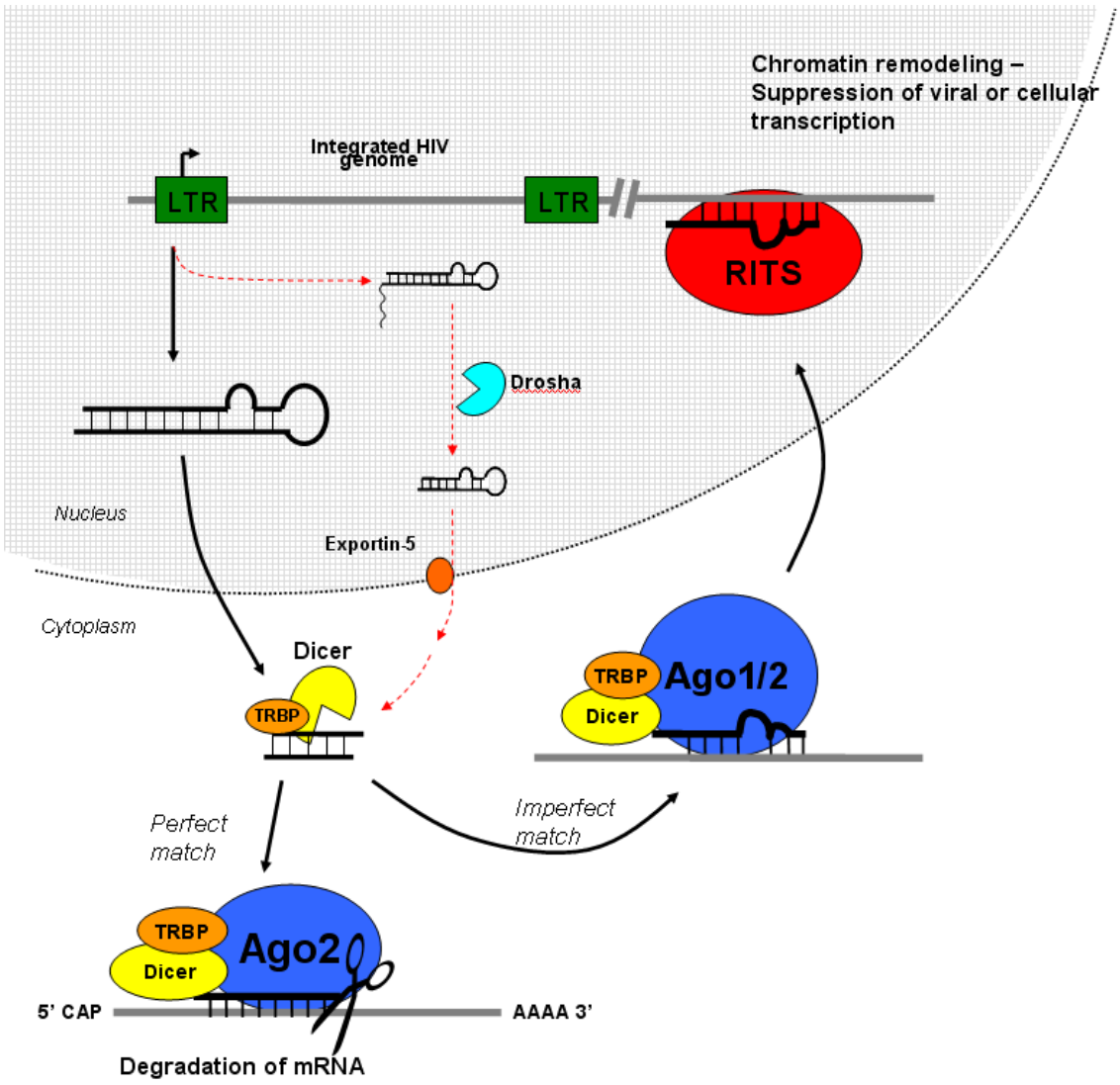
sequence of nucleic acid. The end result is the down regulation of protein expression through either transcriptional silencing, cleavage of target mRNA or inhibition of translation [66]. From this information, we can say that a single microRNA may regulate the expression of multiple proteins.

There are a few mechanisms involved in creating microRNA. MicroRNA is produced from genomic DNA that is transcribed by Pol II (same as mRNA). Exogenously introduced dsRNA is recognized by Dicer, the cellular ribonuclease III enzyme, and cleaved into 21 nucleotide segments called microRNA. Endogenously expressed RNA can be involved in RNAi through a slightly different pathway involving Drosha mediated cleavage of RNA stem-loops in the nucleus, followed by exportation to the cytoplasm by Exportin-5, and finally cleavage by Dicer to generate a small RNA duplex approximately 22 nucleotides in length with a two nucleotide 3' overhang on each strand [67] (Figure 2). One strand of the microRNA duplex is incorporated into an Argonaute containing effector complex which silences gene expression through two different mechanisms. In the first, the small RNA associates with the RNA-induced silencing complex (RISC) and guides the complex to a complementary sequence of mRNA where a member of the Argonaute family of proteins cleaves the target mRNA [68-71]. This perfect matching leads to a silencing of the gene. Alternatively, the microRNA may guide the RISC complex to a somewhat complementary region in the 3'UTR of the mRNA. This association inhibits protein translation without degrading the target mRNA [69, 72, 73] . This non-perfect matching leads to a downregulation of gene expression. In addition to attaching the RISC complex, the RNA can associate with the RNA-induced initiation of transcriptional silencing (RITS) complex. Similar to the RISC

mechanism, the microRNA guides this complex to a complementary region of chromosomal DNA and recruits factors that modify the chromatin structure and induce transcriptional silencing [74-77]. RNAi mechanisms have the potential to be viable therapeutics against HIV-1 infection.

**Figure 2: Model for the generation and action of HIV-1 TAR derived microRNA**

Basal transcription of the HIV-1 LTR produces short RNA transcripts containing the TAR element. Short RNA elements are directed to the cytoplasm where they are processed by Dicer to yield a viral microRNA. Longer viral transcripts can be processed into pre-microRNAs by Drosha. Viral microRNA associates with the RISC or RITS complex and through homology to sequence contained in the viral RNA, as well as the proviral genome, may direct silencing of viral gene expression. Alternatively, the viral microRNA may contain sufficient homology to mediate silencing of cellular genes. Once microRNAs have been generated, Argonaute proteins associate with the microRNAs. If the microRNA matches an mRNA transcript exactly, Ago2 carries out cleavage of the mRNA transcript. In the case of imperfect matches, target mRNAs are sequestered at ribosomal P-bodies or loaded into the transcriptional silencing complex, RITS [139].


Chromatin remodeling –
Suppression of viral or cellular transcription
Integrated HIV genome
LTR
LTR
RITS
Drosha
Nucleus
Exportin-5
Cytoplasm
Dicer
TRBP
TRBP
Ago1/2
Dicer
Perfect match
Imperfect match
TRBP
Dicer
Ago2
5' CAP
AAAA 3'
Degradation of mRNA

**D. Antiviral Therapeutics**

Various therapeutics for HIV-1 are still being theorized and constructed, despite the success of HAART. However, HAART therapy suppresses HIV-1 infections but cannot cure viral infections. Within the HAART multidrug treatment, the drug maraviroc targets the host's CCR5 surface receptor. Maraviroc forces the CCR5 receptor to misfold and then prevents gp120-mediated viral fusion. Therefore, no new infections can occur, but there are still latent cells creating viral genomes. Also, the virus can quickly generate resistant mutants to evade the drug compounds that are used to target viral proteins [78,79]. The reverse transcriptase of HIV-1 has very low fidelity and creates *de novo* mutations in approximately a third of each generation of new viruses. Sometimes, these resistant strains exhibit improved replication in the presence of HAART [80]. Consequently, new anti-HIV-1 therapies need to be developed that target the host proteins that aid in viral replication.

Cellular mechanisms directly involved with HIV-1 transcription are potential drug targets. The tumor suppressor protein, p53 protects the integrity of the genome and is sometimes referred to as the "guardian of the genome" [81]. Wild-type p53 is usually at low levels during normal cell activity; however, p53 is stabilized and activated when the cell is exposed to various stimuli, including DNA damage complexes [82-87]. Activated p53 is involved in apoptosis via transcription-dependent and transcription-independent mechanisms [88-93] and inducing cell cycle arrest at both the G1/S [94-98] and G2/M checkpoints [99-103]. Even though the exact mechanism of p53 activity is not fully understood, researchers have focused on phosphorylation. p53 can be phosphorylated by numerous kinases including ATM, ATR, DNA PK, and mTOR on various residues [104].

Of the eighteen known phosphorylation sites on p53, eleven sites are located within the transactivation domain/Proline-rich domain, three within the DNA-binding domain, three within the C-terminal domain and one with the tetramerization domain [105]. Phosphorylation at Ser15 is activated in response to different stress signals, such as serum starvation [106-110]. Ser15 phosphorylation stimulates the recruitment of p300, CBP, and p/CAF, which function to acetylate the C-terminus of p53, resulting in decreased ubiquitination and proteasomal degradation [111]. Acetylation at the C-terminus followed by interaction with the ubiquitin ligase MDM2 results in the stabilization of p53 [112-116]. In the end, p53-phospho-Ser15 is stable and able to transcribe its downstream targets. One downstream target that is important because if its roles in cell cycle checkpoints, differentiation, and cellular senescence is p21/waf1 [117, 118]. p21/waf1 is a cdk inhibitor that binds through a N-terminal domain and to its cyclin partner [119]. This inhibition is important since the cdk/cyclin complexes are very important for viral transcription. Therefore p53 activators and cdk inhibitors (9AA, Flavopiridol, Cyc202, etc.) could have significant roles in hindering viral transcription.

A new and upcoming therapeutic for viral infections is siRNA. RNA-mediated interference (RNAi) has become an integral technology for researchers and is progressing as a means for treating diseases [120]. The theory behind RNAi is that small duplexes of RNA, siRNAs are used to silence the expression of specific genes in a sequence specific manner. In order for siRNA to work, the therapy must manipulate the host cell microRNA machinery to reproduce the 21bp sequences used to silence specific genes. Some researchers were able to deliver exogenous mature siRNAs duplexes into cells, followed by direct association with Argonaute proteins without the need for processing

by either Drosha or Dicer [121–123]. Specifically, in mouse models, liposomes carrying siRNA duplexes directed against Fas or its downstream effector, caspase-8 were delivered via tail vein injection into mice with high risk acute liver failure [124, 125]. They were able to efficiently knockdown both Fas and caspase-8 in order to prevent apoptosis of hepatocytes, necrosis and the subsequent invasion of fibroblasts into the lesion. In terms of HIV-1, siRNA therapies are still very experimental. A potential target for siRNA is the TAR hairpin. The TAR hairpin is very important for viral replication and is well-conserved [126]. Short TAR hairpins can be found at high levels during every stage of HIV-1 infection, and these hairpins can be directly processed by Dicer [139]. It seems that siRNA therapies are the most intriguing method to combat viral infections. However, siRNAs could possibly acquire off-target effects, resulting in the silencing of the target gene as well as other, unintended genes. These microRNAs and siRNAs do not need one hundred percent hybridization to have silencing effects and an unintended silencing of certain genes could result in harmful alterations for normal cells. Therefore, before siRNA therapies can be used, researchers must be able to ensure safe and effective delivery.

## CHAPTER II: RESEARCH OBJECTIVES AND SPECIFIC AIMS

The aim of the work described in this manuscript is to study how microRNA mechanisms play a significant role in manipulating both cellular and viral mechanisms to maintain latency. These studies also include potential cdk inhibitors as future therapeutics against HIV-1 infection. This hypothesis takes advantage of the viral manipulation of host cell mechanisms; specifically the TAR-derived viral microRNA is able to regulate various gene expression patterns. These studies focus on how TAR microRNA has the ability to manipulate host cell apoptotic pathways and also viral genes expression. Since cdk/cylin complexes are important for viral transcription, these studies center on the possible cdk inhibitors that inhibit viral transcription, without affecting normal cellular mechanisms. These cdk inhibitors and possibly many other potential drugs are affected by microRNA mechanisms. Future studies would be able to find the specific mechanisms *in vivo,* of the TAR-derived microRNA in maintaining viral latency and drug efficacy. The goals of this study would be to explain the functions of the viral TAR microRNA and its effects on maintaining latency and drug efficacy.

## CHAPTER III: MATERIALS AND METHODS

### A. Cell culture.

HEK293T, TZMBl, HeLaT4 and cMagi cell lines were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (FBS) (10%), 2mM L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml) (cDMEM). HCT116 WT and HCT116 Dicer -/- cell lines were grown in McCoy's medium supplemented with FBS (10%), 2mM L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml). CEM and ACH2 cells were grown in RPMI 1640 supplemented with FBS, L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml). All cell lines were maintained at 37ºC in 5% CO2. Transfections were carried out using Metafectene (Biontex), Attractene (Qiagen) or Lipofectamine (Invitrogen) according to manufacturers instructions. Cells were cultured to confluence and pelleted at 4°C for 15 min at 3,000 rpm. The cell pellets were washed twice with 25 mL of phosphatebuffered saline (PBS) with Ca2+ and Mg2+ (Quality Biological) and centrifuged once more. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na3VO4, 1 mM DTT, one complete protease cocktail tablet/50 mL) and incubated on ice for 20 min, with a gentle vortexing every 5 min. Cell

lysates were transferred to eppendorf tubes and were centrifuged at 10,000 rpm at 4C for 10 min. Supernatants were transferred to a fresh tube where protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA).

**B. Cloning and sequencing of the TAR microRNA**

cMagi cells were infected with $HIV_{IIIB}$ and microRNA enriched libraries were prepared as described using suitable adaptors [127, 128]. RT-PCR amplification with an excess of the reverse primer (1:50 ratio) was employed to produce a cDNA library. Biotinylated capture oligonucleotides were then hybridized to an aliquot (5 ul) of the library in TEN buffer. (CTCTCTGGCTAACTAGGGAACCCACTG and ACTGGGTCTCTCTGGTTAGACCAGATTTGA for HIV-mir-3p and HIV-mir-5p respectively) Hybridized pairs were captured by uMACS Streptavidin Kit and the single-stranded miRNA eluted by adding 150 ul of water preheated to 80°C. Recovered single-stranded cDNA molecules were amplified by PCR, ligated into the pTZ57R/T vector and transformed into JM109 bacteria. Positive colonies were identified and sequenced.

**C. RNA and siRNA molecules**

TAR-WT and TAR-D were transcribed from previously described T7 expression vectors [129]. For *in vitro* transcription reactions 1.5 μg of each plasmid was linearized with HindIII (New England Biolabs), ethanol precipitated and used for *in vitro* transcription via the MegaScript High Yield Transcription Kit (Ambion). After transcription TAR RNA was purified on a 2% agarose gel, eluted from the gel with 0.5 M NaAcetate, 1 mM

EDTA, 0.2% SDS, and ethanol precipitated before re-suspension in DEPC treated water. siERCC1 were obtained from a commercial source (Dharmacon).

**D. Serum starvation**

293T cells were plated 24 hours prior to transfection. Then, 24 hours post-transfection with either TAR-WT or TAR-D RNA, the media was replaced by Dulbecco's modified Eagle's medium with 0.1% FBS. After 48 hours of serum starvation, the cells were cultured for various assays.

**E. Flow cytometry**

Cell cycle analysis was performed on HeLaT4 cells transfected with either TAR-WT, TAR-D or siERCC1. Cells were collected by low speed centrifugation and washed with PBS without Ca2+ and Mg2+ and fixed with 70% ethanol. Following rehydration in PBS, cells were stained in PBS containing 25 ug/ml propidium iodide (Sigma), 10 ug/ml RNase A (Sigma) and 0.1% NP-40. Cells were analyzed on a BD FacsCalibur flow cytometer and analyzed using CELLQuest software (BD Biosciences). Cell cycle analysis and measurement of apoptosis was performed using ModFit LT software. Aggregates and debris were excluded by gating on the FL2W and FL2A parameters. Apoptosis was considered to be the population of cells that were sub-G1. Apoptosis analyses were confirmed with BD Biosciences Annexin V Apoptosis detection kit following the procedure outlined by the company.

**F. Western blots**

Cell extracts were resolved by SDS PAGE on a 4–20% trisglycine gel (Invitrogen). Proteins were transferred to Immobilon membranes (Millipore) at 200 mA for 2 hours. Membranes were blocked with Dulbecco's phosphate-buffered saline (PBS) 0.1% Tween-20 + 5% BSA. Primary antibody against specified antibodies was incubated with the membrane in PBS +0.1% Tween-20 overnight at 4°C. Membranes were washed three times with PBS +0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for one hour. Presence of secondary antibody was detected by SuperSignal West Dura Extended Duration Substrate (Pierce). Luminescence was visualized on a Kodak 1D image station. Primary Antibodies: B-actin, p21/waf1 and MDM2 antibodies were from Santa Cruz Biotech. P53 pSer 15 antibody was from Cell Signaling Technologies.

**G. 2D-gel electrophoresis (2DGE) and MALDI-TOF MS**

Five hundred micrograms of serum starved 293T cell extracts transfected with either TAR-WT or TAR-D RNA were subjected to isoelectric focusing on an IPG strip, pH 3.0-10.0 and further subjected to SDS-PAGE on a 4-20% Criterion Tris-Glycine gel. The gels were stained with Coomassie Blue and protein spots of interest were excised. Spots were vortexed, washed, and equilibrated in 25 mM $NH_4HCO_3$, broken into smaller pieces, and vortexed and washed 3X with 50% ACN/25 mM $NH_4HCO_3$ to remove the Coomassie from the gel. Gel pieces were vortexed and washed with 100% ACN to dehydrate the gel pieces. Gel pieces were re-swelled with up to 200 ng of trypsin (in enough volume to cover the gel) and incubated on ice for 30 min. Residual trypsin was removed, 20 µl of 25 mM $NH_4HCO_3$ or enough to cover the gel was added to the gel

pieces and the reactions were incubated overnight at 37ºC. Peptides were extracted with 1X dH2O wash with brief vortexing and sonication, followed by 3X washes with 60% ACN/5% TFA. Extracted peptides were pooled together and SpeedVac'ed to reduce the volume to approximately 10 µl. Twenty microliters of 0.1% TFA was added to each tube and peptides were desalted using C18 ZipTips (Millipore) according to manufacturer's instructions. Peptides were spotted on MALDI sample plate 1:1 with α-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution: 10 mg CHCA, 500 µl 100% ACN, 500 µl 0.1% TFA. Positive control calibration peptide solution of Bradykinin, Angiotensin II, P14R, and ACTH was spotted along with negative control empty gel slice. Mass peaks obtained were entered into Mascot (http://www.matrixscience.com/) and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) databases for peptide mass fingerprinting analysis.

**H. CAT assays**

Various cells lines were transfected with the HIV-1 LTR-CAT plasmid and in some cases pc-Tat was added to initiate the LTR transcription. Some cells also underwent drug treatments prior to the assay. After 48 hours, cells were lysed and chloramphenicol acetyltransferase (CAT) activity was determined. A standard reaction was performed by adding 4uL of the cofactor acetyl coenzyme A to a microcentrifuge tube containing 10uL cell extract and 1uL of radiolabeled ($^{14}$C) chloramphenicol in a final volume of 50 ul and incubating the mixture at 37°C for 1 hour. The reaction mixture was then extracted with ethyl acetate and separated by thin-later chromatography on silica gel plates (Bakerflex silica gel thin-later chromatography plates) in a chloroform-methanol (19:1) solvent. The

resolved reaction products were then detected by exposing the plate to a PhosphorImager cassette.

**I. Luciferase assays**

TZM-bl cells were transfected with pc-Tat (0.5 ug) using the Attractene reagent (Qiagen) according to the manufacturers' instructions. TZM-bl cells contain an integrated copy of the firefly luciferase gene under the control of the HIV-1 promoter (obtained through the NIH AIDS Research and Reference Reagent Program). The next day, cells were treated with DMSO or the indicated compound. Forty-eight hours post drug treatment, luciferase activity of the firefly luciferase was measured with the BrightGlo Luciferase Assay (Promega). Luminescence was read from a 96 well plate on an EG&G Berthold luminometer.

**J. Chromatin immunoprecipitation assay (ChIP)**

ACH2 cells were treated with 2.5 uM 9AA and processed 48 hours later for ChIP. For ChIP, approximately $5 \times 10^6$ cells were used per IP. Cells were cross-linked with 1.0% formaldehyde at 37°C for 10 minutes, pelleted, washed, and cells lysed using SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, one tablet complete protease inhibitor cocktail per 50 ml) on ice for 10 mins. Cells were sonicated on ice for 6 cycles to obtain an average DNA length of 500 to 1200 bp. Lysate was clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C. Supernatant was then diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and pre-cleared with a mixture of protein A/G agarose

(blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml BSA) at 4°C for 1 hour. Pre-cleared chromatin was incubated with 10 µg of antibody at 4°C overnight. Next day, 60 µl of a 30% slurry of blocked protein A/G agarose was added and complexes incubated for 2 hours. Immune complexes were recovered by centrifugation and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), twice with high salt buffer (0.1% SDS, 1% Triton X-100 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and once with TE buffer. Immune complexes were eluted twice with elution buffer (1% SDS, 0.1 M NaHCO3) and incubating at room temperature for 15 minutes on a rotating wheel. Cross-links were reversed by adding 20 µl of 5 M NaCl and incubating elutes at 65°C overnight. The next day, proteinase K (100 µg/ml final concentration) was added and samples incubated at 55°C for 1 hour. Samples were extracted with phenol:chloroform twice and ethanol precipitated overnight. Pellets were then washed with 70% ethanol, dried, resuspended in 50 µl of TE, and assayed by PCR. Thirty-five cycles of PCR were performed in 50 µl with 10 µl of immunoprecipitated material, 0.1 µM of primers, 0.2 mM dNTPs, and 1.0 unit of Taq DNA polymerase. Finally, PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

# CHAPTER IV: RESULTS

## A. HIV-1 microRNA created from TAR hairpin

The functions of a number of viral miRNA have been determined and they appear capable of regulating both viral and cellular gene expression [130]. The structures of many viral RNAs provide appropriate targets for microRNA machinery. Several viruses have already been identified that produce microRNAs after Dicer processing, including: human cytomegalovirus, human herpesevirus 8, Epstein Barr virus, herpes simplex virus and peach latent mosaic viroid [131-134]. In term of HIV-1, a group has reported that a miRNA is produced from the *nef* RNA [135]. Computer modeling has also predicted the existence of up to five structures in HIV-1 RNA that may be processed by Dicer to yield miRNA, including stem-loop structures within the *env* gene and TAR-region [136]. A viral miRNA generated from full-length, doubly spliced or singly spliced HIV-1 transcripts could potentially inhibit viral replication, block translation of viral proteins or cause chromatin remodeling of the viral genome. Since the integrated HIV genome is associated with chromatin remodeling complexes and proteins with histone acetyl-transferase activity involved with activation of the virus, it makes sense that a viral microRNA could have significant downregulating effects on all of these viral complexes.

In addition to targeting viral genes, a HIV-1 microRNA could also regulate the expression of cellular proteins.

We saw much potential in the TAR hairpin element becoming cleaved by microRNA machinery. The TAR element, about 50 nucleotides long, at the 5' end of the HIV-1 viral mRNA was one of the five structures within HIV that had a possibility of being processed by Dicer. The TAR element has been shown to be involved with induction of transcription. It would be interesting if the TAR mircoRNA, rather than the entire hairpin, is actually the functioning sequence in regulating transcription. Further support of a role for TAR hairpins loaded into microRNA machinery is that TAR-RNA Binding Protein (TRBP) was identified as the human homologue of the Drosophila Loquacious protein which is required for efficient loading of the microRNA into the RISC complex [137]. Interestingly, TRBP was discovered over a decade ago through its association with the TAR element and plays a role in transactivation and inhibition of interferon induced PKR [128]. The fact that RNAi components, such as TRBP, can be found associated with the TAR element is strong evidence that TAR may be processed to yield microRNA. In actuality, our laboratory has successfully detected a TAR derived HIV-1 microRNA using RNase protection – a sensitive assay capable of detecting low copy number microRNA. We have shown the presence of the 5' and 3' TAR microRNA in cell lines and *de novo* infection of primary cells and now have cloned the TAR miRNAs from infected cells (Figure 3) [139]. cMagi cells were infected with $HIV_{IIIB}$ and the RNA was extracted from them. miRNA libraries were constructed from this RNA and used for cloning. Cloned sequences of the TAR-5p and TAR-3p miRNA were obtained and compared to predicted sequence registered with the Sanger miRNA

database (Figure 3A). The microRNA was then illustrated in a schematic of the TAR hairpin (Figure 3B). Using the cloned sequence for TAR, we reconstructed the TAR-WT sequence and created a truncated form of TAR, TAR-D (Figure 3C). 293T cells were transfected with TAR-WT or TAR-D RNA. Northern blots from the transfected 293T RNA extract confirmed the presence of TAR microRNA from the TAR-WT transfection (Figure 3D). From these sequences, the 5'-TAR microRNA seems to have more homology to various cellular and viral genes.

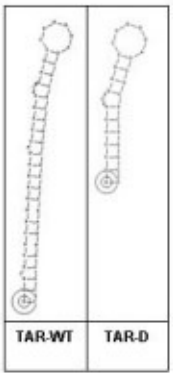
**Figure 3: Determination of the sequence of that HIV-1 TAR microRNA**. RNA from cMagi cells infected with $HIV_{IIIB}$ was used to construct miRNA libraries and used for cloning. A) Cloned sequences of the TAR-5p and TAR-3p (5' and 3' arm) miRNA obtained as compared to predicted sequence registered with the Sanger miRNA database. B) Diagram showing the TAR hairpin and the position of the mature miRNA within the TAR sequence. C) Structure of the TAR-WT and truncated TAR-D mutant used for 293T transfections**.** D) 293T cells were mock transfected (lane 2) or transfected with TAR-WT (lane 3) or TAR-D (lane 4) RNA. Forty-eight hours after transfection RNA was isolated and subjected to Northern blotting for TAR sequence. Numbers to the left indicate the size of the RNA ladder in nucleotides. Diagrams to the right show the positions of the wild-type TAR and the mature TAR miRNA.

A)

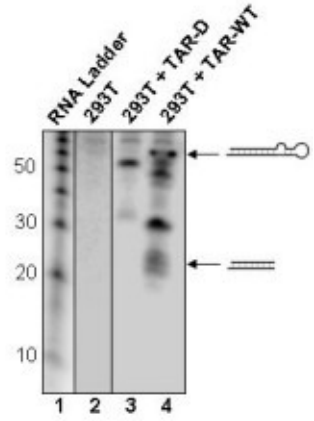
| | # of Clones | Cloned Sequence | nt |
|---|---|---|---|
| TAR-5p | Sanger DB | UCUCUCUGGUUAGACCAGAUCUGA | 24 |
| | 2 clones | GGUCUCUCUGGUUAGACCA | 19 |
| | 1 clones | GGGUCUCUCUGGUUAGACCA | 20 |
| TAR-3p | Sanger DB | UCUCUGGCUAACUAGGGAACCCA | 23 |
| | 10 clones | CUAACUAGGGAACCCAC | 17 |
| | 4 clones | GCUAACUAGGGAACCCAC | 18 |

B)


C)




D)



**B. HIV-1 microRNA Effects on Cellular Mechanisms**

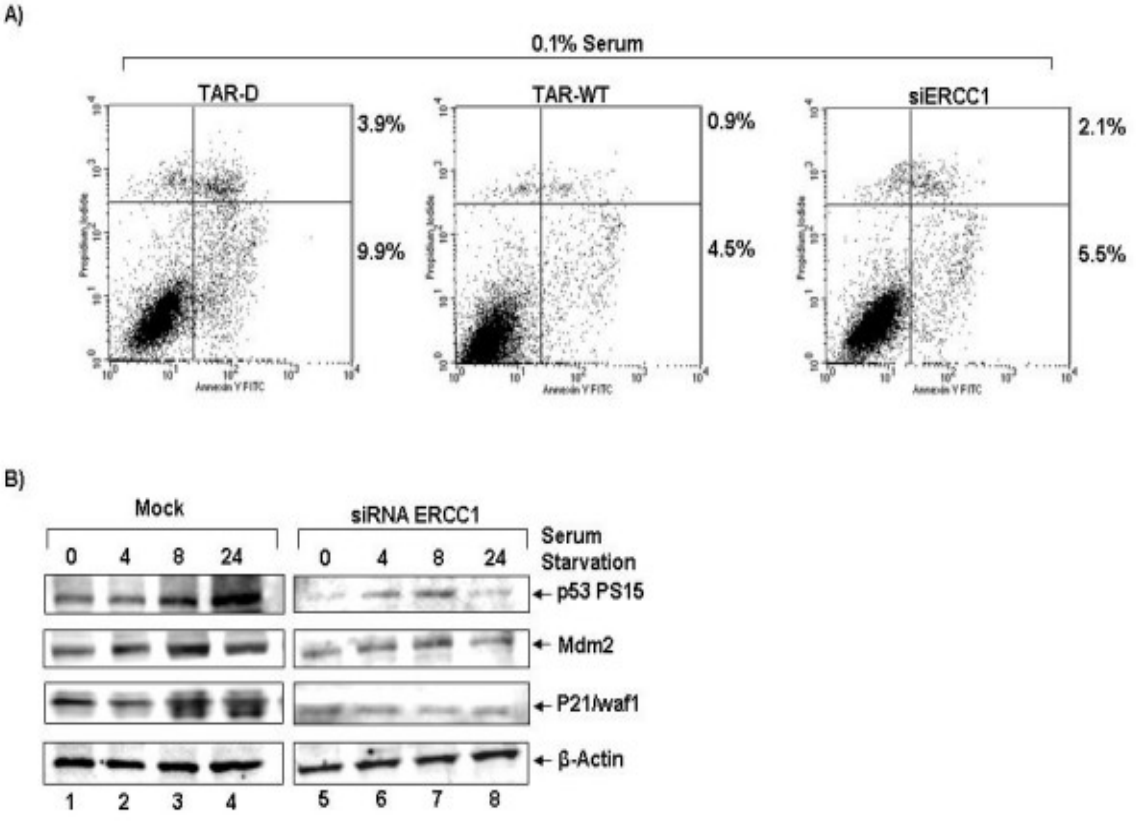
Upon infection, HIV-1 has been proven to downregulate many cellular genes [140-142]. These downregulations could be due to the fact that the TAR hairpin is able to produce mircoRNA that is able to bind to complementary genes and lower expression. From various studies, latent cells have been shown to produce a high amount of short, abortive RNA transcripts only 50-100 nucleotides in length, that contain the HIV-1 TAR hairpin [143]. Of the entire HIV-1 genome, these TAR-containing short transcripts are the only HIV RNA produced in large quantities during latency. Therefore, it is possible that the microRNAs generated from TAR may act to suppress viral gene expression and alter host-cell proteins levels in order to maintain the latent state.

In previous studies, we predicted a few important apoptotic genes altered by HIV-1 infection [139]. The excision repair cross-complementing 1, ERCC1, protein was upregulated in the presence of viral infection, especially in microRNA machinery deficient cells. This finding is not surprising since DNA repair proteins, like ERCC1 are upregulated during infection [146]. We found that serum starvation is a great way to induce apoptosis mechanisms, such as ERCC1, for easy observation. To confirm the role of ERCC1 in protecting cells from serum starvation induced apoptosis, siRNA against ERCC1 was utilized. 293T cells were transfected with TAR-D, TAR-WT or siRNA against EGFP (control) or ERCC1. Cells were serum starved for 48 hours, and the level of apoptosis was determined at 96 hours post serum starvation (Figure 4A). Control transfection of TAR-D mutant showed that 9.9% of the cells were apoptotic. siRNA against ERCC1 prevented the induction of apoptosis at 48 hours, comparable to the transfection with wild type TAR RNA. Therefore, repression of ERCC1 expression

inhibited serum starvation induced apoptosis. Our next step was to find other apoptotic genes involved with the TAR microRNA preventing apoptosis.

The p53 pathway is a very well studied apoptotic pathway that could tie in with ERCC1. This is especially important after cells have been serum starved, causing p53-mediated apoptosis. Activation of p53 induces the expression of Mdm2, p21/waf1 and Bax. Mdm2 and p21/waf1 regulate the cell cycle and feed back on p53, while Bax is translocated to the mitochondria and begins the apoptotic cascade. From here, we wanted to observe the levels of activated p53 and downstream p53 targets after suppression of ERCC1 due to TAR microRNA. 293T cells were mock transfected or transfected with siRNA against ERCC1. Twenty-four hours after transfection the cells were once again cultured in serum starvation conditions for 0, 4, 8 and 24 hours. Cell extracts were prepared at each time point and Western blotted for p53 phosphorylated on serine 15 (activated), Mdm2, p21/waf1 and β-actin (Figure 4B). Serum starvation of the control cells triggered an increase in p53 phosphorylation and a subsequent increase in the expression of Mdm2 and p21/waf1 (lanes 1–4). The phosphorylation of p53 was highest at the 24 hour time point, but increased phosphorylation can be seen from 4 hours on. Interestingly, 293T cells transfected with the siRNA against ERCC1 showed only a small increase in p53 phosphorylation that peaked at 8 hours (lane 7), a slight increase in Mdm2 at 8 hours and no increase in p21 expression (lanes 5–8). Also, the levels of p53 phsophorylation, Mdm2 and p21 in the ERCC1 knockdown before serum starvation all appeared lower than in the control cells (compare lane 1 to lane 5). These results indicated a new possibility that p53 activation is tied to ERCC1 response to cellular stress.

**Figure 4: Anti-apoptotic effect validated through the repression of ERCC1**. A) HeLaT4 were transfected with TAR-D, TAR-WT, siEGFP, or siERCC1 RNA. Twenty-four hours after transfection media were replaced with low serum (0.1%) media, and the cells were cultured for 96 hours. Apoptosis was measured at 96 hours after serum starvation using FACs analysis. Data are representative of two experiments. B) 293T cells were transfected with siRNA against ERCC1 (lanes 5–8) or mock (lanes 1–4) for twenty-four hours, and then the media was replaced with low serum media. Cells were harvested for Western blot analysis at 0, 4, 8 and 24 hours after serum starvation and Western blotted for phosphor-p53 Ser15, Mdm2, p21 and β-actin. Pictured Western blots utilized 20 micrograms of total protein per lane.

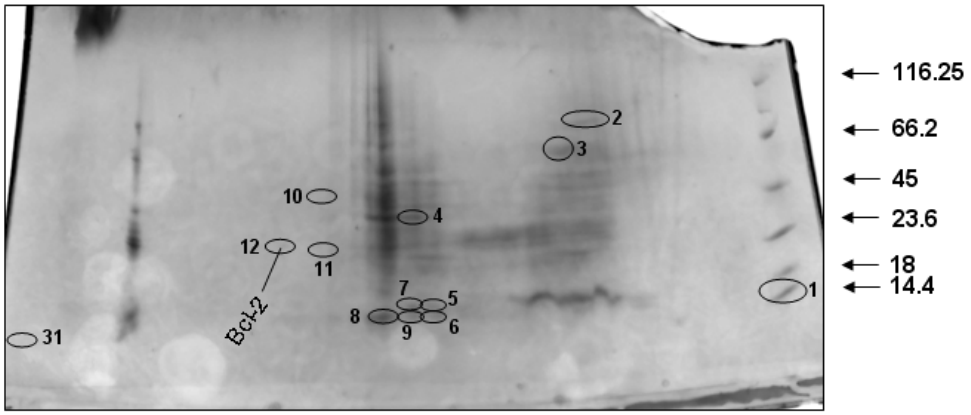

A)
0.1% Serum
TAR-D
3.9%
9.9%
TAR-WT
0.9%
4.5%
siERCC1
2.1%
5.5%
B)
Mock
siRNA ERCC1
0 4 8 24
0 4 8 24
Serum Starvation
p53 PS15
Mdm2
P21/waf1
β-Actin
1 2 3 4
5 6 7 8

To determine other proteins that are altered in the presence of the TAR microRNA we performed a proteomic analysis. As in previous studies, 293T cells were transfected with the TAR-WT and TAR-D mutant RNA. Twenty-four hours post-transfection, the cells were serum starved in 0.1% FBS and allowed to grow 48 hours before collection. The samples were prepared and run through 2-D electrophoresis (PI and molecular weight separation). The gels were stained with Coomasie blue and we determined a few spots that were different between the TAR-WT and TAR-D cells. The spots in the gel were cut out and prepared for MALDI-TOF analysis.

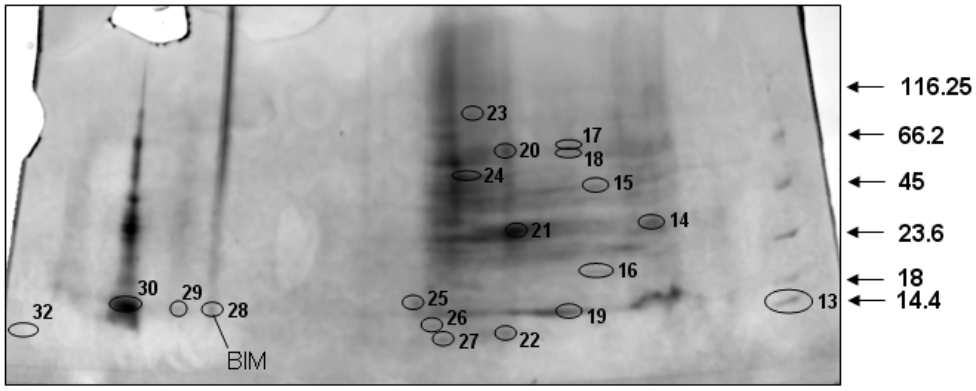
After the mass fingerprinting anaylsis, we were able to determine that the Bcl-2 proteins were differentially regulated between the two samples (Figure 5). In the cells lacking the functional TAR microRNA, the pro-apoptotic protein BIM was detected (Figure 5, Spot 28). However in cells that were transfected with the TAR-WT RNA, the anti-apoptotic protein Bcl-2 was detected (Figure 5, Spot 12). The Bcl-2 family of proteins regulates a pathway that has the ability to translocate the Bax protein to the mitochondria and therefore initiating apoptosis. Bcl-2, Bcl-XL, and Mcl-1 are a few members of the Bcl-2 family of proteins that inhibits Bax-mediated apoptosis. The BIM protein is a pro-apoptotic complex that binds to Bcl-2 and allows the Bax mediated apoptosis to occur. These results are another interesting finding that the viral TAR microRNA inhibits apoptosis mechanisms in order for the virus to have time to replicate before cell killing.

**Figure 5: HIV-1 TAR Proteome.** 293T cells were transfected with TAR-D control or TAR-WT RNA. Twenty-four hours post transfection the media was replaced with DMEM with 0.1% FBS. Cells were harvested 48 hours post serum starvation and prepared for 2-D gel electrophoresis. Spot #12 shows Bcl-2 upregulated in the TAR-WT transfected cells; while, spot #28 shows BIM upregulated in the TAR-D transfected cells.

TAR-WT


116.25
66.2
45
23.6
18
14.4
1
2
3
4
5
6
7
8
9
10
11
12
31
Bcl-2

TAR-D

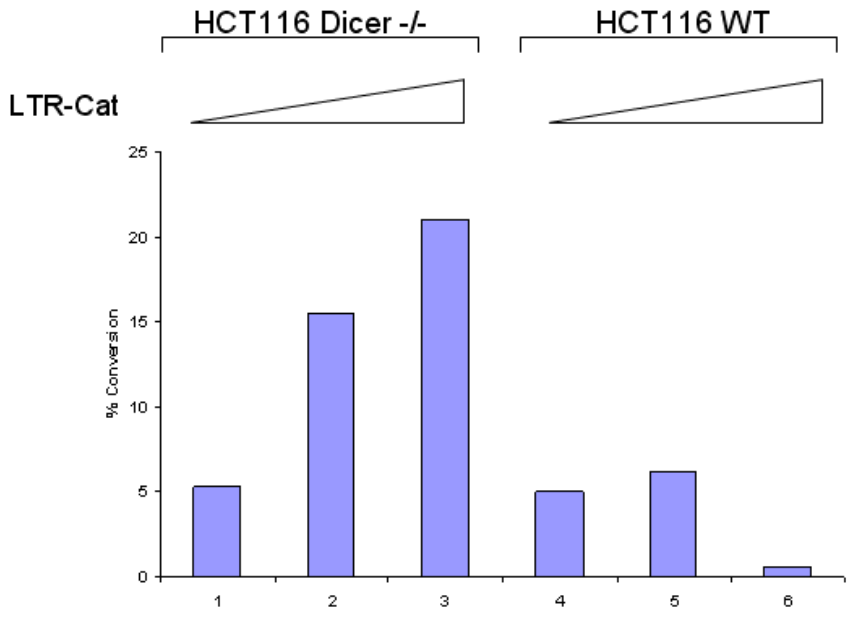

116.25
66.2
45
23.6
18
14.4
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
32
BIM

### C. microRNA Mechanism Effects on Viral Transcription

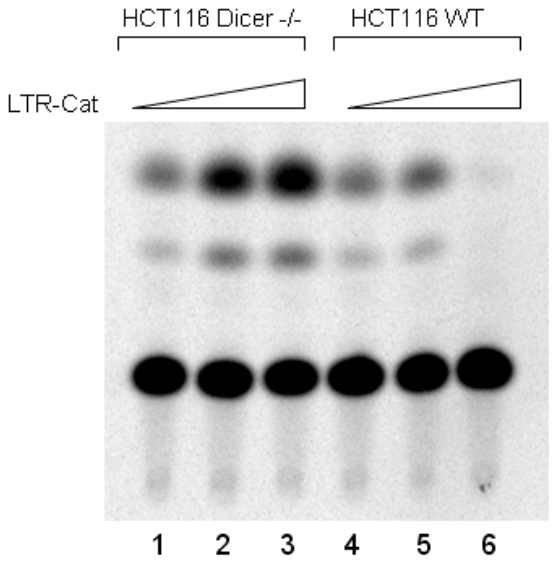
The TAR microRNA has been shown to play a significant role in suppressing cellular apoptosis mechanisms to aid the virus in replication. We also wanted to observe the effects of viral mircoRNA on its own viral genes, specifically transcription of the HIV-1 LTR. We implemented the use of HCT116 cells that either contained a WT Dicer (HCT116 WT) or lacked the Dicer protein (HCT116 Dicer-/-). These cell lines were transfected with the HIV-1 LTR-CAT plasmid. After 48 hours, cells were collected and chloramphenicol acetyltransferase (CAT) activity was determined (Figure 6). We can see that there is a higher amount of basal HIV-1 LTR transcription in the cells that lacked Dicer compared to cells that expressed Dicer (Figure 6, Lanes 1-3 vs Lanes 4-6). It seems that the presence of viral microRNA can also lead to the suppression of viral genes.

**Figure 6: HIV-1 LTR-CAT Expression Differs with microRNA Machinery Presence.** A) Graphic representation of panel B. B) HCT116 WT and HCT116 Dicer-/- cell lines were transfected with HIV-1 LTR-CAT. After 48 hours, cells were collected and chloramphenicol acetyltransferase (CAT) activity was determined. The chloramphenicol acetyltransferase reaction mixture was then extracted with ethyl acetate and separated by thin-later chromatography on silica gel plate in a chloroform-methanol (19:1) solvent. The resolved reaction products were then detected by exposing the plate to a PhosphorImager cassette.

A)


HCT116 Dicer -/-
HCT116 WT
LTR-Cat
% Conversion
25
20
15
10
5
0
1
2
3
4
5
6

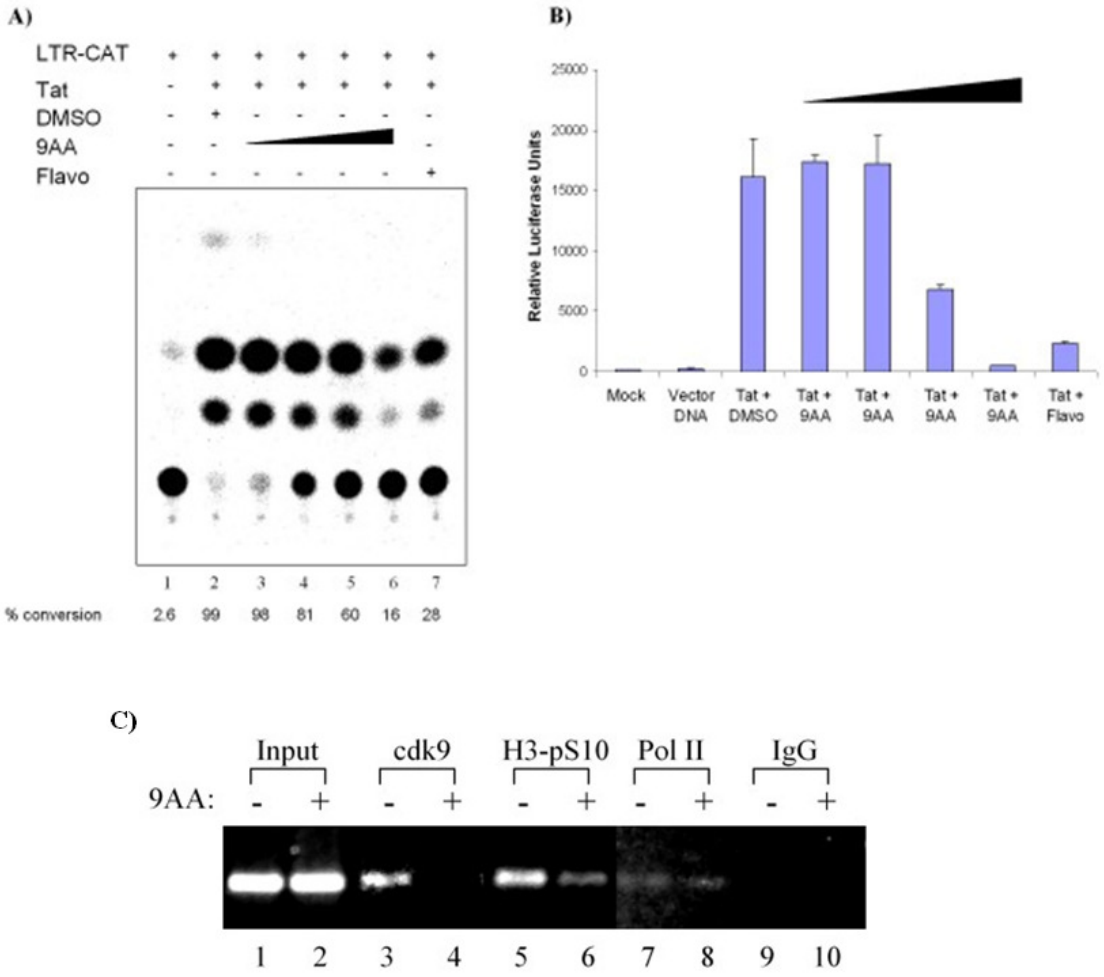
B)


HCT116 Dicer -/-
HCT116 WT
LTR-Cat
1
2
3
4
5
6

**D. Cdk Inhibitors as Potential Antiviral Therapeutics**

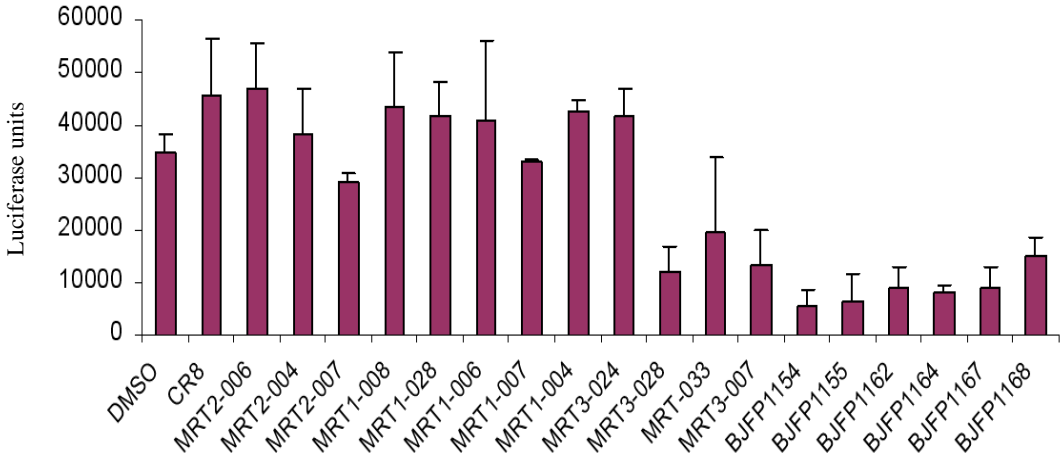
Even with the success of combined HAART therapies, drugs need to be developed that will target latently infected cells. Besides specific siRNA therapies, cdk inhibitors have emerged as the next generation of potential therapeutics against HIV-1 infection. Previously, a member of our laboratory found that 9AA drug was able to reactivate the p53 mechanisms in HIV-1 infected cells [144]. However, we decided to go further to find exactly how 9AA inhibits the virus. First, CEM cells were transfected with HIV-1 LTR-CAT and pc-Tat by electroporation. Twenty-four hours post-transfection, cells were treated with DMSO, 9AA or Flavopiridol. Cells were harvested 48 hours post transfection and processed for CAT activity (Figure 7A). Flavopiridol showed significantly decreased viral transcription, as expected. 9AA also shows decreased viral transcription with increasing amounts of drug. We then decided to try these drugs in a cell line with an integrated HIV-1 LTR. TZM-bl cells contain an integrated HIV-1 LTR-Luc transcript and can be activated by addition of pc-Tat. After transfection of pc-Tat, the TZM-bl cells were treated with DMSO, 9AA and Flavo. Cells were harvested 48 hours post transfection and processed for Luciferase activity (Figure 7B). Once again, Flavopiridol showed significantly decreased viral transcription, as well as 9AA with increasing amounts of drug. From here we decided to observe the effects of 9AA in an HIV-1 infected cell line, ACH2. Cells were drug treated with DMSO or 9AA for 48 hours and then collected for chromatin immunoprecipitation (ChIP) analysis (Figure 7C). We were able to determine that with 9AA treatment, cdk9 (important for HIV-1 transcription) is no longer bound to the HIV-1 LTR. Therefore, 9AA seems to be a cdk inhibitor that significantly downregulates viral transcription.

**Figure 7: 9AA inhibits HIV-LTR transcription via loss of cdk9 from the HIV-LTR**. A) CEM cells were transfected with 2.5 μg HIV-LTR CAT and 0.5 μg of pc-Tat by electroporation. Twenty-four hours post-transfection, cells were treated with DMSO, 9AA (0.1, 0.5, 1, or 2.5 μM), or 100 nM Flavo (flavopiridol). Cells were harvested 48 hours post transfection and processed for CAT assays. CAT assays were performed with 4 mM acetyl CoA, 5 μl of 14C-chloramphenicol (40 mCi/mmole), 10 μl of protein extracts, and 18 μl of water. Reactions were carried out at 37°C for 30 minutes. Samples were extracted with ethyl acetate, dried, and separated by TLC. B) TZM-bl cells were transfected with 1.0 μg of pc-Tat and treated the next day with DMSO, 9AA (0.1, 0.5, 1, or 2.5 μM), or 100 nM flavopiridol. Cells were processed 48 hours post drug treatment for luciferase assays. Assays were performed in triplicate and an average value is shown plus standard deviation. C) ACH2 cells were treated with either DMSO or 2.5 μM 9AA for 48 hours, cross-linked and collected for ChIP analysis. Antibodies against cdk9, histone H3-phospho-Ser10 (H3-pS10), RNA Polymerase II (Pol II), and rabbit IgG were utilized [147].


A)
LTR-CAT
Tat
DMSO
9AA
Flavo
1 2 3 4 5 6 7
% conversion 2.6 99 98 81 60 16 28
B)
Relative Luciferase Units
25000
20000
15000
10000
5000
0
Mock
Vector DNA
Tat + DMSO
Tat + 9AA
Tat + 9AA
Tat + 9AA
Tat + 9AA
Tat + Flavo
C)
Input
cdk9
H3-pS10
Pol II
IgG
9AA: - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10

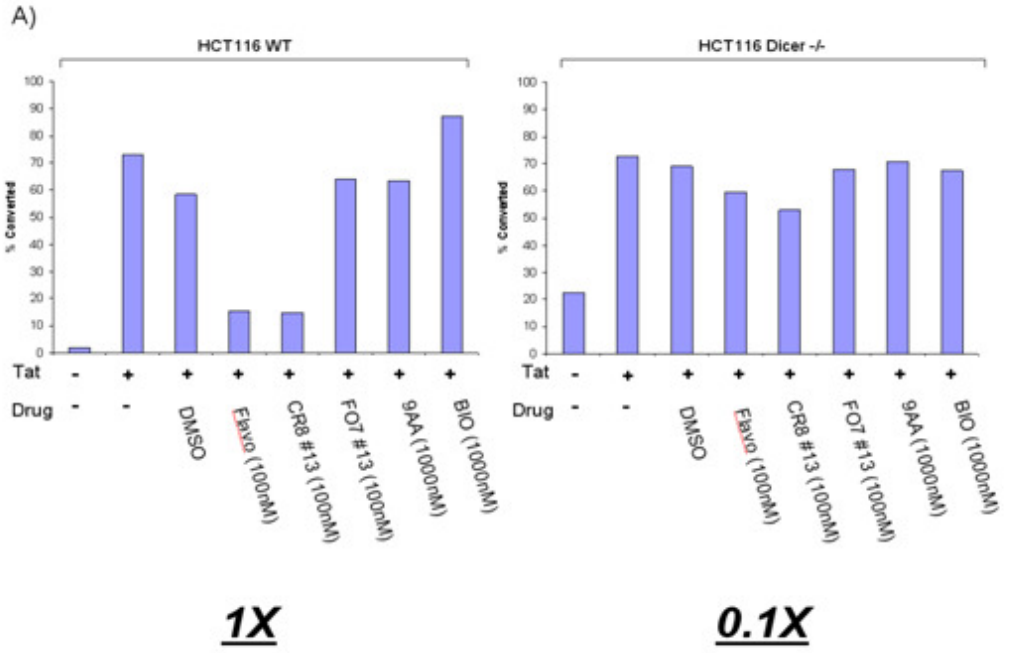
Another family of cdk inhibitory drugs that we have tested is the Roscovitine family. More specifically, Cyc202 (R-roscovitine) is a second generation drug that targets the cdk2/cyclin E complex and allows apoptosis to occur in HIV-1 infected T-cells, monocytes, and peripheral blood mononuclear cells [145]. We also decided to see if a derivative of Cyc202 was better at inhibition of viral transcription. A slight alteration at the purine ring of Cyc202 resulted in CR8. CR8 exhibited better inhibition of viral replication (Data not shown); however, we received aid from Laurent Meijer to further generate a few more derivatives of CR8. TZM-bl cells were used to transfect with Tat and treated with eighteen CR8 derivatives. Cells were harvested 48 hours post transfection and processed for Luciferase activity (Figure 8). Among the many possible derivatives, about nine derivatives showed inhibition of the HIV-1 LTR. We decided to further study the effects of the BJFP1154 (CR8#13) analog, since it exhibited the most viral transcription inhibition in TZM-bl cells.

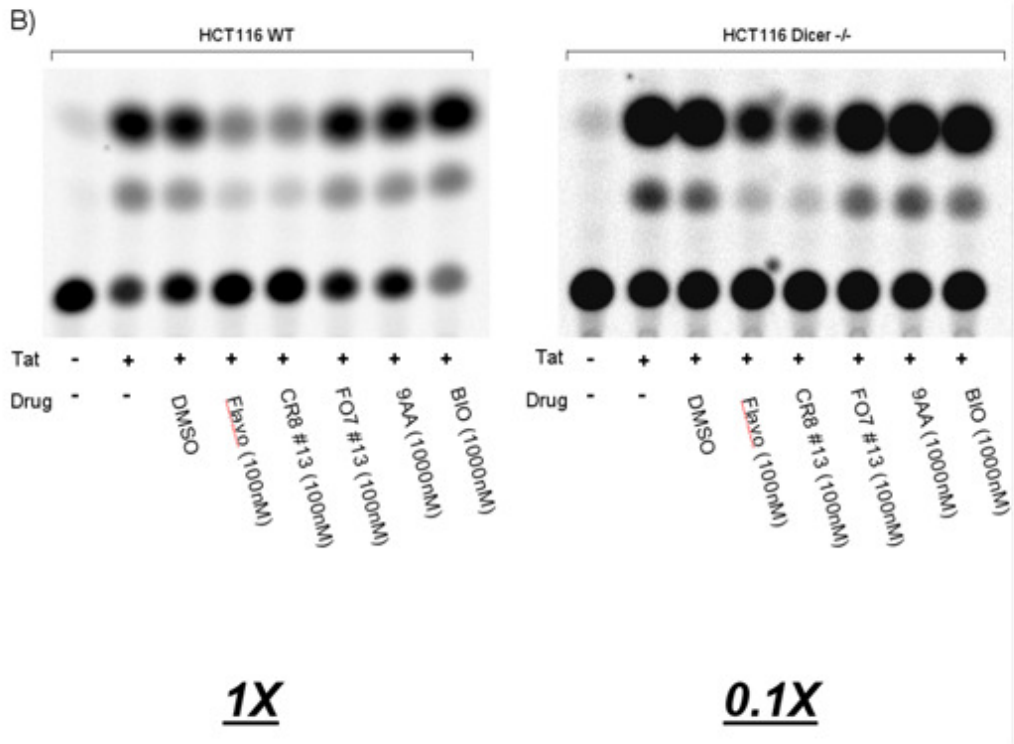
**Figure 8: Screening of CR8 derivatives in Tat-dependent transcription of the HIV-1 LTR.** TZM-bl cells were transfected with 1 µg of Tat and treated the next day with DMSO or the indicated CR8 derivative compound at 50 nM. 48 hours post drug treatment, luciferase activity of the firefly luciferase was measured with the BrightGlo Luciferase Assay and luminescence was read from a 96 well plate on an EG&G Berthold luminometer. Assays were performed in triplicate, average and standard deviations are shown.


Luciferase units
60000
50000
40000
30000
20000
10000
0
DMSO
CR8
MRT2-006
MRT2-004
MRT2-007
MRT1-008
MRT1-028
MRT1-006
MRT1-007
MRT1-004
MRT3-024
MRT3-028
MRT-033
MRT3-007
BJFP1154
BJFP1155
BJFP1162
BJFP1164
BJFP1167
BJFP1168

After discovering a few compounds that exhibited transcriptional inhibition, we decided to study the efficacy of the drugs in the presence or absence of Dicer, an integral part of cellular microRNA machinery. Once again, we implemented the use of HCT116 cells that either contained a WT Dicer (HCT116 WT) or lacked the Dicer protein (HCT116 Dicer-/-). These cells were first transfected with HIV-1 LTR-CAT and then pc-Tat plasmid to activate the HIV-1 LTR transcription. The addition of pc-Tat initiates the creation of many TAR microRNA transcripts. Six hours later, the cells were treated with DMSO, Flavo, CR8#13, F07#13, 9AA, or BIO. To the previously described drugs, we added a flavone derivative, F07#13 and a 6-bromoindirubin derivative, BIO. F07 has been shown to decrease the amount of cancer cells in G0/G1 phase, increase S phase cells, and stimulate cell death pathways [148]. BIO has been shown to be a potent GSK-3 inhibitor, which can be important in reversing the effects of Tat in terms of neurotoxicity and Tat-dependent viral transcription [149, 150]. Cells were harvested 48 hours post transfection and processed for CAT assays (Figure 9). Similar to previous results, the activation of the HIV-1 LTR in cells lacking Dicer exhibited significantly more viral transcription than cells containing Dicer. Also, we see that two drugs, Flavo and CR8#13 inhibit viral transcription significantly better in cells that have Dicer. The other drugs may not have an effect on viral transcription because they do not utilize the TAR microRNA or that the effects of Tat overpower the effects of the drugs. These results show that microRNA machinery may be very important in the efficacy of certain drugs.

**Figure 9: Effect of Drugs on Tat-mediated trans-activation in HCT116 WT versus HCT116 Dicer-/- cells.** A) Graphic representation of panel B. B) HIV-1 LTR-CAT (1μg) construct was transfected in 2 × 10^6 cells using Lipofectamine in the absence or presence of Tat expression vector (1ug). 6 hour later, the transfected cells were treated with DMSO, Flavopirodol (100nM), CR8#13 (100nM), F07#13 (100nM), 9AA (1000nM), or BIO (1000nM). Transfected and treated cells were plated in 6-well plates of complete DMEM for 48 h incubation at 37 °C. Cells were harvested and cell extracts were used for CAT analysis. One tenth the amount of HCT116 Dicer-/- extract compared to HCT116 WT was used for CAT analysis. Values represent the percentage of conversion of the [$^{14}$C]chloramphenicol substrate in the CAT assay.


A)
HCT116 WT
HCT116 Dicer -/-
% Converted
Tat
Drug
DMSO
Flavo (100nM)
CR8 #13 (100nM)
FO7 #13 (100nM)
9AA (1000nM)
BIO (1000nM)
1X
0.1X


B)
HCT116 WT
HCT116 Dicer -/-
Tat
Drug
DMSO
Flavo (100nM)
CR8 #13 (100nM)
FO7 #13 (100nM)
9AA (1000nM)
BIO (1000nM)
1X
0.1X

## CHAPTER V: DISCUSSION

While current anti-retroviral drugs can halt viral replication, latent HIV-1 infections persist in infected patients. Any halt or interruption to the therapy quickly results in a resurgence of viral titers due to the reservoir of latent infections. Consequently, research into the mechanisms underlying viral latency has taken on increasing significance and investigators have developed numerous models of latent infections. Understanding how latently infected cells function, enable virus replication, and avoid immune response is the key to finding a cure for HIV-1 infection.

RNA interference has become an integral part of research. Small RNAs are able to specifically target genes and suppress their effects. In the medical research field, small RNAs could possibly be used to suppress viral, bacterial, or any other infectious genes. Many infectious agents are also able to use the cellular microRNA machinery to create its own small RNA. For example, HIV-1 creates its own microRNA from the TAR hairpin structure of the viral genome. After export from the nucleus, the TAR element becomes bound to a microRNA processing protein called Dicer. Dicer is the main producer of viral microRNA, specifically TAR microRNA. The TAR microRNA could possibly be binding to the cdk genes and suppress their expression. For example, the cdk inhibitors

that are used could be recruiting TAR microRNA to bind to cdk9 and remove it from the HIV-1 LTR, resulting in decreased viral transcription. By creating microRNA, HIV-1 proves once again to be able to manipulate cellular mechanisms for its own benefit.

The HIV-1 induced regulation of cellular genes could now be explained by the presence of viral microRNA. ERCC1 is a protein that was found to be upregulated during HIV-1 infection, especially in cells that lack the TAR microRNA. From our studies, we have found that when there is minimal TAR microRNA being produced, there is a very high level of ERCC1 being produced. By knocking down ERCC1, we also found that high levels of ERCC1 leads to more activated and stabilized p53 mechanisms. Therefore the cell can undergo apoptosis. However, in infected cells, the virus does not want apoptosis to occur in order to give it time for the virus to mature in latently infected cells. The presence of the viral microRNA allows levels of ERCC1 to be diminished, therefore reducing the amount of activated p53 mechanisms. Apoptosis decreases and the virus is allowed to replicate. Another apoptotic mechanism that HIV-1 microRNA manipulates is the BAX- induced mitochondrial pathway. From our studies, we have found that in the presence of TAR microRNA, the Bcl-2 anti-apoptotic protein is upregulated. Without the viral microRNA present, the BIM pro-apoptotic protein is upregulated. Since Bcl-2 is able to prevent BAX from translocation to the mitochondria, the mitochondrial apoptotic cascade is prevented. Once again, TAR microRNA is able to suppress another apoptotic mechanism. Overall, it seems that the TAR microRNA is integral for the virus to suppress apoptosis and allow the virus to mature in these latently infected cells.

The HIV-1 microRNA also has the ability to manipulate viral mechanisms. From our studies, we have shown that in the presence of TAR microRNA, the virus is able to suppress the transcription of its own viral genome. Latent cells produce massive amounts of abortive transcripts between 50-100 nucleotides in length that contain the HIV-1 TAR hairpin. These hairpin structures can be cleaved by Dicer to produce viral microRNA. After TAR microRNA is produced, it can act as a negative feedback mechanism to suppress viral transcription and maintain latency. Basal levels of transcription, rather than activated viral transcription, were studied to gain a better understanding of latency. During latency, the viral genes are not being transcribed at high levels. Therefore if we transfect in an HIV-1 LTR sequence into a cell, we are able to study how the cell reacts in a latent model. We are able to study the mechanisms of how the TAR microRNA manipulates both cellular and viral genes to maintain latency. The viral protein, Tat, is able to activate HIV-1 LTR transcription. Activated transcription of the HIV-1 LTR allows researchers to study how the TAR microRNA reacts when cells leave latency to enter active infection. We believe that comparing and understanding how the TAR microRNA manipulates the cellular and viral pathways is the key to finding effective theraputics towards eliminating HIV-1 infection.

After learning that the HIV-1 TAR microRNA plays significant roles in manipulating both cellular and viral mechanisms, this viral microRNA could also be involved with drug efficacy. As previously reported, cdk/cyclin complexes play an important role in viral replication. Effective cdk inhibitors could play a significant role in suppressing viral replication and possibly better therapeutics for HIV-1 infection. We first started to look at a p53 activator. As previously stated, the presence of activated p53

enabled an infected cell to undergo apoptosis and prevent the virus from replicating. The 9AA drug was able to activate p53 at significant levels and also p21/waf1 to therefore suppress viral transcription in various cell types. Another important finding is that cdk9 (important for increased viral transcription) bound to the HIV-1 LTR was significantly decreased. 9AA seemed like not only a p53 activator, but also a cdk inhibitor. This led to our studies with other cdk inhibitors. We examined the effects of the Roscovitine derivative, Cyc202. Cyc202 was able to prevent cdk2/cyclin E complex binding to the HIV-1 LTR [145]. The end result of Cyc202 may be similar to the 9AA treatment by decreasing cdk2/cyclin E activity. Elevated cdk2/cyclin E activity results in loss of the $G_1$/S checkpoint and increased virus production. We found that CYC202 effectively inhibits wild type and resistant HIV-1 mutants in T-cells, monocytes, and peripheral blood mononuclear cells at a low IC(50) and sensitizes these cells to enhanced apoptosis resulting in a dramatic drop in viral titers [145]. We then wanted to study potentially more effective cdk inhibitors. A derivative of Cyc202, named CR8, was able to suppress viral transcription better than Cyc202 (Data not shown). However, we once again attempted to find a derivative of CR8 that could potentially eliminate HIV-1 transcription completely without affecting essential cellular genes that incorporate the use of certain cdks. The most promising was CR8#13, which decreased viral transcription significantly more than the other roscovitine derivatives. Not only did CR8 downregulate viral transcription, it also did not affect downstream cdk9 effector genes (Data not shown). Once again, we decided to see if the presence of the TAR microRNA has an effect on the efficacy of CR8#13, as well as other drug derivatives that seemed to be effective at downregulating viral transcription. In fact, both Flavopiridol and the CR8#13 exhibited

much dependence on the presence of the viral microRNA. These two drugs exhibited significantly more downregulation of viral transcription compared to other drugs when Dicer is present. Dicer is non-specific in terms of creating microRNA from a small RNA. The drugs that seem to be more effective with Dicer present need the use of microRNA to target various genes in order to downregulate viral transcription. Therefore, these drugs may be manipulating the cellular and even viral microRNA in order to effectively decrease HIV-1 replication.

Overall, the HIV-1 field has progressed rapidly in developing an effective therapy, HAART, to prevent infection from spreading to other healthy cell. However, new treatments need to be developed in order to destroy latently infected cells and cure infection. To do this, researchers must gain a full understanding of what causes viral transcription and replication. It seems that the production of its own viral TAR microRNA, allows HIV-1 to both suppress apoptotic cellular mechanisms and also keep viral genome activation at a minimum until these latently infected cells are stimulated to infect other healthy cells. These microRNA pathways also enable certain drugs to have greater efficacy and can play vital roles in drug functional pathways. Understanding latency and how microRNA mechanisms contribute is integral to finding a therapeutic to cure HIV-1 infection.

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## CHAPTER VI: REFERENCES


1) de la Fuente, C., et al., *Pharmacological cyclin-dependent kinase inhibitors as HIV-1 antiviral therapeutics.* Curr HIV Res, 2003. **1**(2): p. 131-52.

2) McGovern SL et al., *A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening*. J Med Chem. 2002. **45**(8): 1712–22.

3) Coffin, J.M., S.H. Hughes, and H.E. Varmus, *Retroviruses*. 1997, Plainview, NY: Cold Spring Harbor Laboratory Press.

4) Greene, W.C. and B.M. Peterlin, *Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy.* Nat Med, 2002. **8**(7): p. 673-80.

5) Chan, D.C., et al., *Core structure of gp41 from the HIV envelope glycoprotein.* Cell, 1997. **89**(2): p. 263-73.

6) Freed, E.O. and M.A. Martin, *The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection.* J Biol Chem, 1995. **270**(41): p. 23883-6.

7) O'Neill, E., *et al*., *Dynamic Evolution of the Human Immunodeficiency Virus Type 1 Pathogenic Factor Nef.* Journal of Virology, 2006. **80**(3): p. 1311-1320.

8) Roeth, J.F. and K.L. Collins, *Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways.* Microbiol Mol Biol Rev, 2006. **70**(2): p. 548-63.

9) Geyer, M., *et al*, *Structure-Function Relationships in HIV Nef.* EMBO Reports, 2001. **2**(7): p. 580-585.

10) Arold, S.T.a.B., A.S., *Dynamic Nef and Nef Dynamics: How Structure Could Explain the Complex Activities of this Small HIV Protein.* TRENDS in Biochemical Sciences, 2001. **26**(6): p. 356-363.

11) Das, S.R., and Jamel, S., *Biology of the HIV Nef Protein.* Indian J Med Res, 2005. **121**: p. 315-332.

12) Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.

13) Gummuluru, S. and M. Emerman, *Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed Tcell lines.* J Virol, 1999. **73**(7): p. 5422-30.

14) Jowett, J.B., et al., *The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle.* J Virol, 1995. **69**(10): p. 6304-13.

15) Freed, E.O., *HIV-1 gag proteins: diverse functions in the virus life cycle.* Virology, 1998. **251**(1): p. 1-15.

16) Tasara, T., M.O. Hottiger, and U. Hubscher, *Functional genomics in HIV-1 virus replication: protein-protein interactions as a basis for recruiting the host cell machinery for viral propagation.* Biol Chem, 2001. **382**(7): p. 993-9.

17) Sorin, M. and G.V. Kalpana, *Dynamics of virus-host interplay in HIV-1 replication.* Curr HIV Res, 2006. **4**(2): p. 117-30.

18) Bachand, F., et al., *Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 gag precursor.* J Biol Chem, 1999. **274**(13): p. 9083-91.

19) Wang, L., et al., *Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and trans-activation of viral long terminal repeat.* J Biol Chem, 1995. **270**(43): p. 25564-9.

20) Felzien, L.K., et al., *HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 co-activator.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5281-6.

21) Kino, T., et al., *Human immunodeficiency virus type 1 (HIV-1) accessory protein*

*Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators.* J Virol, 2002. **76**(19): p. 9724-34.

22) Sawaya, B.E., et al., *Transdominant activity of human immunodeficiency virus type 1 Vpr with a mutation at residue R73.* J Virol, 2000. **74**(10): p. 4877-81.

23) Misumi, S., et al., *Three isoforms of cyclophilin A associated with human immunodeficiency virus type 1 were found by proteomics by using twodimensional gel electrophoresis and matrix-assisted laser desorption ionizationtime of flight mass spectrometry.* J Virol, 2002. **76**(19): p. 10000-8.

24) Bannwarth, S. and A. Gatignol, *HIV-1 TAR RNA: the target of molecular interactions between the virus and its host.* Curr HIV Res, 2005. **3**(1): p. 61-71.

25) Bieniasz, P.D., et al., *Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both necessary and sufficient for full activation of transcription.* Proc Natl Acad Sci U S A, 1999. **96**(14): p. 7791-6.

26) Bryant, M. and L. Ratner, *Myristoylation-dependent replication and assembly of human immunodeficiency virus 1.* Proc Natl Acad Sci U S A, 1990. **87**(2): p. 523-7.

27) Ciborowski, P.a.G., H, *Human Immunodeficiency Virus-Mononuclear Phagocyte Interactions: Emerging Avenues of Biomarker Discovery, Modes of Viral Persistence and Disease Pathogenesis.* Current HIV Research, 2006. **4**(3): p. 279-291.

28) Freed, E.O., *Viral late domains.* J Virol, 2002. **76**(10): p. 4679-87.

29) Hope, T.J., *Viral RNA export.* Chem Biol, 1997. **4**(5): p. 335-44.

30) Jeang, K.T., H. Xiao, and E.A. Rich, *Multifaceted activities of the HIV-1 transactivator of transcription, Tat.* J Biol Chem, 1999. **274**(41): p. 28837-40.

31) Bohan, C.A., et al., *Analysis of Tat transactivation of human immunodeficiency virus transcription in vitro.* Gene Expr, 1992. **2**(4): p. 391-407.

32) Feinberg, M.B., D. Baltimore, and A.D. Frankel, *The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation.* Proc Natl Acad Sci U S A, 1991. **88**(9): p. 4045-9.

33) Laspia, M.F., A.P. Rice, and M.B. Mathews, *HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation.* Cell, 1989. **59**(2): p. 283-92.

34) Campbell GR et al. *The glutamine-rich region of the HIV-1 Tat protein is involved in T-cell apoptosis.* J. Biol. Chem. 2004. **279**(46): 48197–204.

35) Kato, H., et al., *HIV-1 Tat acts as a processivity factor in vitro in conjunction with cellular elongation factors.* Genes Dev, 1992. **6**(4): p. 655-66.

36) Marciniak, R.A., et al., *HIV-1 Tat protein trans-activates transcription in vitro.* Cell, 1990. **63**(4): p. 791-802.

37) Marciniak, R.A. and P.A. Sharp, *HIV-1 Tat protein promotes formation of more processive elongation complexes.* Embo J, 1991. **10**(13): p. 4189-96.

38) Kim, Y.K., et al., *Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation.* Mol Cell Biol, 2002. **22**(13): p. 4622-37.

39) Kashanchi, F., et al., *Interaction of human immunodeficiency virus type 1 Tat with a unique site of TFIID inhibits negative cofactor Dr1 and stabilizes the TFIIDTFIIA complex.* J Virol, 1996. **70**(8): p. 5503-10.

40) Chiang, C.M. and R.G. Roeder, *Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators.* Science, 1995. **267**(5197): p. 531-6.

41) Dal Monte, P., et al., *TAR and Sp1-independent transactivation of HIV long terminal repeat by the Tat protein in the presence of human cytomegalovirus IE1/IE2.* Aids, 1997. **11**(3): p. 297-303.

42) Garcia-Martinez, L.F., D. Ivanov, and R.B. Gaynor, *Association of Tat with purified HIV-1 and HIV-2 transcription preinitiation complexes.* J Biol Chem, 1997. **272**(11): p. 6951-8.

43) Roebuck, K.A., M.F. Rabbi, and M.F. Kagnoff, *HIV-1 Tat protein can transactivate a heterologous TATAA element independent of viral promoter sequences and the trans-activation response element.* Aids, 1997. **11**(2): p. 139-

44) Veschambre, P., P. Simard, and P. Jalinot, *Evidence for functional interaction between the HIV-1 Tat transactivator and the TATA box binding protein in vivo.* J Mol Biol, 1995. **250**(2): p. 169-80.

45) Kashanchi, F., et al., *Direct interaction of human TFIID with the HIV-1 transactivator tat.* Nature, 1994. **367**(6460): p. 295-9.

46) Herrmann, C.H. and M.A. Mancini, *The Cdk9 and cyclin T subunits of TAK/PTEFb localize to splicing factor-rich nuclear speckle regions.* J Cell Sci, 2001. **114**(Pt 8): p. 1491-503.

47) Deng, L., et al., *Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones.* Virology, 2000. **277**(2): p. 278-95.

48) Deng, L., et al., *Enhancement of the p300 HAT activity by HIV-1 Tat on chromatin DNA.* Virology, 2001. **289**(2): p. 312-26.

49) Zhu, Y., et al., *Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro.* Genes Dev, 1997. **11**(20): p. 2622-32.

50) Herrmann, C.H. and A.P. Rice, *Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor.* J Virol, 1995. **69**(3): p. 1612-20.

51) Yang, X., et al., *TAK, an HIV Tat-associated kinase, is a member of the cyclindependent family of protein kinases and is induced by activation of peripheral*

*blood lymphocytes and differentiation of promonocytic cell lines.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12331-6.

52) Bres, V., et al., *Differential acetylation of Tat coordinates its interaction with the co-activators cyclin T1 and PCAF.* Embo J, 2002. **21**(24): p. 6811-9.

53) Bres, V., et al., *A non-proteolytic role for ubiquitin in Tat-mediated transactivation of the HIV-1 promoter.* Nat Cell Biol, 2003. **5**(8): p. 754-61.

54) Boulanger, M.C., et al., *Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression.* J Virol, 2005. **79**(1): p. 124-31.

55) Athanassiou, Z., et al., *Structural mimicry of retroviral tat proteins by constrained beta-hairpin peptidomimetics: ligands with high affinity and selectivity for viral TAR RNA regulatory elements.* J Am Chem Soc, 2004. **126**(22): p. 6906-13.

56) Nekhai, S., et al., *HIV-1 Tat-associated RNA polymerase C-terminal domain kinase, CDK2, phosphorylates CDK7 and stimulates Tat-mediated transcription.* Biochem J, 2002. **364**(Pt 3): p. 649-57.

57) Parada, C.A. and R.G. Roeder, *Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain.* Nature, 1996. **384**(6607): p. 375-8.

58) Pumfery, A., et al., *Chromatin remodeling and modification during HIV-1 Tatactivated transcription.* Curr HIV Res, 2003. **1**(3): p. 343-62.

59) Zhou, M., et al., *Coordination of transcription factor phosphorylation and histone methylation by the P-TEFb kinase during human immunodeficiency virus type 1 transcription.* J Virol, 2004. **78**(24): p. 13522-33.

60) Nekhai, S., R.R. Shukla, and A. Kumar, *A human primary T-lymphocyte-derived human immunodeficiency virus type 1 Tat-associated kinase phosphorylates the C-terminal domain of RNA polymerase II and induces CAK activity.* J Virol, 1997. **71**(10): p. 7436-41.

61) Nekhai, S., et al., *Cell cycle-dependent stimulation of the HIV-1 promoter by Tatassociated CAK activator.* Virology, 2000. **266**(2): p. 246-56.

62) Ammosova, T., et al., *Phosphorylation of HIV-1 Tat by CDK2 in HIV-1 transcription.* Retrovirology, 2006. **3**: p. 78.

63) Deng, L., et al., *HIV-1 Tat interaction with RNA polymerase II C-terminal domain (CTD) and a dynamic association with CDK2 induce CTD phosphorylation and transcription from HIV-1 promoter.* J Biol Chem, 2002. **277**(37): p. 33922-9.

64) Ammosova, T., et al., *RNA interference directed to CDK2 inhibits HIV-1 transcription.* Virology, 2005. **341**(2): p. 171-8.

65) Malumbres, M., et al., *Driving the cell cycle to cancer.* Adv Exp Med Biol, 2003. **532**: p. 1-11.

66) Agrawal N et al. *RNA interference: biology, mechanism, and applications.* Microbiol Mol Biol Rev, 2003. **67**:657–85.

67) Hannon GJ. *RNA interference.* Nature, 2002. **418**:244–51.

68) Agrawal, N., et al., *RNA interference: biology, mechanism, and applications.* Microbiol Mol Biol Rev, 2003. **67:**657-85.

69) Bartel, D. P. *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**:281-97.

70) Hannon, G. J. *RNA interference*. Nature, 2002. **418:**244-51.

71) Liu, J., et al., *Argonaute2 is the catalytic engine of mammalian RNAi.* Science, 2004. **305**:1437-41.

72) Doench, J. G., and P. A. Sharp. *Specificity of microRNA target selection in translational repression.* Genes Dev, 2004. **18**:504-11.

73) Olsen, P. H., and V. Ambros. *The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation.* Dev Biol, 1999. **216**:671-80.

74) Hall, I. M., et al., *Establishment and maintenance of a heterochromatin domain.* Science, 2002. **297** 2232-7.

75) Jenuwein, T. *Molecular biology. An RNA-guided pathway for the epigenome.* Science, 2002. **297**:2215-8.

76) Matzke, M. A., and J. A. Birchler. *RNAi-mediated pathways in the nucleus.* Nat Rev Genet, 2005. **6**:24-35.

77) Volpe, T. A., et al., *Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi.* Science, 2002. **297**:1833-7.

78) Shafer RW, Schapiro JM. *HIV-1 drug resistance mutations: an updated framework for the second decade of HAART.* AIDS Rev, 2008. **10**(2):67–84.

79) Chen R, Quinones-Mateu ME, Mansky LM. *Drug resistance, virus fitness and HIV-1 mutagenesis.* Curr Pharm Des, 2004. **10**(32):4065–70.

80) Mansky LM, Temin HM. *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase.* J Virol, 1995. 69(8):5087–94.

81) Lane DP. *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381):15–6.

82) Janus F, et al. *The dual role model for p53 in maintaining genomic integrity.* Cell Mol Life Sci, 1999. **55**(1):12–27.

83) Albrechtsen N, et al. *Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53.* Oncogene, 1999. **18**(53):7706–17.

84) Somasundaram K. *Tumor suppressor p53: regulation and function.* Front Biosci, 2000. **5**:D424–37.

85) Striteska D. *[The tumor supressor gene p53].* Acta Medica (Hradec Kralove) Suppl, 2005. **48**(1):21–5.

86) Rezacova M, Vavrova J, Cerman J. *[A cell and genotoxic stress: a reaction to double strand breaks of DNA].* Cas Lek Cesk, 2005. **144**(Suppl 3):13–7.

87) Attardi LD. *The role of p53-mediated apoptosis as a crucial anti-tumor response to genomic instability: lessons from mouse models.* Mutat Res, 2005. **569**(1–2):145–57.

88) Yu J, Zhang L. *The transcriptional targets of p53 in apoptosis control.* Biochem Biophys Res Commun, 2005. **331**(3):851–8.

89) Liu Y, Kulesz-Martin M. *P53 regulation and function in normal cells and tumors.* Medicina (B Aires), 2000. **60**(Suppl 2):9–11.

90) Erster S, et al. *In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation.* Mol Cell Biol, 2004. **24**(15):6728–41.

91) Kojima K, et al. *Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia.* Blood, 2006. **108**(3):993–1000.

92) Kelley ST, et al. *Tumor response to neoadjuvant chemoradiation therapy for rectal adenocarcinoma is mediated by p53-dependent and caspase 8-dependent apoptotic pathways.* Clin Colorectal Cancer, 2005. 5(2):114–8.

93) Strachan GD, et al. *E2F1 induces cell death, calpain activation, and MDMX degradation in a transcription independent manner implicating a novel role for E2F1 in neuronal loss in SIV encephalitis.* J Cell Biochem, 2005. **96**(4):728–40.

94) Meek DW. *The role of p53 in the response to mitotic spindle damage*. Pathol Biol (Paris), 2000. **48**(3):246–54.

95) Bartek J, Lukas J. *Pathways governing G1/S transition and their response to DNA damage.* FEBS Lett, 2001. **490**(3):117–22.

96) Bartek J, Lukas J. *Mammalian G1- and S-phase checkpoints in response to DNA damage.* Curr Opin Cell Biol, 2001. **13**(6):738–47.

97) Bartkova J, et al. *Deregulation of the G1/S-phase control in human testicular germ cell tumours.* Apmis, 2003. **111**(1):252–65.

98) Honma M. *Generation of loss of heterozygosity and its dependency on p53 status in human lymphoblastoid cells*. Environ Mol Mutagen, 2005. **45**(2–3):162–76.

99) Taylor WR, Stark GR. *Regulation of the G2/M transition by p53*. Oncogene, 2001. **20**(15):1803–15.

100) Stark GR, Taylor WR. *Analyzing the G2/M checkpoint.* Methods Mol Biol, 2004. **280**:51–82.

101) Stark GR, Taylor WR. *Control of the G2/M transition.* Mol Biotechnol, 2006. **32**(3):227–48.

102) Houtgraaf JH, Versmissen J, van der Giessen WJ. *A concise review of DNA damage checkpoints and repair in mammalian cells.* Cardiovasc Revasc Med, 2006. **7**(3):165–72.

103) Sancar A, et al. *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. **73**:39–85.

104) Kruse JP, Gu W. *Modes of p53 regulation.* Cell, 2009. **137**(4):609–22.

105) Olsson A, et al. *How important are post-translational modifications in p53 for selectivity in target gene transcription and tumour suppression?* Cell Death Differ, 2007. **14**(9):1561–75.

106) Khanna KK, et al. *ATM associates with and phosphorylates p53: mapping the region of interaction.* Nat Genet, 1998. **20**(4):398–400.

107) Banin S, et al. *Enhanced phosphorylation of p53 by ATM in response to DNA damage.* Science, 1998. **281**(5383):1674–7.

108) Canman CE, et al. *Activation of the ATM kinase by ionizing radiation and phosphorylation of p53.* Science, 1998. **281**(5383):1677–9.

109) Saito S, et al. *Phosphorylation site interdependence of human p53 post-translational modifications in response to stress.* J Biol Chem, 2003. **278**(39):37536–44.

110) Lee JH, et al. *Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death.* J Cell Sci, 2007. **120**(Pt 13):2259–71.

111) Lavin MF, Gueven N. *The complexity of p53 stabilization and activation.* Cell Death Differ, 2006. **13**(6):941–50.

112) Michael D, Oren M. *The p53-Mdm2 module and the ubiquitin system.* Semin Cancer Biol, 2003. **13**(1):49–58.

113) Yang Y, Li CC, Weissman AM. *Regulating the p53 system through ubiquitination.* Oncogene, 2004. **23**(11):2096–106.

114) Brooks CL, Gu W. *Dynamics in the p53-Mdm2 ubiquitination pathway.* Cell Cycle, 2004. **3**(7):895–9.

115) Brooks CL, Gu W. *p53 ubiquitination: Mdm2 and beyond.* Mol Cell, 2006. **21**(3):307–15.

116) Marchenko ND, Moll UM. *The role of ubiquitination in the direct mitochondrial death program of p53.* Cell Cycle, 2007. **6**(14):1718–23.

117) Ohata M, et al. *Prognostic implications of p21 (Waf1/Cip1) immunolocalization in multiple myeloma.* Biomed Res, 2005. 26(3):91–8.

118) de Oliveira RM. Klotho *RNAi induces premature senescence of human cells via a p53/p21 dependent pathway.* FEBS Lett, 2006. **580**(24):5753–8.

119) Child ES, Mann DJ. *The intricacies of p21 phosphorylation: protein/protein interactions, subcellular localization and stability.* Cell Cycle, 2006. **5**(12):1313–9.

120) Castanotto D, Rossi JJ. *The promises and pitfalls of RNA-interference-based therapeutics.* Nature, 2009. **457**(7228):426–33.

121) He L, Hannon GJ. *MicroRNAs: small RNAs with a big role in gene regulation.* Nat Rev Genet, 2004. 5(7):522–31.

122) Ouellet DL, et al. *MicroRNAs in Gene Regulation: When the Smallest Governs It All.* J Biomed Biotechnol, 2006. **2006**(4):69616.

123) Perron MP, Provost P. *Protein components of the microRNA pathway and human diseases.* Methods Mol Biol, 2009. **487**:369–85.

124) Zender L, et al. *Caspase 8 small interfering RNA prevents acute liver failure in mice.* Proc Natl Acad Sci USA, 2003. **100**(13):7797–802.

125) Song E, et al. *RNA interference targeting Fas protects mice from fulminant hepatitis.* Nat Med, 2003. **9**(3):347–51.

126) Leonard JN, et al. *HIV evades RNA interference directed at TAR by an indirect compensatory mechanism.* Cell Host Microbe, 2008. **4**(5):484–94.

127) Barad O, et al. *MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues.* Genome Res., 2004. **14**:2486–94.

128) Bentwich I, et al. *Identification of hundreds of conserved and nonconserved human microRNAs.* Nat Genet., 2005. **37**:766–70.

129) Rounseville MP, Kumar A. *Binding of a host cell nuclear protein to the stem region of human immunodeficiency virus type 1 trans-activation-responsive RNA.* J Virol., 1992. **66**:1688–94.

130) Dykxhoorn DM. *MicroRNAs in viral replication and pathogenesis*. DNA Cell Biol**.,** 2007. **26**:239–49.

131) Grey, F., et al. *Identification and characterization of human cytomegalovirus-encoded microRNAs.* J Virol, 2005. **79:**12095-9.

132) Landry, P., and J. P. Perreault. *Identification of a peach latent mosaic viroid hairpin able to act as a Dicer-like substrate.* J Virol, 2005. **79**:6540-3.

133) Pfeffer, S., et al. *Identification of microRNAs of the herpesvirus family*. Nat Methods, 2005. **2**:269-76.

134) Umbach, J. L., et al. *MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs*. Nature 2008.

135) Omoto, S., et al. *HIV-1 nef suppression by virally encoded microRNA.* Retrovirology, 2004. **1**:44.

136) Bennasser, Y., et al. *HIV-1 encoded candidate micro-RNAs and their cellular targets.* Retrovirology, 2004. **1**:43.

137) Chendrimada, T. P., et al. *TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing.* Nature, 2005. **436**:740-4.

138) Dorin, D., M. et al. *The TAR RNA-binding protein, TRBP, stimulates the expression of TAR-containing RNAs in vitro and in vivo independently of its ability to inhibit the dsRNA-dependent kinase PKR*. J Biol Chem, 2003. **278**:4440-8.

139) Klase Z et al. *HIV-1 TAR miRNA protects against apoptosis by altering cellular gene expression.* Retrovirology**,** 2009. **6**:18.

140) Greenway, A. L., et al. *HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication*. J Biosci, 2003. **28**:323-35.

141) Liang, W. S., et al. *Therapeutic targets for HIV-1 infection in the host proteome*. Retrovirology, 2005. **2**:20.

142) Zhao, R. Y., M. Bukrinsky, and R. T. Elder. *HIV-1 viral protein R (Vpr) & host cellular responses.* Indian J Med Res, 2005. **121**:270-86.

143) Adams, M., L. et al. *Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts.* Proc Natl Acad Sci U S A, 1994. **91**:3862-6.

144) Wu W et al., *Drug 9AA reactivates p21/Waf1 and Inhibits HIV-1 progeny formation.* Virol J, 2008. **5**:41.

145) Agbottah E, et al. *Antiviral activity of CYC202 in HIV-1-infected cells.* J Biol Chem., 2005. **280**(4):3029–42.

146) Cummings M, et al. *XPA versus ERCC1 as chemosensitising agents to cisplatin and mitomycin C in prostate cancer cells: role of ERCC1 in homologous recombination repair.* Biochem Pharmacol, 2006. **72**:166–75.

147) Guendel I et al. *9-Aminoacridine inhibition of HIV-1 Tat dependent transcription*. Virol J, 2009. **6**:114.

148) Choi SU et al. *Effects of flavonoids on the growth and cell cycle of cancer cells.* Anticancer Res, 1999. **19**(6B):5229-33.

149) Meijer, L., et al., *GSK-3-selective inhibitors derived from Tyrian purple indirubins.* Chem Biol, 2003. **10**(12): p. 1255-66.

150) Maggirwar, S.B., et al., *HIV-1 Tat-mediated activation of glycogen synthase kinase-3beta contributes to Tat-mediated neurotoxicity.* J Neurochem, 1999. **73**(2): p. 578-86.

151) Strebel K. *Virus-host interactions: role of HIV proteins Vif, Tat, and Rev.* AIDS, 2003. **17 Suppl 4**: S25–34.