TARGETING THE CLASS I HDACs TO DISRUPT QUIESCENT HIV-1 PROVIRUSES

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Infection with the human immunodeficiency virus type 1 (HIV-1) was first described in a report in the Morbidity and Mortality Weekly Report in June of 1981. Since that time millions of people around the world have been affected by HIV-1 infection. In the last three decades, significant advances have been made in the treatment of people who are infected with HIV-1. However, there is still no cure for HIV-1 infection. The primary obstacle to viral clearance is the existence of long-lived cells harboring replication competent quiescent proviruses. One approach that has been proposed for elimination of this replication competent virus from infected patients is called the "induce and clear" method. This dissertation focuses on identifying factors that could potentially be used as therapeutic targets for induction of quiescent HIV-1 proviruses.

c-Myc and YY1 are two well-known transcription factors that have been demonstrated to bind to the HIV-1 long terminal repeat (LTR) and recruit repressive histone deacetylase (HDAC) enzymes. Therefore, the hypothesis was that specifically targeting factors that recruit HDAC to the HIV-1 promoter would be sufficient to disrupt occupancy of the HDACs and induce expression from quiescent proviruses. The results demonstrate that depletion of YY1 is sufficient to induce
expression from quiescent proviruses in two models of HIV-1 latency; however, it did not affect the residence of HDACs at the HIV-1 LTR.

To further identify specific targets for disruption of quiescent HIV-1, the next step was to elucidate which of the class I HDACs was important for the strong induction of transcription from HIV-1 promoters following treatment with class I selective HDAC inhibitors. Individual depletion of HDAC1, -2, and -3 demonstrated that depletion of HDAC1 and HDAC2 does not affect transcription from the HIV-1 promoter; however, depletion of HDAC3 does significantly induce transcription from quiescent HIV-1 promoters, indicating that it may be possible to reverse latency using therapeutics targeting HDAC3.

The overall conclusions of this thesis indicate that redundant mechanisms of HDAC recruitment are present at HIV-1 LTRs and therefore depletion of HDAC recruiters may not be sufficient to disrupt latency. However, HDAC3 is a potential therapeutic target for induction of transcription from the HIV-1 promoter.
This dissertation is dedicated to my ever-supportive husband and my sweet, happy little girl.
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TABLE OF CONTENTS

LIST OF TABLES........................................................................................................ x

LIST OF FIGURES..................................................................................................... xi

LIST OF ABBREVIATIONS........................................................................................ xii

I. BACKGROUND AND SIGNIFICANCE† ................................................................17
   Transmission of HIV..........................................................................................18
   HIV-1 infection ..................................................................................................18
   Prevention of HIV-1 infection ...........................................................................21
   Anti-retroviral therapy ......................................................................................22
   HIV-1 vaccine ..................................................................................................24
   Latent HIV-1 ....................................................................................................25
   HIV-1 eradication strategies ............................................................................28
   The human immunodeficiency virus ...............................................................29
   Establishment of Latency ..................................................................................34
   HIV-1 persistence ............................................................................................35
   Models for the study of latent HIV-1 ...............................................................35
   HDAC inhibitors as inducers of latent HIV-1 ....................................................37
   Histone deacetylases .........................................................................................45
   c-Myc ...............................................................................................................46
   YY1 ....................................................................................................................47
Selective targeting of HDACs to induce HIV-1 transcription ...................... 48

REFERENCES ............................................................................................................ 52

II. SELECTIVE TARGETING OF THE REPRESSIVE TRANSCRIPTION FACTORS YY1 AND cMYC TO DISRUPT QUIESCENT HUMAN IMMUNODEFICIENCY VIRUSES ................................. 63

Materials and Methods ......................................................................................... 67

Results ...................................................................................................................... 72

Discussion .................................................................................................................. 81

Contributions ............................................................................................................ 85

REFERENCES ............................................................................................................ 94

III. HDAC3 IS A KEY THERAPEUTIC TARGET FOR THE DISRUPTION OF LATENT HIV-1 INFECTION ................................................................. 97

Overview .................................................................................................................... 97

Materials and methods ............................................................................................ 100

Discussion .................................................................................................................. 109

Contributions ............................................................................................................ 116

REFERENCES ............................................................................................................ 123

Introduction ............................................................................................................... 126

Findings and implications .......................................................................................... 129

Future directions ....................................................................................................... 133

Concluding Remarks ................................................................................................. 134

REFERENCES ............................................................................................................ 135
LIST OF TABLES

Table

1.1 Clinical trials for treatment of latent HIV-1 .......................................................... 50
LIST OF FIGURES

Figure

1.1 Induction and clearance strategies ........................................................... 51

2.1 Depletion of cMyc or YY1 does not affect Jurkat cell viability ........................................................... 86

2.2 Depletion of cMyc, YY1 or cMyc and YY1 does not significantly affect expression of HDAC1, HDAC2 or HDAC3 in 2D10 cells ........................................................... 87

2.3 Depletion of YY1 significantly increases HIV-1 expression in 2D10 cells ........................................................... 88

2.4 HDAC occupancy of the HIV-1 promoter is not affected by depletion of cMyc or YY1 ........................................................... 89

2.5 Histone 3 and acetylated histone 3 levels on the HIV-1 promoter did not significantly change following depletion of cMyc or YY1 in Jurkat cells ........................................................... 90

2.6 Concurrent knockdown of cMyc and YY1 did not significantly affect HDAC1, -2, or -3 recruitment to the HIV-1 promoter or the levels of histone 3 ........................................................... 91

2.7 Targeting YY1 and HDAC activity significantly increases expression from the HIV-1 LTR ........................................................... 92

2.8 Expression from the HIV-1 LTR does not require cMyc or YY1 ........................................................... 93

3.1 Depletion of HDAC3 significantly increases expression from the HIV-1 promoter ........................................................... 118

3.2 Co-depletion of HDAC2 and HDAC3 significantly increases expression from the HIV-1 promoter ........................................................... 119

3.3 Depletion of HDAC1, -2, and -3 significantly increases expression from the HIV-1 promoter ........................................................... 120

3.4 Chemical inhibition of HDACs following depletion of HDAC3 significantly increases expression from the HIV-1 promoter ........................................................... 121
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Aza-CdR</td>
<td>5-aza-2'-deoxycytidine</td>
</tr>
<tr>
<td>7sk-snRNP</td>
<td>7sk-small nuclear ribonucleoprotein complex</td>
</tr>
<tr>
<td>acH3</td>
<td>Acetylated histone 3</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Akt</td>
<td>Ak thyoma</td>
</tr>
<tr>
<td>APOBEC3G</td>
<td>Apolipoprotein B mRNA-editing-enzyme-catalytic, polypeptide-like 3G</td>
</tr>
<tr>
<td>ART</td>
<td>Anti-retroviral therapy</td>
</tr>
<tr>
<td>BRG1</td>
<td>Brahma-related gene 1</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CBF1</td>
<td>C-promoter binding factor 1</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine (C-C motif) receptor 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>coREST</td>
<td>Co-repressor of repressor element 1-silencing transcription factor</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DDTC</td>
<td>Diethyldithiocarbamate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>EZH2</td>
<td>Enhancer of zeste homolog 2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Gag</td>
<td>Group specific antigen</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H3</td>
<td>Histone 3</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active anti-retroviral therapy</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone Deacetylase inhibitor</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lack cells</td>
</tr>
<tr>
<td>HEXIM</td>
<td>Hexamethylene bisacetamide inducible 1</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HMBA</td>
<td>Hexamethylene bisacetamide</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyl transferase</td>
</tr>
<tr>
<td>HMTi</td>
<td>Histone methyl transferase inhibitor</td>
</tr>
<tr>
<td>HP1-α</td>
<td>Heterochromatin protein 1 alpha</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>In</td>
<td>Integrate</td>
</tr>
<tr>
<td>LSF</td>
<td>Late SV40 Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MRK</td>
<td>Merck</td>
</tr>
<tr>
<td>mRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>N-CoR</td>
<td>Nuclear hormone receptor co-repressor</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid protein</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative factor</td>
</tr>
<tr>
<td>NF-κb</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NS</td>
<td>Non-specific</td>
</tr>
<tr>
<td>NURD</td>
<td>Nucleosome remodeling deacetylase</td>
</tr>
<tr>
<td>P6</td>
<td>Spacer peptide 6</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>pTEF-b</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of virion expression</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park memorial institute culture medium</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev response element</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription-quantitative PCR</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>SIN3A</td>
<td>SWI-independent 3A</td>
</tr>
<tr>
<td></td>
<td>Silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>SUV39</td>
<td>Suppressor of variegation 3-9 homolog 1</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWIitch/sucrose non-fermentable</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans-activation response</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>UNAIDS</td>
<td>Joint United Nations programme on HIV and AIDS</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
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<tr>
<td>Vor</td>
<td>Vorinostat</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein unique</td>
</tr>
<tr>
<td>XFIM</td>
<td>X-linked mental retardation</td>
</tr>
<tr>
<td>YY1</td>
<td>Ying and yang 1</td>
</tr>
</tbody>
</table>
CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Introduction

Over the last three decades HIV has become a worldwide epidemic that has claimed the lives of millions of people, and more people are currently living with the infection. As of 2011, the World Health Organization estimated that 34 million people were infected with HIV, and 2.5 million new infections occurred during the previous year (100). Additionally, a total of 1.7 million people died from AIDS associated infections in 2011 (100).

The highest burden of HIV infection is on the continent of Africa where, in 2011, UNAIDS estimated that over 23 million people were infected with HIV, which is more than the rest of the world combined (93). Furthermore, HIV is the number one cause of death in Africa. Although the prevalence of HIV in high-income countries is significantly less than in low- to middle-income countries, it is still a significant problem. Approximately 1.4 million people are infected with HIV in North America. In the United States, most new infections are occurring in young adults between the ages of 20 and 30 (15). The most common route of HIV transmission is through men having sex with men followed by heterosexual intercourse. However, the age group

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that comprises the highest number of people living with HIV in the United States is between 45-54, which is likely due to a population of infected individuals that were infected during the initial outbreak and are now aging due to the availability of ART. In the United States, minority communities are disproportionately affected by HIV with an estimated 47% of new infections occurring in the African American population in the United States in 2010 (15). Both in the United States and worldwide, HIV is a significant public-health challenge.

Transmission of HIV

HIV-1 is transmitted through contact of bodily fluids between infected and uninfected persons. The majority of infections occur through sexual contact or through needle sharing during drug use (15). A small number of infections occur due to transmission from HIV-infected mothers to their infants in utero, during birth, or later through breast-feeding. However, a combination of providing pre-natal prophylaxis and formula can reduce the rate of infant infections to less than 2% in some hospitals (24). In the past, there were instances where people were infected through blood transfusions. However, increased screening of blood products has effectively eliminated new infections through blood transfusions. Overall, the majority of preventable new infections occur through sexual contact.

HIV-1 infection

HIV-1 primarily infects cells of the immune system that express CD4 on their surface and also have the co-receptors CCR5 or CXCR4, which include T cells,
macrophages, and a few other subsets that have recently been defined. CD4+ T cells have many roles in the immune system and differentiate into several lineages. One of the primary roles of T cells is in the response of the immune system to bacterial infection. CD4+ T cells help fight bacterial infection by binding to MHC class II molecules on the surface of antigen presenting cells that are presenting bacterial peptides and stimulating fusion of the vesicles and lysosomes within the infected cell to destroy the bacteria (48). Additionally, when CD4+ T cells recognize their target antigens on B cells, they stimulate the B cells to proliferate and produce antibodies. Antibodies that are specific for a pathogen can protect the host from infection either by coating the pathogen and preventing it from being able to attach to and infect cells or by targeting the pathogen for destruction by macrophages (48). Depletion of the CD4+ T cells results in severe immune depression and leaves the host susceptible to infection.

Macrophages and dendritic cells are both antigen-presenting cells, which means that they sample the environment for infectious agents, break them down and display foreign peptides on their cell surface to alert the immune system to infection. Macrophages are located throughout the body in the various tissues where they remove foreign microorganisms by phagocytosis (48). Additionally, macrophages clear cell debris from cells that have undergone apoptosis. Macrophages can also secrete signaling peptides that recruit immune cells to the site of infection. Dendritic cells are located in tissues that come in contact with the external environment such as the nose, lung, stomach and intestines. Dendritic cells sample their environment for potentially infectious microbes by phagocytosing large amounts of particulate
matter and displaying the contents on their surface for recognition by T cells (48). Both macrophages and dendritic cells are antigen-presenting cells that display class II MHC molecules to activate helper T cells. Although macrophages and dendritic cells are susceptible to HIV-1 infection, they have a limited amount of CD4 on their cell surface and it is unclear how commonly they are infected by HIV-1 in vivo.

Soon after infection with HIV-1, the number of CD4+ T cells undergoes a drastic reduction that corresponds to an initial peak in viremia. At this stage, patients experience symptoms similar to a cold and sometimes have enlarged lymph nodes. After the initial peak in viremia, the CD4+ T cell counts stabilize as the host’s immune system attains a level of control. This stage of the disease is referred to as the asymptomatic period. Patients typically remain in the asymptomatic period for between two to twelve years. New guidelines suggest that ART be offered to all HIV-1 infected patients regardless of their infection status and CD4+ T cell counts (92). The current recommendations indicate that the patient should remain on ART indefinitely (92). The transmitted virus is generally able to use the CCR5 chemokine receptor as a co-receptor for viral entry, which is referred to as being CCR5 trophic. However, virus that is able to use the CXCR4 co-receptor has the ability to be transmitted but is only transmitted rarely. In patients that are infected with CCR5 trophic virus, as the disease progresses, new HIV-1 virions emerge with the ability to use the CXCR4 co-receptor (29). As HIV-1 infection progresses, the number of CD4+ T cells continues to decline and severe immune suppression develops. At this point, patients begin to develop opportunistic infections and are considered to have acquired immunodeficiency syndrome (AIDS). The immune suppression caused by
HIV-1 infection does not directly cause mortality in the patients. Opportunistic infections that do not commonly cause significant disease in immune competent individuals are significantly worse in patients whose immune systems are compromised due to HIV-1 infection and can lead to mortality if they are not treated. Opportunistic infections that are prevalent in patients that have progressed to AIDS in high-income countries are thrush, pneumonia, cervical cancer, infection with *mycobacterium* and infection with cytomegalovirus (12). Tuberculosis, infection with enteric parasites, pneumonia and malaria are common opportunistic infections in regions were they are prevalent, and tuberculosis is the number one cause of death in HIV-1 infected patients in Africa. The inability of the immune system to combat opportunistic infections that are generally innocuous to immune competent individuals eventually causes mortality due to those infections.

During the early years of ART, HIV-1 developed resistance to many of the drugs that were first developed as they were used singly or in pairs. Since the introduction of combination therapies, the development of resistance has become less of a problem. However, transmission of resistant species may limit therapy options for some patients.

**Prevention of HIV-1 infection**

During the last two decades, the number of new HIV-1 infections has significantly decreased due to many factors including campaigns to educate the public on how to prevent transmission of the virus (65). However, many new infections still occur each year. Recently, a great deal of focus has been on
developing additional prophylactic methods to prevent transmission of HIV-1. Cohen et al. found that treating the infected patient with ART significantly decreased the rate of transmission to their partner (19). Another study by Karim et al. in which they tested intravaginal gels containing the anti-retroviral drug Tenofovir demonstrated a 39% reduction in HIV-1 acquisition (1). Condoms have traditionally been a very effective means of preventing new infections; however, in many cultures and relationships, placing the burden/responsibility of prevention on the male in the relationship has been unsuccessful. Developing tools to allow women to control their exposure risk is essential. Trials like the early treatment study and the intravaginal tenofovir study mentioned above are a positive step toward helping women to gain control over their own sexual health. However, neither of these studies had a 100% efficacy rate, and it is impractical and possibly unethical to ask people to rely on prevention methods that are not completely protective.

**Anti-retroviral therapy**

Currently, there is no cure for HIV-1 infection. However, ART is able to extend the life span of infected individuals to near that of non-infected individuals (96). Yet, ART is not a complete resolution to the epidemic for several reasons, the primary reason being that the quality of life for patients that are on ART is significantly affected. The associated adverse effects of the regimens are numerous and include diarrhea, nausea, rash, depression and headaches (44). Furthermore, the stigma associated with HIV-1 infection can be socially isolating. In addition to affecting the quality of life, another downside of ART is that the cost of these drugs is extremely
high. A typical month of first line ART in 2006 cost $1,140, and fourth line drugs cost upwards of $3,700 a month (84). Furthermore, current guidelines recommend that patients continue therapy indefinitely. The average life expectancy of patients after beginning ART is 24.7 years (84). In total, with doctors visits, tests and ART the lifetime cost of HIV-1 is upwards of $380,000 per patient (84). Although the advances in treatment of HIV-1 have significantly increased the quality of life for patients there is still significant room for improvement.

In countries with patent law, to access drugs that cost less, patients generally have to wait 20 years until the drug is no longer protected by patent law. Furthermore, the trade-off is that the older drugs have more associated adverse effect and many strains of HIV-1 have developed resistance to these drugs. Although these drugs can reduce the cost of treatment, the disadvantages are still significant.

In recent years, significant efforts have been made by several countries and organizations to provide anti-retroviral treatment to HIV-1 infected individuals in low- and middle-income countries. According to the World Health Organization 54% of people in low- and middle-income countries now have access to ART (99). However, there is still a significant need for additional access. In addition to the financial restraints, the physical access to a medical facility to obtain the ART and the social stigma associated with taking it prevents many people from getting the treatment they need. Furthermore, the economic burden of providing these drugs is largely shouldered by developed countries that are currently struggling with their own
economic crises. Therefore, providing ART to these countries may not be feasible or sustainable for the long-term.

Efforts to develop new therapies are being pursued (and to a large extent have been successful) that would have to be taken less often, are less susceptible to the development of resistance and that have fewer adverse effects (44). However, several downsides to the development of new therapies exist. Like current regimens, new therapies would have to be taken for the rest of the patient’s life. Although many of the newer drugs have fewer adverse effects, the associated adverse effects, both short term and long term, are still a concern (44). One of the most alarming aspects for the development of new drugs is that the cost is still significant, sometimes more than the current regimens. Therefore, the development of new therapies for HIV-1 is a good intermediate strategy, but is not a suitable approach for long-term management of the epidemic. Furthermore, studies such as the Cohen et al. study, which revealed a significant decrease in the transmission of HIV-1 in couples in which the index patient was taking ART have opened the discussion regarding how early ART should be initiated (19). Initiating ART earlier would significantly increase the cost and prolong the exposure of patients to the adverse effects that are associated with therapy.

**HIV-1 vaccine**

A significant amount of effort has been put into the development of a vaccine for HIV-1. However, even the most successful trial, RV144, only saw an efficacy of 31.2% (79). Follow-up studies found that while that vaccine used in the RV144 trial
generated IgG antibodies against the variable regions 1 and 2 of envelope, IgA antibodies were also produced that may have diminished the effect of the IgG antibodies (45). As in the RV144 trail, the reasons for the lack of protection of many of the vaccines that have been developed are complicated. One of the primary reasons that development of a vaccine for HIV-1 has been difficult is the high genetic variability of HIV-1 populations. Successful design of an antibody-based vaccine may require the identification of an epitope that is broadly neutralizing across several HIV-1 clades. The morphology of the Env trimer and the genetic variability of HIV-1 make the development of such an antibody difficult. Regions of Env that are highly immunogenic are sequestered in the trimer, making them inaccessible to antibodies. Furthermore, due to conformational changes, antibody targets on gp120 can become inaccessible following binding to CD4. Furthermore, many antibodies target the variable regions of Env, which are highly glycosylated, and a simple mutation that results in the loss or addition of a glycosylation site can prevent binding of antibodies (47). Vaccine technology is continually improving and an effective vaccine may be developed in the future. While a vaccine would be a huge contribution to preventing new HIV-1 infections, it would not address the needs of people that are already infected with HIV-1. Therefore, it is important to pursue additional strategies in conjunction with vaccine research.

Latent HIV-1

Each of the above strategies to control HIV-1 is important and has significantly influenced the epidemic. However, none is completely efficacious, cost-
effective or without limitations. Recently, a substantial amount of effort has been
directed toward developing a short-term therapy that would eliminate all replication
competent virus from infected individuals. Following such an eradication therapy,
some remnants of defective proviruses may still be detectable in patients. Such a
sterilizing cure would address many of the shortcomings of the above-mentioned
approaches. The quality of life of the patients would be significantly improved over
current ART regimens because they would not have to endure the ART-associated
adverse effects. The cost of treatment to eliminate the virus would be limited to the
duration of therapy. Furthermore, individuals that are in situations where they are
unable to control their exposure would not have to endure a life-long infection.
Overall, a cure would be the best solution for communities, patients and health care
providers.

Before moving on to how an effective cure for HIV-1 would be achieved it is
important to explain why the current ARTs are not curative. Over the years since the
beginning of the epidemic many therapies targeting different stages of the HIV-1
replication cycle have been developed including drugs that target entry, reverse
transcription, integration, and protease cleavage. These therapies are very effective
at inhibiting HIV-1 replication. In fact, patients that are faithfully taking ART and have
not developed resistance have viral loads that are undetectable by standard
methods for many years. However, a very small population of HIV-1 infected cells
remain. The majority of this latent virus resides in the CD4+ resting memory T cells
of the immune system. Other reservoirs have been suggested to exist in the brain,
gut-associated lymphoid tissue, bone marrow, and genital tract (14). Furthermore,
additional undiscovered reservoirs may also exist, and studies to elucidate these reservoirs are currently underway. Cells are considered to harbor latent virus when the virus has entered the cell, reverse transcribed its viral RNA into a DNA copy and inserted its DNA provirus into the host cell’s genome; instead of continuing through the replication cycle, the proviral genome becomes transcriptionally silent. These transcriptionally silent but replication competent viral genomes can collectively be referred to as quiescent or latent proviruses, meaning that they are not making viral proteins or full length RNA, which would be required for the virus to continue through the replication cycle. Unfortunately, these viral genomes are virtually indistinguishable from the genome of the host and are therefore very difficult to target. Virus that is not going through the stages of the replication cycle that are targeted by ART are resistant to treatment with current regimens. If the virus were to remain in this state indefinitely, it would not be a problem for patients. However, the virus has the ability to spontaneously and unpredictably resume transcription and if the patient is not on ART, the virus can resume replication and renew infection. Therefore, patients must remain on ART indefinitely to control spontaneous reactivation of these transcriptionally silent viruses.

Until recently, few researchers were in serious pursuit of strategies to cure HIV-1 infection, but this effort has recently gained widespread acceptance and attention. Because of this, many novel strategies have been proposed to complement traditional drug therapies. However, regardless of the approach, not all therapies are created equal. Indeed, for a therapy to be effective and feasible for worldwide use, it will have to meet several requirements. First and most importantly,
the therapy must be safe and have manageable side effects. Second, it must not extensively activate the immune system, as activated T cells are more susceptible to HIV-1 infection and are more difficult to protect with ART. Third, the treatment must have a finite duration that will allow the patient to live a healthy life without the need for ongoing treatment. Fourth, if the goal is eradication, the treatment must be able to access all reservoirs of persistent infection throughout the body. Finally, to ensure global utility and availability, the therapy must be economically and logistically accessible to the developing world, as this is where the burden of lifelong therapy is least sustainable. Initially, drugs that have already been pharmacologically characterized and approved for use in humans for treatment of other diseases will have an advantage over newer approaches, as the time from initial testing to implementation will be drastically reduced. That said, many novel approaches to treating HIV-1 infection, some of which are detailed below, are receiving a great deal of attention from the research community and may prove invaluable in our pursuit of a cure.

**HIV-1 eradication strategies**

Eradication is what most often comes to mind when people think about a “cure” for HIV-1. The goal of this approach is clearance of all replication competent virus from the patient. Purging HIV-1 provirus from latent reservoirs is crucial to any HIV-1 eradication strategy, and interest in this area has spurred major efforts to identify therapeutics that can do so. Most eradication studies have focused on identifying small molecule drugs that elicit proviral expression with the notion that
ART will prevent new infections while the immune system, possibly with the help of other therapeutics, will clear infected cells. Indeed, several small molecule drugs that have been developed for the treatment of HIV-1 latency are currently in clinical trials (table 1). Beyond these drugs, other conceptual options for eradication include gene therapy approaches such as HIV-1-specific recombinases that destroy proviral DNA or HIV-1-dependent suicide genes that selectively kill HIV-1-infected cells.

The human immunodeficiency virus

To understand how a cure could possibly be achieved, it is important to first understand more about HIV-1 and its replication cycle. HIV-1 is a positive stranded enveloped RNA virus in the family Retroviridae and the genus lentivirus. The HIV-1 genome encodes the core proteins (Gag, Pro, Pol, and Env), two regulatory proteins (Tat and Rev), and four auxiliary proteins (Nef, Vif, Vpu, and Vpr) (33).

The HIV-1 virion initially makes contact with the target host cells by binding to the CD4 receptor and either the CCR4 or CXCR5 co-receptors on the cell surface. The viral gene env codes for the HIV-1 glycoproteins gp41 and gp120, which bind to CD4 and the CCR5 and CXCR4 co-receptors and facilitate viral entry (102). The polyprotein Pol encodes several proteins that are important for many stages of the viral replication cycle including protease, reverse transcriptase, RNase H and Integrase. Pol is cleaved into its components by protease, which also cleaves the Env and Gag polyproteins in to their individual viral proteins, which will be discussed later during the discussion of maturation of the virions (46, 60). After the virus has bound to the receptors and fused with the cell, one of the two RNA genomes is
reverse transcribed by viral Reverse Transcriptase (RT), which creates a double-stranded DNA proviral genome from the genomic RNA. During this process an RNA/DNA intermediate product is briefly produced that is composed of the genomic RNA and the first strand of the DNA provirus. The viral protein RNase H quickly degrades the RNA in this hybrid to allow the second strand of DNA to be synthesized (86). Following synthesis, the pre-integration complex, which contains the HIV-1 provirus, is shuttled into the nucleus where it integrates into the host’s DNA. The ability of the HIV-1 pre-integration complex to enter the nucleus allows HIV-1 to infect non-dividing cells (32). Once the proviral DNA is integrated into the host’s genome the integrated provirus serves as a transcription template for viral RNAs. The viral protein Tat, through its interaction with pTEF-b, is a potent activator of HIV-1 transcription. HIV-1 transcripts contain several splice donor and acceptor sites that are recognized and cleaved by the cellular splicing machinery. The initial viral RNAs are fully spliced and produce the early proteins such as Tat, Nef and Rev. However, HIV-1 also needs to produce partially spliced and full-length genomic RNA. The viral protein Rev binds to unspliced or partially spliced HIV-1 RNA and facilitates their nuclear export (32). The Gag polyprotein is cleaved during virion maturation into its individual components that include matrix (MA), capsid (CA), nucleocapsid (NC) and p6, which are important for the assembly and budding of new viruses. The matrix protein is important for localization of the Gag polyprotein to the cell membrane where the virus assembles prior to budding from the cell (32). Once the virus buds from the cell, it undergoes further maturation through proteolytic
cleavage of Gag-Pro-Pol into its constitutive components, which facilitates condensation of NC-RNA and maturation of the CA core (60).

The final viral particle has many components including a conical shaped viral core that is composed of the capsid protein, RT and two copies of the genomic RNA, which is bound to nucleocapsid to facilitate encapsidation into the virion (31). Nucleocapsid accelerates reverse transcription by facilitating binding of the R sequence of the ss-cDNA region to tar during the transfer of the first strand (23, 39). The viral protein p6 is involved in budding of the virus from cells and in incorporation of Vif and VPR into the virion (40).

Retroviruses have been around for thousands of years. In fact, a large percentage of the human genome is composed of remnants of retroviruses called retroelements (25). Over time, human cells have evolved protective mechanisms to defend themselves from retroviruses including TRIM5α, Tetherin and APOBEC3G. TRIM5α is a cellular protein that interacts with the capsid protein and accelerates decoating of the virus, which results in an unstable viral core that is unable to advance through the HIV replication cycle (91). Tetherin is another host factor that is restrictive to HIV-1. Tetherin homodimers are located in lipid rafts in the cellular membrane. When HIV-1 buds from the cell, regions of the tetherin protein are embedded in the membrane of the budding virus and in the membrane of the cell, which effectively tethers the virion to the cell and prevents budding (110). APOBEC3G is a DNA cytosine deaminase that is packaged into HIV-1 virions and is able to mutate HIV-1 DNA during subsequent infection as it is being reverse
transcribed (105). HIV-1 encodes the Vif accessory protein, which counteracts the cellular factor APOBEC3G (87). Vif binds to and promotes degradation of the cellular antiviral protein APOBEC3G (69). Viruses that lack Vif are susceptible to mutation and inactivation by APOBEC3G.

Another accessory protein, Vpr is involved in many processes during the HIV-1 replication cycle from induction of HIV-1 transcription to nuclear import of the HIV-1 pre-integration complex. However, the most well-known function of Vpr is its ability to arrest cells in the G2 phase of the cell cycle, which is important for the early stages of HIV-1 infection (41, 56). The Vpu protein binds to and induces degradation of the CD4 protein and is involved in release of viral particles from the cellular membrane. Nef induces the degradation of several cellular proteins including MHC-1 and CD4, which prevents superinfection and interferes with the ability of the immune system to control infection (2, 8). Additionally, Nef is important for the pathogenicity of HIV-1, which was most elegantly demonstrated by the Sydney blood bank cohort of HIV-1 infected patients who were infected with a virus containing a deletion in Nef and remained asymptomatic for over 15 years (28, 34). The many virally encoded proteins interact with cellular proteins to facilitate successful replication of HIV-1.

Several noncoding regulatory regions including the Trans-Activating Response element (TAR) and the Rev Response Element (RRE) are important parts of the HIV-1 genome. As the proviral genome is transcribed, a secondary structure called TAR forms toward the beginning of the elongating RNA that is able to bind the viral protein Tat (7). Tat in turn recruits pTEF-b, which phosphorylates the C-terminal
tail of RNA pol II and promotes transcriptional elongation (66). The RRE is another non-coding region that is very important for HIV-1 replication. The RRE is recognized by the viral protein Rev, which facilitates nuclear export of partially spliced and unspliced genomic RNAs (78). Partially spliced RNAs are important for translation of Env, Vpu, Vpr, and Vif. The non-coding regions TAR and RRE are involved in many steps of the HIV-1 replication cycle.

One of the most important non-coding regions of HIV-1 is the long terminal repeat (LTR) or the promoter. The LTR controls viral transcription and is thought to mediate the development of HIV-1 latency. During HIV-1 replication, transcription is very robust. However, occasionally, the long terminal repeat becomes occupied by repressive transcription factors that inhibit transcription from the viral promoter (95). When viral mRNAs are not transcribed or viral proteins not translated, infected cells are not detectable by the immune system and the provirus is not susceptible to ART. The long terminal repeat has been extensively characterized and can be occupied by many transcription factors including c-Myc, YY1, NF-κB and CBF-1 (21, 49, 67, 104). Many of these factors have been demonstrated to both positively and negatively regulate transcription depending on the context of the promoter. Furthermore, c-Myc, YY1, NF-κB and CBF-1 have all been shown to be important for recruitment of Histone deacetylase 1 (HDAC1) to the HIV-1 LTR in many different contexts (21, 49). The restriction of transcription at the HIV-1 promoter due to the development of heterochromatin is thought to play an important role in the maintenance of the quiescent proviral state.
Establishment of Latency

Occupancy of the LTR by the repressive factors discussed in the previous section is thought to maintain latency. However, how latency initially develops is still somewhat debatable. Several mechanisms have been proposed for the establishment of the quiescent proviruses. One theory is that as the infected active CD4+ T cell transitions to the resting state the availability of factors that are required for transcription become limited within the cell and are not available at the HIV-1 promoter (59). Other experts believe that latency may be a random state that is established in active CD4+ T cells that become long lived resting CD4+ T cells. The site of viral integration may also affect the development of latency. Studies have shown that HIV-1 preferentially integrates into actively transcribing genes (42). Insertion downstream of an active promoter may inhibit the ability of the HIV-1 genome to initiate transcription due to interference by active transcriptional machinery from the upstream promoter (43). Additionally, although HIV-1 genomes favor sites of active transcription, it is still possible for them to insert into sites that contain heterochromatin, which can restrict access of transcription factors to the promoter (64). Others have proposed that some HIV-1 latency may be due to the persistence of pre-integration complexes in cells. However, research has indicated that these pre-integrated complexes have a half-life of approximately one day and therefore may not account for a significant amount of the replication latency found in patients (111). It is currently unknown which of these mechanisms are primarily responsible for the majority of latent HIV-1. It is likely, however, that all of the above mechanisms may contribute to maintenance of the quiescent proviral pool.
HIV-1 persistence

The mechanism of maintenance of the latent reservoir has also been debated. Some believe that there is a low level of ongoing replication in patients that are on suppressive HAART that continually reseeds the latent viral reservoir (109). However, studies looking at evolution of virus do not see any evidence of viral evolution, which indicates that the viruses are not replicating (74). Therefore, some have proposed that the latent population may be maintained by replication and clonal expansion of infected cells (88). Others believe that there may be pockets of infected cells that occasionally reseed the viral reservoir (52, 88). Eventually, it may be necessary to determine which of these scenarios most accurately mimics the in vivo state to tailor latency treatments.

Models for the study of latent HIV-1

Lack of a truly representative model system is a significant obstacle to HIV-1 latency research. Currently, the best model is the cells of aviremic patients. The gold standard for monitoring the effectiveness of small molecules on inducing viral production is the patient cell assay, which is labor, time and resource intensive (18). Many important research goals cannot be achieved using human cells due to technological or ethical restraints. Furthermore, access to latently infected patient cells is limited. SIV models are available, but are expensive and do not accurately represent the latent state in humans. Humanized mouse models have been developed for latency (16). These models are relatively new, but may prove to be a good system to screen therapies in prior to introduction into humans. So far, our lab
and Dr. Victor Garcia’s lab have been able to stably suppress viral infection in humanized mice using ART (16, 26). The next step in this model will be to test the induction and clearance strategies by administering HDAC inhibitors or other compounds that activate HIV-1 transcription in the presence of ART and by monitoring the percentage of latently infected cells.

For many years, cell line models of HIV-1 latency have been the workhorses of the field. Initially, many HeLa cell lines carrying HIV-1 LTRs with a reporter were used (50, 54, 58, 82). More recently, several Jurkat, monocytic and glial cell lines have been developed that contain full-length HIV-1 genomes with a reporter inserted into the genome to facilitate detection of transcription from the viral promoter (111). Jurkat cells are derived from T lymphocytes, which are the target cells of in vivo infection. These cell lines have been very useful for the careful characterization of the range of factors that bind to the HIV-1 LTR, and preliminary studies on small molecules and their ability to induce transcription from the HIV-1 LTR (104). However, each cell line is a clone that has only one insertion site. In vivo the virus integrates randomly into each cell creating a diverse population of insertion sites that cannot be accurately mimicked by the current cell line models (85). Furthermore, the cell lines are composed of actively replicating cells, whereas resting memory T cells, where the majority of latent virus is thought to exist, are in a quiescent state. Several of these lines are available, but alone none are truly representative of the in vivo environment where there are many different insertion sites and little cellular replication. However, cell lines have proven to be a good starting point to test the effectiveness of compounds prior to moving them to the patient cell assays.
Recently, several labs have developed primary cell models of latency where CD4+ T cells are isolated from uninfected donors and subsequently infected with HIV-1 and allowed to enter a resting state (10, 83). These models mirror the range of insertion sites that are observed \textit{in vivo} and the non-dividing phenotype of resting memory CD4+ T cells. Therefore, labs are now incorporating use of one or more of these models into their research.

\textbf{HDAC inhibitors as inducers of latent HIV-1}

Histone deacetylase inhibitors (HDACis) were identified as candidate anti-latency drugs following the discovery that their target HDACs were specifically recruited to the HIV-1 promoter and maintained latency (51, 94). Corroborating this, HDACi were identified in several screens for compounds that induce transcription from quiescent HIV-1 proviruses (57). While HDACs might be better termed lysine deacetylases, as they can remove acetyl groups from both histone and non-histone proteins, their removal of acetyl groups from the lysine residues within histone tails is associated with reduced transcription from cellular and viral promoters. Inhibition of HDACs allows histone lysine acetylation to persist, which then results in recruitment of transcriptional activators and facilitates transcription from the HIV-1 promoter (Figure 1.1). Suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) are HDACis that activate HIV-1 transcription in both cell line models of latent HIV-1 and in \textit{ex vivo} cultured patient cells. Both drugs are currently approved for clinical use, SAHA for oncology and VPA for a variety of indications, and many of their pharmacological characteristics are known. HIV-1-centered clinical trials have been
conducted for VPA, and two such clinical trials are ongoing for SAHA (4, 61). Past studies testing VPA using varying designs and endpoints have had mixed results, but uniform and exposure-dependent depletion of the latent viral reservoir has not been observed (61, 89). Recent work suggests that alternative HDACis, such as SAHA, are able to induce expression from the HIV-1 promoter at lower concentrations than VPA (70). Notably, the initial results from the SAHA clinical trials are promising. In a trial being conducted by our group, HIV-1-positive patients were given a 400 mg dose of SAHA and resting CD4+ T cells were isolated to measure viral RNA production and global histone H3 acetylation to determine whether SAHA is able to upregulate viral transcription. To date, all patients in the study have demonstrated increased viral RNA production and histone H3 acetylation, which indicates that HIV-1 latency was disrupted following a single 400 mg dose of SAHA (clinical trial NCT01319383) (Table 1)(4). A second study at Monash University in Melbourne is underway to assess a multi-doses regimen of SAHA administered as 400 mg of SAHA daily for 14 days (Table 1) (63). The results from this trial are currently pending. If the results from these studies verify the ability of SAHA to activate quiescent HIV-1 in patients, the next step will be to determine whether this activation has an effect on the viral reservoir.

In addition to SAHA and VPA, several additional HDACis that activate transcription of quiescent proviruses including Belinostat, Panobinostat, Givinostat, and Entinostat, are currently in phase II clinical trials for treatment of cancers or juvenile arthritis (70, 101). Furthermore, a clinical trial for juvenile arthritis in which children ranging from 6-17 years of age were administered daily Givinostat revealed
only minor adverse drug effects, suggesting the drug’s potential utility for treating youth and adolescents (98). In addition to activation of HIV-1, Givinostat and SAHA have also been demonstrated to transiently reduce expression of a subset of cytokines at a clinically relevant dose in ex vivo studies (37, 62). Because activating the inflammatory response may activate the immune system, making cells susceptible to HIV-1 infection, suppression of the inflammatory response may prevent infection of additional cells upon reactivation. Depending upon the safety of these drugs and their pharmacological suitability, these drugs may also be tested in future clinical trials addressing HIV-1 latency.

Conceptually, it is important to note that the most desirable qualities of the ideal HDACi for clinical application in HIV-1 remains to be defined. Obviously, such a drug must be clinically well tolerated. Current evidence suggests that inhibitors selective for the Class I HDACs 1, 2, and 3 are the most relevant (5, 51), and recent studies suggest that HDAC3 may be the most important target. However, highly selective inhibitors for HDAC3 are only recently available. Although it is widely assumed that a more potent HDAC inhibitor will have a better clinical effect, this is unproven. Of central importance, the optimal duration and schedule for dosing that is required to achieve sufficient HIV-1 induction without impairing the immune response or inducing toxicity is unknown.

Disulfiram

Disulfiram is a zinc-chelating agent that is approved for use in humans to treat alcoholism. Its toxicities are managable. Dilsulfiram and its metabolite
diethyldithiocarbamate (DDTC) were recently demonstrated to activate HIV transcription from a primary cell model of latent HIV (107). A study by Doyon and colleagues suggests that disulfiram causes depletion of Phosphatase and tensin homolog (PTEN), which is an inhibitor of the Akt pathway. Once inhibition of Akt is removed it phosphorylates HEXIM1, which releases p-TEFb. Free p-TEFb is then recruited to the HIV promoter where it can activate transcription (Figure 1.1). In further support of this model, dilsulfiram mediated activation of quiescent HIV proviruses was blocked by an inhibitor of the Akt pathway, which indicates that disulfiram may modulate HIV expression through its effects on PTEN and the Akt pathway (27). A clinical trial (NCT01286259) with dilsulfiram has been undertaken to determine its effect on persistent HIV infection in patients. The pilot study enrolled 14 participants who took 500 mg of disulfiram daily for 14 days (Table 1). The initial results from the pilot study revealed a modest but statistically significant increase in plasma HIV RNA in the group as a whole, largely driven by transient and temporally dispersed increases in viral load in only a few patients and a minimal absolute decrease in the frequency of infected resting CD4+ T cells that was within the range of variation seen in viral outgrowth assays (90). At this time, it is unclear if disulfiram can contribute to clinically significant reductions in the viral reservoir.

**PD-1 inhibition**

PD-1, which is a receptor that is best known for its role in immune exhaustion, is currently the focus of several studies aimed at treating latent HIV. PD-1 antibodies are being investigated for their ability to activate HIV transcription and/or reverse
immune exhaustion caused by HIV infection while specifically targeting HIV infected cells. To date, a single abstract has been presented, which suggests that exposure to PD-1 antibodies induces expression of HIV. However, the relevance and validity of this observation was weakened by the fact that the cells studied were obtained from viremic patients (22). A clinical trial in development to determine the effect of PD-1 antibodies on persistent infection in ART-treated patients is currently delayed by unanswered safety concerns arising from observed toxicities following the use of the drug in oncology trials (Table 1).

**Vaccination**

Several studies have reported increases in viral expression following routine vaccination against other pathogens in HIV-infected individuals, which may be attributable to mild activation of the immune system (13). However, the increased viral loads and T cell activation after immunization were not associated with better viral control when ART was interrupted (13), indicating that this therapy may need to be performed in the context of ART or other therapeutics. In an interesting and provocative study, Persaud and colleagues immunized patients with a poxvirus vaccine engineered to express HIV antigens and observed a significant, albeit transient, decrease in replication-competent HIV in the resting T cell reservoir (Table 1) (77). These results suggest that such approaches might activate latent virus and/or induce an immune response to low-level antigen production in some cells, thereby resulting in a decline of the number of latently infected T cells. Such
vaccines have been studied and well tolerated in HIV infected patients, and so this strategy has potential for use in treating latent HIV (38).

Other potential targets

While HDACis are currently the most promising candidates for the “kick and kill” approach, many other cellular pathways are involved in the maintenance of latent HIV, and compounds targeting these pathways may enter clinical trials in the near future. Here, we briefly discuss examples of these pathways and a few of the promising compounds under study.

Activation of the protein kinase C (PKC) and NF-κB pathways induces expression of quiescent HIV proviruses. Once activated, PKC phosphorylates IκBα, which results in release of NF-κB. NF-κB then translocates to the nucleus where it binds to the HIV LTR and promotes transcriptional activation (Figure 1.1). Drugs targeting these pathways, such as tumor necrosis factor-alpha (TNF-α), prostratin, and bryostatin are currently under study for treatment of latent HIV infection (11, 75, 103). Prostratin, a phorbol ester that targets both the PKC and NF-κB pathways, activates HIV transcription in several J-lat cell line models of HIV latency (103). Additionally, prostratin in combination with the HDACis VPA and SAHA synergistically increases the amount of virus produced from cell line models of HIV latency (80). Bryostatin, a macrocyclic lactone, is another candidate that targets the PKC and NF-κB pathways. In addition to modulating the PKC and NF-κB pathways, bryo-1 also downregulates the CD4 receptor on T cells, which limits the ability of HIV to infect these cells (Figure 1.1) (11). Importantly, Bryostatin does not activate T-
cells and has low cytotoxicity (71). Bryostatin has entered a phase II clinical trial for
treatment of ovarian cancer (73). However, in this trial, despite a partial response of
some patients to the treatment, severe myalgia developed in all study participants
(73). While studies to advance bryostatin for future treatment of latent HIV are
planned, testing will have to be cautious in light of these side effects.

Hexamethylene bisacetamide (HMBA), a modulator of the HMBA inducible 1
(HEXIM1) component of the 7SK snRNP, also stimulates HIV transcription (17, 55).
HMBA activates the PI3K/Akt pathway, resulting in HEXIM1 phosphorylation and
release of the transcriptional elongation factor P-TEFb from the transcriptionally
repressive 7SK snRNP/HEXIM complex (20). Following its release, P-TEFb is
recruited to HIV TAR by HIV Tat, which facilitates productive transcription elongation
(Figure 1.1). HMBA reached phase II clinical trials for acute myelogenous leukemia
(3), and while the drug was tolerable in this setting, further studies have not been
pursued due to lack of efficacy in cancer. HMBA was also rapidly metabolized,
eventually requiring continuous IV infusion in studies to achieve detectable drug
levels. Clinical trials for treatment of latent HIV have not been pursued.

In addition to histone deacetylation and signaling cascades, other possible
targets for therapy are being investigated. Among these are enzymes that methylate
histone proteins or DNA. Depending on the site of methylation and the number of
methyl groups present, these modifications can recruit proteins that activate or
repress transcription. 5-aza-2’-deoxycytidine (5-Aza-CdR), an inhibitor of DNA
methyltransferases, activates transcription from the HIV LTR in several cell line
models of latent HIV (Figure 1.1)(30). Furthermore, several histone methyltransferases (HMTs), including SUV39, G9a, EZH2 among others, have been demonstrated to be involved in transcriptional modification of HIV (35). When the HIV promoter is in the quiescent state high levels of the repressive chromatin modifications trimethylated lysine 9 and lysine 27 of histone 3 and HP1-α can be detected at the HIV promoter by ChIP (76). Following induction of transcription from the HIV LTR using TNF-α the levels of trimethylated lysine 9 and lysine 27 of histone 3 as well as the levels of associated HP1-α decrease. Significantly, the HMT inhibitors (HMTis) Chaetocin, BIX-01294 and DZNep reactivate HIV transcription in cell line models of HIV latency. However, the relevance of these observations to clinical applications remains unproven and awaits the assessment of these compounds in authentic resting cells from patients (35). Further, the clinical safety of these drugs has not been assessed outside of limited studies in advanced malignancies, and so the development and clinical validation of safe and effective HMTis and DNMTis remains an important research goal.

Additional compounds that may relieve HIV latency are currently under investigation. Xing and colleagues conducted a drug library screen to identify compounds that activate HIV transcription in a primary cell model of HIV latency, and derivatives of quinolin-8-ol, a bivalent cation chelator, were identified (Figure 1.1)(106). Furthermore, Micheva-Viteva and colleagues also performed a high throughput screen of 200,000 compounds on a lymphoid cell line model of HIV latency and found that AV6, a 4-3’, 4’-dichloroanilino-6-methoxyquinoline compound, was able to induce transcription of HIV (72). While the mechanisms of viral activation
are not known for these newly identified compounds, they did not significantly activate T cells. Thus, these new compounds represent additional classes of drugs that show potential as a treatment for latent HIV infection (72).

**Histone deacetylases**

As discussed in a previous section, small molecules that are able to inhibit HDACs are potent activators of transcription from quiescent proviruses in almost every model they have been tested in. Therefore, the role of histone deacetylases in establishment and maintenance of HIV-1 latency has been extensively characterized. The HDAC family of enzymes contains 11 different members that are divided into four classes (9). Class I is composed of HDAC1, HDAC2, HDAC3 and HDAC8. HDACs are lysine deacetylases that were originally identified as proteins that deacetylate the tails of histone proteins; however, several non-histone targets of HDACs have also been identified including the p65 subunit of NF-κb and CyclinT1 (9), which is a component of p-TEFb (9, 53). Deacetylation of histone tails removes signals that recruit factors including bromodomain proteins and transcription factors that initiate transcription and elongation from the HIV-1 promoter. HDAC1, HDAC2, HDAC3 and HDAC8 make up the class I HDAC family. HDAC1 and HDAC2 are 85% homologous at the DNA sequence level, while HDAC3 only shares 52% and 53% similarity with HDAC1 and HDAC2, respectively (108). HDAC1 and HDAC2 are components of the Sin3, CoREST/REST, XFIM and the NuRD complexes (97), while HDAC3 is found in a separate complex, the NCoR/SMRT complex (97). HDAC3 has specifically been demonstrated to deacetylate two factors that are present at the
HIV-1 promoter in addition to histone tails, namely p65 and CyclinT1 (36, 53). Keedy et al. demonstrated that the HIV-1 long-terminal repeat is specifically occupied by HDAC1, HDAC2 and HDAC3 (51). Furthermore, Archin et al. demonstrated that small molecule inhibitors of HDAC1, HDAC2 and HDAC3 are potent inducers of transcription from the HIV-1 promoter, while inhibitors of HDAC1 and HDAC2 do not significantly activate transcription from the HIV-1 promoter (6), indicating that HDAC3 is required for HDAC inhibition mediated expression from the HIV-1 promoter. However, these studies did not address whether HDAC3 was sufficient to induce transcription from the HIV-1 LTR. HDAC1 and HDAC3 transcripts are expressed at a four and eight log fold increase relative to an internal control gene in CD4+ T cells from patients, respectively, while HDAC2 is expressed to a lesser extent of approximately a one log fold increase over the internal control gene (51). Additionally, HDAC1, HDAC2 and HDAC3 proteins are detectable by Western blot in nuclear extracts of aviremic CD4+ patient cells (51).

c-Myc

HDACs do not contain DNA binding domains and must be recruited to promoters through association with local transcription factors. The lab has extensively characterized the recruitment and binding of two repressive transcription factors that associate with the HIV-1 LTR and HDACs, c-Myc and YY1. The lab initially became interested in c-Myc when it was detected in a screen to determine which mRNA transcript levels were affected by treatment with the HDAC inhibitor valproic acid (VPA) (49). The expression of c-Myc is significantly downregulated by
treatment with VPA in CD4+ T cells from aviremic patients (49). Furthermore, overexpression of c-Myc in HeLa cells inhibited the ability of Tat to induce transcription from the HIV-1 promoter (49). Additional investigation of cMyc revealed that it associates with the HIV-1 LTR through an interaction with Sp1 and that it interacts with HDAC1, suggesting a mechanism for its role in repression of HIV-1 transcription (49).

**YY1**

Several studies have been performed demonstrating that YY1 binds to the HIV-1 LTR and represses HIV-1 transcription. YY1 is recruited to the HIV-1 LTR through an interaction with LSF (81). Overexpression of YY1 inhibited the basal level of expression from the HIV-1 promoter in a HeLa cell line containing an HIV-1 driven reporter (68); however, overexpression of LSF does not have an effect on HIV-1 expression. Co-overexpression of LSF and YY1 significantly decreases basal transcription from the HIV-1 LTR (81). Later studies revealed that YY1 associates with and recruits HDAC1 to the HIV-1 promoter (21). These findings highlight the role of YY1 at the HIV-1 LTR.

The following work was performed to further extend the previous findings regarding c-Myc and YY1 and recruitment of HDAC1 to the HIV-1 LTR and to determine whether c-Myc and YY1 also recruit HDAC2 and HDAC3 to the HIV-1 promoter.
Selective targeting of HDACs to induce HIV-1 transcription

This dissertation is focused on identifying targets for the induction and clearance method of disrupting latent HIV. When this project was initially started Keedy et al. had recently determined that of the 11 HDACs, HDAC1, HDAC2, and HDAC3 were specifically bound to the HIV-1 promoter. Furthermore, Archin et al. had found that HDAC inhibitors that target HDAC1, HDAC2, and HDAC3 were able to effectively induce transcription from quiescent viral promoters in cell line models of latent HIV-1 and in cells from infected, suppressed patients. These two important studies indicated that HDAC1, HDAC2 and HDAC3 were important for reactivation of quiescent HIV-1 proviruses. However, HDACs affect the expression of many cellular genes. Therefore, the goal of this project was to identify methods to specifically target transcription from the HIV-1 promoter. Previous work had determined that two transcription factors, cMyc and YY1, both repressed expression from the HIV-1 promoter and that they were both important for recruitment of HDAC1 to the HIV-1 promoter. Because of the high similarity between HDAC1, HDAC2, and HDAC3 and because HDAC1 and HDAC2 are found in several complexes together (97), the hypothesis for the first project in this dissertation was that cMyc and YY1 may recruit HDAC2 and HDAC3 to the HIV-1 promoter. Furthermore, targeting recruitment of cMyc or YY1 to the HIV-1 promoter may relieve HDAC mediated repression of viral transcription. While depletion of YY1 was sufficient to induce transcription from the HIV-1 promoter, it did not affect the occupancy of HDAC2 or HDAC3 at the HIV-1 promoter. Furthermore, depletion of cMyc did not affect transcription from the HIV-1 promoter in the Jurkat cell line models that were studied nor did it significantly affect
occupancy of HDAC2 or HDAC3. Therefore, redundant mechanisms at the HIV-1 promoter may compensate for the loss of a single or subset of transcription factors.

To follow up on the initial study, the goal of the second project was to determine whether targeting a single or subset of HDACs would be sufficient to induce transcription from the HIV-1 promoter. However, each of the HDACs control transcription from many cellular promoters. Therefore, it was reasonable to hypothesize that targeting only one or two of the HDACs may affect the transcription of fewer cellular genes. To determine whether targeting a subset of the HDACs was sufficient to relieve repression of the HIV-1 promoter shRNAs were used to deplete HDAC1, HDAC2, and HDAC3 individually or in combination. Depletion of HDAC1 or HDAC2 did not affect transcription from the HIV-1 promoter. Importantly, depletion of HDAC3 increased both protein and mRNA expression from the HIV-1 promoter, indicating that HDAC3 may be a potential therapeutic target for the treatment of latent HIV.
<table>
<thead>
<tr>
<th>Strategy</th>
<th>Clinical trial</th>
<th>Approach</th>
<th>Endpoint</th>
<th>Summary of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral induction</td>
<td>NCT01319383</td>
<td>Vorinostat; one 400 mg dose; additional doses are planned.</td>
<td>Cell associated HIV gag RNA in resting CD4+ T cells.</td>
<td>A mean increase of 4.5 fold unspliced gag mRNA was detectable in all eight patients 4-7 h after administration of Vorinostat.</td>
</tr>
<tr>
<td>Viral induction</td>
<td>NCT01365065</td>
<td>Vorinostat; 400 mg per day for 14 days.</td>
<td>Cell associated HIV RNA and DNA in total CD4+ T cells using the single copy assay.</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Viral induction</td>
<td>NCT01286259</td>
<td>Disulfiram, 500 mg per day for 14 days.</td>
<td>Cell associated HIV RNA using the single copy assay and reservoir size (IUPM) using the outgrowth assay.</td>
<td>A mean increase of 4.5 fold of RNA and a modest but not significant decrease in viral reservoir size.</td>
</tr>
<tr>
<td>Viral clearance</td>
<td>NCT0017549</td>
<td>Therapeutic vaccination with HIV-pox-based vaccine; 3-4 vaccinations; weeks 0, 4, 8 and 24.</td>
<td>Reservoir size (IUPM) by outgrowth assay at weeks 2, 4, 6, 24, 26, 40, and 72.</td>
<td>Decrease in the number of latently infected resting CD4+ T cells at 40 weeks after vaccination that was no longer detectable at 70 weeks.</td>
</tr>
<tr>
<td>Viral induction and clearance</td>
<td>ACTG5301</td>
<td>anti-PD-1 antibodies</td>
<td>In development</td>
<td>N/A</td>
</tr>
<tr>
<td>Creation of HIV resistant cells</td>
<td>NCT0042634</td>
<td>SB-728-T; infusion with 10^10 zinc-finger nuclease modified CD4+ T cells followed by a 12 week treatment interruption at week 4.</td>
<td>CD4+ T cell counts, HIV DNA, persistence and trafficking of SB-728-T, and HIV RNA during treatment interruption in immune responders.</td>
<td>Increase in CD4+ T cell counts, modified T cells traffic to the GALT, and modified T cells persist for longer than one year.</td>
</tr>
<tr>
<td>Creation of HIV resistant cells</td>
<td>NCT01045454, NCT01252641</td>
<td>SB-728-T; Infusion with 1x10^9, 2x10^9 or 3x10^10 zinc finger nuclease modified CD4+ T cells.</td>
<td>CD4+ T cell counts and viral load.</td>
<td>Ongoing</td>
</tr>
<tr>
<td>RNA-based therapeutics</td>
<td>NCT01153646</td>
<td>Transplantation of HSCs that were transduced with a shRNA targeting tat/rev, a CCR5 ribozyme, and a TAR decay into patients with lymphoma.</td>
<td>Monitoring of vector expressed RNAs.</td>
<td>Vector expressed RNA was detected for up to 24 months after transplantation.</td>
</tr>
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</table>

IUPM, Infectious units per million cells; GALT, Gut associated lymphoid tissue; HSC, Hematopoietic stem cells.
Figure 1.1. Induction and clearance strategies. (I) Induction. Several small molecules have been identified that are able to induce transcription of quiescent HIV-1 proviruses. Prostratin, TNF-α, and bryostatin have been demonstrated to act on the PKC/NF-κB pathway. Activated protein kinase C (PKC) phosphorylates IκBα causing release of NF-κB. NF-κB then translocates to the nucleus where it binds to the promoter of HIV-1 and promotes transcription. HDAC inhibitors directly inhibit deacylation of histones at the HIV-1 LTR, which is associated with induction of transcription. Similarly, histone methyl transferase inhibitors (HMTi) inhibit the methylation of histone tails, which is associated with transcription from the HIV-1 promoter in some model systems. HMBA causes activation of the Akt pathway, which may result in phosphorylation of HEXIM. This may result in the release of pTEF-b from the inhibitory HEXIM complex, allowing pTEF-b access to the HIV-1 LTR where it phosphorylates RNA pol II and primes the LTR for transcription. Disulfiram has been shown to act upstream of HMBA, perhaps by causing degradation of PTEN, an inhibitor of AKT. Activated AKT then phosphorylates HEXIM and LTR induction ensues. Vaccination may activate some T cells, inducing transcription from quiescent HIV-1 LTRs. (II) Clearance. Once viral transcription has been induced, cells containing HIV-1 must be cleared. There are several mechanisms that may contribute to clearance. If HIV-1 transcription is sufficient to produce HIV-1 protein, HIV-1 antigens may be displayed on the cell surface, which may allow the immune system to target and destroy the infected cells. Alternatively, the cell may undergo apoptosis in response to HIV-1 production. Anti-retroviral therapy (ART) will be used to prevent de novo infection. Figure reproduced with permission from Barton, K. M., B. D. Burch, N. Soriano-Sarabia, and D. M. Margolis. 2013. Prospects for Treatment of Latent HIV. Clinical Pharmacology and Therapeutics 93:46-56.
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CHAPTER 2

SELECTIVE TARGETING OF THE REPRESSIVE TRANSCRIPTION FACTORS YY1 AND cMYC TO DISRUPT QUIESCENT HUMAN IMMUNODEFICIENCY VIRUSES

Overview

Quiescent HIV-1 infection of resting CD4+ T cells is an obstacle to eradication of HIV-1 infection. These reservoirs are maintained, in part, by repressive complexes that bind to the HIV-1 long terminal repeat (LTR) and recruit histone deacetylases (HDACs). cMyc and YY1 are two transcription factors that are recruited as part of well-described, distinct complexes to the HIV-1 LTR and in turn recruit HDACs. In prior studies, depletion of single factors that recruit HDAC1 in various cell lines was sufficient to upregulate LTR activity. Short hairpin RNAs (shRNAs) were used to test the effect of targeted disruption of a single transcription factor on quiescent proviruses in T cell lines. In this study, it was found that depletion of YY1 significantly increases transcription of mRNA and protein expression from the HIV-1 promoter in some contexts, but does not affect HDAC1, HDAC2, HDAC3 or acetylated histone 3 occupancy of the HIV-1 LTR. Conversely, depletion of cMyc or cMyc and YY1 does not significantly alter the level of transcription from the LTR or affect recruitment of HDACs to the HIV-1 LTR. Further, global inhibition of HDACs with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) enhanced the increase in LTR transcription in cells that were depleted of YY1. These findings show that, despite

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prior isolated findings, redundancy in repressors of HIV-1 LTR expression will require selective targeting of multiple restrictive mechanisms to comprehensively induce the escape of quiescent proviruses from latency.

**Introduction**

The transcription of quiescent proviral genomes is repressed by the presence of facultative heterochromatic structures, which contain high levels HDAC1, trimethylated lysine 9 of histone 3, trimethylated lysine 27 of histone 3, HP1-α, and low levels of acetylated histone 3 (26). Additionally, the quiescent HIV-1 long terminal repeat (LTR) is occupied by the repressive NURD and the NCoR histone deacetylase (HDAC) complexes in Jurkat and monocyte-derived macrophage cell lines, respectively (9, 14). These HDAC complexes repress transcription by removing acetyl groups from histones, which results in a repressive chromatin structure at the HIV-1 LTR that is not permissive for transcription. HDAC inhibitors are consistently able to reactivate quiescent HIV-1 in cell line models and in patients’ cell (1-3, 15). However, HDACs regulate the expression of a broad range of cellular factors, and HDAC inhibitors affect the acetylation of lysines on many non-histone proteins. Therefore, the aim of this study was to determine whether factors that specifically recruit HDACs to the HIV-1 promoter could be directly targeted to reverse HIV-1 quiescence. To date, several transcription factors have been described that recruit HDAC1 to the HIV-1 LTR, including cMyc, YY1, CBF1, NF-κb and Sp1 (15, 16, 25, 28). Surprisingly, although each of these factors binds to the LTR at a distinct location and individually recruits HDACs, previous studies have
found that single depletion of any one of these factors is sufficient to disrupt quiescent HIV-1 proviruses and occupancy of HDAC1 at the HIV-1 promoter. Furthermore, these findings indicate that in the absence of one HDAC recruiting complex, HDAC occupancy cannot be maintained at a level sufficient to prevent reactivation of HIV-1 transcription.

YY1 and cMyc are two transcription factors that are recruited to the HIV-1 promoter and are involved in maintenance of transcriptional repression. YY1 can both activate and repress transcription depending on the promoter context (8). YY1 is recruited to the HIV-1 LTR by the transcription factor LSF and represses transcription by recruiting HDAC1 in HeLa cells (11, 15). cMyc is a transcription factor that is most commonly known for its roles in regulating cellular proliferation and cell growth. Like YY1, it is able to act as both a transcriptional activator and repressor depending on the context of the promoter (12). The mbIII domain of cMyc is important for its ability to repress transcription and mediates the interaction between cMyc and HDAC3 (19). cMyc is repressive when recruited to the HIV-1 LTR by the transcription factor Sp1 (16). However, cMyc is required for Tat activated HIV-1 transcription (7). Because of their well-characterized role in the maintenance of quiescent HIV, cMyc and YY1 are promising targets for therapies to disrupt the silencing of integrated HIV-1 proviruses.

Many of the initial studies that were instrumental in elucidating the mechanisms of HIV-1 transcriptional repression have been performed in HeLa cell lines. HeLa cells are an epithelial cell line that were originally isolated from cervical
cancer tissue (21). In vivo, HIV-1 infects CD4+ T cells of the immune system. The cell environment and transcriptional profiles of epithelial and T cells are very different. Therefore, mechanisms that maintain transcriptionally quiescent proviral genomes in T cells are of paramount relevance. Jurkat cells are a cell line derived from T cells with biochemical features similar to resting CD4+ T cells. Several cell lines that are derived from Jurkat cells and contain quiescent, but inducible HIV-1 proviruses have been created as models of HIV-1 latency, including 2D10 and J89 cells (18, 20). Studies were performed in 2D10 and J89 cells because unique proviral insertion sites, and perhaps subtle differences in cellular gene programming, result in widely distinct sensitivity to viral induction strategies. These studies more accurately reflect the diversity of HIV-1 latency in vivo than those performed in a single clonal model.

The goal of this study was to determine whether the selective targeting of transcription factors, such as cMyc and YY1, that recruit HDACs to the LTR and maintain transcriptional repression of HIV-1, could disrupt latency in T cell lines. Further, to determine whether these factors play a unique or general role in the individual recruitment of HDAC1, HDAC2 and HDAC3 to the HIV-1 LTR. The mechanisms that maintain proviral quiescence are high-value therapeutic targets for the development of anti-latency therapies.
Materials and Methods

Cell culture

J89 (a kind gift from Dr. David Levy at the University of Alabama at Birmingham) and 2D10 (a kind gift from Dr. Jonathan Karn at Case Western Reserve University) cells were maintained in RPMI supplemented with 10% FBS and 1% Pen/strep in a 37˚C incubator containing CO₂. Cells were passaged every three to four days, and all experiments were performed on cells that had been passaged fewer than twelve times. For the experiments in which suberoylanilide hydroxamic acid (SAHA, Merck Research Laboratories, West Point Pennsylvania) or phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St Louis, MO), was used, 500 nM of SAHA or 10 nM of PMA was added to the cell media 54 hours after transduction with short-hairpin RNAs (shRNAs) and 18 hours prior to collection for flow cytometry.

Chromatin Immunoprecipitation (ChIP) assays

Ten million cells that had been transduced with shRNAs were collected, washed once with PBS, and then fixed with 1% formaldehyde for 10 min. The cell nuclei were isolated according to the manufacture’s instructions and sonicated for 24 minutes in a bioruptor (Diagenode, Denville, NJ) with intervals that consisted of 30 seconds of sonication and 15 seconds of rest. To bind the antibodies to the beads, protein A and protein G Dynal beads (Invitrogen, Grand Island, NY) were incubated
with 5-10 μg of antibody. The following antibodies were used for the ChIP experiments: HDAC1 ChIP grade (Abcam, Cambridge, MA), HDAC2 ChIP grade (Abcam, Cambridge, MA), HDAC3 ChIP grade (Abcam, Cambridge, MA), histone 3 ChIP grade (Abcam, Cambridge, MA) and acetylated histone 3 (Millipore, Billerica, MA). Then, the sonicated product was added and incubated at 4°C overnight. The next day, the beads were washed once for five minutes with each of the following buffers: ChIP dilution buffer, low salt ChIP buffer, high salt ChIP buffer, LiCl ChIP buffer, and TE buffer. The samples were then eluted from the beads and incubated at 68°C for two hours to decrosslink the proteins from the DNA and to degrade the protein. The DNA was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The enrichment of the HIV-1 LTR in the ChIP samples was assessed using qPCR (quantitative PCR). Independent transductions were performed for each ChIP replicate.

**Quantitative PCR**

One microliter of the DNA from the ChIP assay was used in a final volume of 20 μl containing 10 μl of SYBR green master mix (Applied Biosystems, Foster City, CA) and 0.5 μM each of the primers LTRrt8 (5’-TAGCCAGAGAGCTCCCAGGCTCAGA-3’) and LTRrt9 (5’-AGCCCTCAGATGCTACATATAAGCA-3’). The reaction was run on a 7900 quantitative PCR machine (Applied Biosystems, Foster City, CA) with the following parameters: 50°C for 2 min; 95.0°C for 10 min; and 40 replicates of 95.0°C for 15 sec and 60°C for 1 min. A standard curve ranging from 50% to .0008% of the total ChIP
input was run for each condition, and the data is displayed as the percent input with the background from the IgG condition subtracted. Experiments with less DNA than the IgG control sample were assigned zero quantity. Data is shown as the mean ± the standard error of the mean (SEM) from at least three independent ChIP experiments. The student’s t-test was used to assess significance, and a p-value of less than 0.05 was considered significant.

**Quantification of gene expression**

2D10 or J89 cells that had been transduced with shRNAs were collected 72 hours after transduction, and RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). The RNA was treated with DNase, and then 380 ng of RNA was reverse transcribed using the SuperScript III First-Strand synthesis kit (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. The reverse transcription quantitative PCR assay (RT-qPCR) was used to determine gene expression. The following primers and probes were used to amplify the cDNA: Gag-F: 5’ ACATCAAGCAGCCATGCAAAT, GAG-R: 5’ TCTGGCCTGGTGCAATAGG, GAG FAM PROBE: 5’ CTATCCCATTCTGCAGCTTCATTGATG; EGFP-F: 5’ GGAGCGCACCATCTTTTCTCA, EGFP-R: 5’ AGGGTGTCGCCCTCGCAA, EGFP FAM Probe: CTACAAGACCCGCACGGGAGTG; HDAC1-F: 5’ TGAGGACGAAGACGACCCT, HDAC1-R: 5’ CTCACAGGCAATTCGTTTGTC, and HDAC1 FAM probe: 5’ CAAGCGCATCTCGATCTGCTCCTC (23); HDAC2-F: 5’ CTTTCCTGGCACAGCGCTCTC, and HDAC2 FAM probe: 5’ CTCATTGGAAAAATGTGACAGCATAGT, and HDAC2 FAM probe: 5’
AGGGATATTGGTGCTGGAAAAGGCAA; and HDAC3-F: 5’
GGTGGTTATACTGTCCGAAATGTT, HDAC3-R: 5’
GCTCCTCACTAATGGCCTCTTC, and HDAC3 FAM probe: 5’
AGCAGCGATGTCTCATATGTCCAGCA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified for each sample and used for normalization. The primers and probes used were as follows: GAPDH-F: 5’
GCACCACCAACTGCTTAGCACC, GAPDH-R: 5’ TCTTCTGGGTGGCAGTGATG,
and GAPDH HEX probe: 5’ TCGTGGGAAGGACTCATGACCACAGTCC (27).
Results are displayed as the fold increase over the control condition, which was calculated using the $\Delta\Delta^{\text{ct}}$ method. The values shown represent the mean of three independent experiments ± the SEM.

**Western Blots**

J89 or 2D10 cells were collected 72 hours after being transduced with lentiviruses carrying shRNAs. Protein was extracted with radioimmunoprecipitation assay buffer containing 10 µl Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO) and 10 mM NaF (Sigma-Aldrich, St Louis, MO). The quantity of protein was determined using a Bradford protein assay according to the manufacture’s instructions (Bio-Rad, Hercules, CA). Five to ten micrograms of protein was loaded onto a 4%-12% bis-tris gel (Invitrogen, Grand Island, NY). The western blot was then run as previously described (17). The following primary antibodies were used: HDAC1, HDAC2, HDAC3, cMyc, and YY1 (all from Santa Cruz Biotechnology, Santa Cruz, CA) and alpha tubulin (Abcam, Cambridge, MA) was used as a loading
control. The band intensity of the Western blots was analyzed using Image J software (NIH, Bethesda, MD). Each Western blot was performed using cells from independent transductions at least three times. The values shown represent the mean percent protein knockdown ± the SEM from three independent experiments.

**Transduction of shRNAs**

Three million cells were split into 12 mL of RPMI media containing 10% FBS and 1% pen/strep and incubated overnight. At 24 hours the cells were transduced with lentiviral vectors carrying cMyc or YY1 shRNAs, and the cells were incubated at 37°C overnight. shRNA plasmids and lentiviruses were obtained from the UNC Lent-shRNA core facility, which has the Open Biosystems shRNA library (Open Biosystems, Lafayette, CO). The plasmid TRCN0000039642 was used to deplete cMyc, and plasmid TRCN0000019898 was used to deplete YY1. A plasmid containing a shRNA to eGFP was used as the non-specific (NS) control in the ChIP experiments (catalog number RHS4459, Open Biosystems Lafayette, CO). The pLKO.1 empty vector control (catalog number RHS4080, Open Biosystems, Lafayette, CO) was used as the negative control for the RT-qPCR and flow cytometry experiments. Twenty-four hours after the addition of the shRNA, new media and 2 μg/mL of puromycin was added to select for cells that had been transduced. The cells were then incubated at 37°C for 48 hours before collection for downstream applications. New transductions were performed for each experiment, and cells were never frozen down and thawed for subsequent experiments. Knockdown was assessed by qPCR and Western blot as described above.
Flow cytometry

Cells were collected 72 hours after transduction with lentiviruses, washed once with 1X PBS, and then fixed in 3.2% paraformaldehyde. The GFP expression of the cells in the fixed samples was measured using an Attune flow cytometer (Applied Biosystems, Foster City, CA) or a CyAn ADP analyzer (Beckman Coulter, Inc., Brea, CA, UNC flow cytometry core facility). At least 10,000 cells were collected for each condition. The analysis was performed using FlowJo flow cytometry analysis software (FlowJo, Ashland, OR). The values shown are the mean of three independent experiments ± the SEM from at least three independent experiments.

Results

Depletion of cMyc and YY1 in Jurkat cells does not affect cell viability

To determine whether cMyc and YY1 are required to maintain transcriptional repression of the HIV-1 provirus in Jurkat cells, the RNA interference pathway was used to deplete the mRNA transcripts of cMyc and YY1, and the downstream effects were monitored.

In this study, shRNAs were used instead of siRNAs for three important reasons. First, Jurkat cells are very difficult to transfect, which is the primary method that is used to introduce siRNAs into cells and the transfection procedure can cause sufficient cell stress to activate expression from the HIV promoter, which makes it difficult to interpret the results from these types of experiments. Jurkat cells can be transduced with lentiviruses carrying shRNA expressing vectors relatively efficiently.
Secondly, siRNAs are rapidly used up, which results in a short duration of mRNA depletion that may not correspond to a significant protein depletion depending on the half-life of the protein. shRNAs are continually expressed from a lentiviral vector resulting in a sustained depletion. Thirdly, the shRNA vector contains a puromycin resistance gene that allows for selection of cells carrying the shRNA containing vector.

Specifically, transduction was used to deplete the transcription factors cMyc and YY1. Transduction of cMyc specific shRNAs reduced the amount of cMyc in 2D10 cells by 85% and in J89 cells by 72% (Fig. 2.1 and data not shown). Single depletion of YY1 with shRNAs reduced the amount of YY1 protein by 87% in 2D10 cells and 56% in J89 cells (Fig. 2.1 and data not shown). Furthermore, the transduction of both cMyc and YY1 shRNAs reduced protein levels by 57% and 83% in 2D10 cells and by 86% and 62% in J89 cells, respectively (Fig. 2.1 and data not shown). The transduced cells were then selected with puromycin to ensure that the study population of cells homogenously carried the shRNA-expressing vector. The CellTiter-Blue cell viability assay (Promega, Madison, WI) was used to determine the effect of YY1, cMyc or YY1 and cMyc depletion on the viability of Jurkat cells. Transducing Jurkat cells with cMyc, YY1 or cMyc and YY1 shRNAs does not significantly affect the viability of 2D10 or J89 cells as compared to the cells that were transduced with the vector control plasmid (Fig. 2.1d and data not shown). Therefore, lentivirus transduction of shRNAs is an effective technique for the depletion of cMyc and YY1 in Jurkat cells and does not significantly affect cell viability.
Knockdown of the transcription factors cMyc, YY1 or both does not affect protein or mRNA expression of HDAC1, HDAC2 or HDAC3

CMyC and YY1 both affect the expression and post-transcriptional regulation of a significant number of cellular genes (8, 12). Because cMyc and YY1 are both broadly acting transcription factors, it is possible that they could affect HIV-1 transcription by regulating the expression of HDACs. Furthermore, cMyc has recently been shown to regulate the transcription of HDAC2 in mesenchymal stem cells (6). Therefore, to ensure that the effects of depleting cMyc and YY1 were not related to an effect on HDAC expression, the levels of the HDAC proteins were measured using Western blot and mRNA using RT-qPCR after depletion of cMyc, YY1 or cMyc and YY1 (Fig. 2.2). No significant changes in the protein levels of HDAC1, HDAC2 or HDAC3 were observed by Western blot after knockdown of cMyc, YY1, or cMyc and YY1 in 2D10 or in J89 cells (Fig. 2.2a and data not shown). Furthermore, depletion of cMyc, YY1 or cMyc and YY1 did not significantly affect HDAC1, HDAC2 or HDAC3 mRNA expression when compared to the cells transduced with the vector control (Fig. 2.2). Therefore, the effects of depleting cMyc, YY1 or cMyc and YY1 with shRNAs is not mediated by a secondary depletion of HDAC1, HDAC2 or HDAC3 in 2D10 or J89 cells (Fig. 2.2 and data not shown).

Individual depletion of the transcription factor YY1 significantly increases expression from the HIV-1 LTR.

Targeting cMyc or YY1 containing complexes upregulates expression of HIV-1 mRNA in HeLa cell line models of HIV-1 latency (11, 16, 24). To determine
whether these transcription factors are also important for maintaining HIV-1 transcriptional repression in a T cell line, cMyc, YY1 or cMyc and YY1 were depleted from J89 or 2D10 Jurkat T cells, and expression from the HIV-1 LTR was monitored using RT-qPCR and flow cytometry. The proviral genomes in both J89 and 2D10 cells contain GFP, which can be used to monitor expression from the HIV-1 LTR. In J89 cells, the GFP gene is inserted between the env and nef genes of the HIV-1 provirus (20). In 2D10 cells, the GFP gene is inserted between env and the 3'LTR and nef is deleted (18). Additionally, 2D10 cells contain an attenuated tat and a truncated gag (18). Because the provirus in the 2D10 cells does not contain the complete gag sequence, RT-qPCR was used to monitor GFP mRNA expression in the 2D10 cells as a measure of HIV-1 LTR activity. Following depletion of YY1 from the 2D10 cells there was a significant 4.2 fold increase in GFP mRNA expression from the HIV-1 LTR (Fig. 2.3a, p-value < 0.05). However, GFP mRNA expression did not increase significantly following depletion of cMyc in the 2D10 cells (Fig. 2.3a). Furthermore, depletion of cMyc or YY1 did not result in a significant increase in expression of gag mRNA in the J89 cells (Fig. 2.3c).

To further determine whether the effects seen at the mRNA level were reflected at the protein level, the percentage of cells expressing GFP was measured using flow cytometry. The percentage of 2D10 cells expressing GFP that were depleted of YY1 significantly increased over six fold when compared to the cells transduced with the vector control, which correlates with the observed increase in GFP mRNA that was observed (Fig. 2.3b, p-value < 0.05). Following knockdown of cMyc there was not a significant increase in the percentage of 2D10 or J89 cells
expressing GFP (Fig. 2.3b and 3d). Additionally, no increase in the percentage of J89 cells expressing GFP was observed following YY1 depletion (Fig. 2.3d).

Because cMyc and YY1 can both act as transcriptional activators as well as repressors, next it was important to determined whether cMyc or YY1 were required for HIV-1 transcription by adding PMA to 2D10 or J89 cells that were depleted of YY1, cMyc or both for 24 hours and measuring the percentage of GFP positive cells by flow cytometry. PMA induced a significant percentage of cells to express HIV-1 driven GFP over the cells that were treated with DMSO in the cells depleted of cMyc, YY1 or cMyc and YY1. Furthermore, there was no significant difference in the percentage of GFP positive cells after exposure to PMA in the cells depleted of cMyc, YY1 or cMyc and YY1 as compared to the cells transduced with the control shRNA, which indicates that cMyc nor YY1 is required to induce transcription from the HIV-1 LTR (Fig 2.8).

Together, these results suggest that YY1 is involved in repression of the HIV-1 LTR, but that its effects predominate in select cellular contexts. The different response of the two cell lines to YY1 depletion may be due to differences between the locations of the proviral genomes in these two cell lines or the degree of repressive chromatin structures that have developed at the LTR. These results also indicate that depletion of cMyc or cMyc and YY1 is not sufficient to induce expression from the HIV-1 LTR in these Jurkat cell lines. Furthermore, neither cMyc nor YY1 is required for expression from the HIV-1 LTR. Of relevance to the development of translational strategies to depleted persistent HIV-1 infection, these
findings demonstrate some redundancy or overlap in epigenetic mechanisms that maintain proviral latency.

**Depletion of cMyc or YY1 does not significantly affect HDAC occupancy of the HIV-1 LTR.**

Of the 11 human HDACs, only HDAC1, HDAC2, and HDAC3 have been shown to play a critical, direct role in the regulation of HIV-1 LTR expression (17). In HeLa cell lines containing quiescent proviral genomes, depletion of cMyc or YY1 leads to transcription from the HIV-1 LTR through depletion of HDAC1 occupancy (11, 16). cMyc and YY1 are both associated with recruitment of HDACs and HDAC complexes to LTR of HIV-1 and to the promoters of some cellular genes (19). Furthermore, HDAC inhibitors are consistently able to activate HIV-1 transcription in both cell line models and in patients’ cells (2, 3, 10). Therefore, the next step of this study was to determine whether depleting factors that recruit HDACs to the HIV-1 promoter, cMyc and YY1, had an effect on the recruitment of HDACs to the HIV-1 LTR. Following depletion of cMyc or YY1, chromatin immunoprecipitation (ChIP) was used to measure the occupancy of HDAC1, HDAC2, and HDAC3 in 2D10 and J89 Jurkat cells (Fig. 2.4). Depletion of cMyc or YY1 from 2D10 and J89 cells did not significantly alter HDAC1, HDAC2 or HDAC3 occupancy of the HIV-1 LTR (Fig. 2.4). Although cMyc and YY1 are known to interact with HDACs and HDAC complexes, this result indicates that depletion of these factors is not sufficient to measurably block HDAC recruitment to the HIV-1 LTR in these Jurkat cell lines. This finding is consistent with earlier results that found that depletion of cMyc or cMyc and YY1 did
not significantly affect HIV-1 expression from these cell lines. However, the lack of change in HDAC occupancy of the HIV-1 LTR following depletion of YY1 suggests that the induction of HIV-1 transcription after YY1 knockdown is induced by an effect that does not affect HDAC occupancy at the HIV-1 promoter.

Acetylation of histone 3 increases at the promoters of activated genes, and given sufficient gene expression and chromatin remodeling, H3 histone occupancy may decrease (13, 22). Therefore, to further examine the landscape of the HIV-1 LTR following depletion of cMyc, YY1 or cMyc and YY1 the occupancy of histone 3 and acetylated histone 3 was assessed (22). A significant increase in histone acetylation was observed following depletion of cMyc in 2D10 cells (Fig. 2.5b, p-value < 0.05). This upregulation in histone acetylation was not accompanied by a significant increase in HIV-1 expression or a significant change in HDAC occupancy of the HIV-1 LTR. No significant changes in histone acetylation were observed in J89 cells following cMyc depletion or in either cell line following YY1 depletion (Fig. 2.5). The lack of change in histone 3 acetylation at the HIV-1 LTR following YY1 depletion is surprising given the increase in mRNA expression that was observed and indicates that depletion of YY1 alone is sufficient to disrupt repression of the HIV-1 LTR even in the presence of HDACs. The lack of change in HDAC occupancy following selective disruption of the YY1/LSF complex or the cMyc/Sp1 complex indicates that other factors are present at the HIV-1 LTR that are able to recruit HDACs in the absence of these complexes (11, 16).
Depletion of cMyc and YY1 does not affect quiescent HIV-1 expression in Jurkat cells

Given the possibility that LTR quiescence is maintained by redundant systems capable of recruiting HDACs to the HIV-1 LTR, both cMyc and YY1 were depleted from J89 and 2D10 cells. However, after knockdown of both cMyc and YY1, there was not a significant increase in HIV-1 mRNA expression in J89 or 2D10 cells (Fig. 2.3a and 2.3b). Correspondingly, there was not a significant change in the percentage of cells expressing GFP following depletion of both factors from either cell line (Fig. 2.3c and 2.3d). Therefore, depletion of these two HDAC recruiting factors in not sufficient to disrupt the maintenance of quiescent HIV.

Furthermore, after depletion of both cMyc and YY1 there was not a significant change in the occupancy of HDAC1, HDAC2 or HDAC3 at the HIV-1 LTR as measured by ChIP assay (Fig. 2.6). This suggests that derepression of the HIV-1 LTR in T cell lines, unlike in HeLa cells, may require targeting of additional or different transcription factors. Furthermore, there were no significant changes in histone 3 occupancy or acetylation at the HIV-1 LTR in either 2D10 or J89 cells following depletion of cMyc and YY1 (Fig. 2.6). Altogether, this data indicates that a complex network of factors are involved in the maintenance of HIV-1 quiescence in Jurkat cells and that in the absence of cMyc and YY1, other mechanisms maintain recruitment of HDACs to the HIV-1 LTR.
The effect of YY1 depletion is enhanced by HDAC inhibition.

The modest effects of cMyc and YY1 depletion on HDAC recruitment to the HIV-1 LTR were surprising because both have previously been shown to repress transcription of the HIV-1 LTR through HDAC recruitment. However, as ChIP assays are only semi-quantitative, an alteration of HDAC occupancy that is functionally important might not be detected. Therefore, because RNAi depletion did not achieve complete inhibition of protein expression and to further determine whether cMyc and YY1 were involved in repression of the HIV-1 promoter, 2D10 and J89 cells were treated with the HDAC inhibitor SAHA and the effects of depleting cMyc, YY1 or cMyc and YY1 from 2D10 and J89 cells was assayed. Eighteen hours after the addition of a maximal concentration of SAHA (500 nM) to cells that had been transduced with the vector control, approximately 9.23% of the 2D10 cells were found to be expressing GFP. However, the addition of SAHA in combination with depletion of YY1 significantly increased the effect of YY1 knockdown and induced GFP expression in approximately 32% of the 2D10 cells (Fig. 2.7, p-value < 0.05). Because the concentration of SAHA used is able to completely inhibit HDACs, the significant increase in expression when SAHA is added to YY1 depleted cells indicates that YY1 mediates repression of HIV-1 transcription through a non-HDAC mechanism.

Inhibition of HDACs with 500 nM of SAHA did not augment the effects of depleting cMyc or cMyc and YY1 in 2D10 cells (Fig. 2.7). Furthermore, no significant changes in LTR expression were observed in J89 cells that were treated with SAHA.
in combination with depletion of cMyc, YY1 or cMyc and YY1 (data not shown). This finding indicates that there are indeed multiple mechanisms involved in the repression of HIV-1 transcription and that targeting multiple factors can result in increased HIV-1 expression.

Discussion

cMyc and YY1 bind to the HIV-1 LTR and regulate transcription through recruitment of HDACs (11, 16). To determine whether selective anti-latency therapy could be specifically targeted to transcription factors that recruit HDACs to the HIV-1 LTR. Depletion of cMyc, YY1 or cMyc and YY1 did not significantly affect transcription or protein levels of the HDAC proteins, which indicates that the effects on HIV-1 transcription are not a secondary effect of changes in HDAC expression. Depletion of YY1 resulted in a significant four-fold increase in mRNA expression and a significant increase from approximately .7% of the cells in the control shRNA condition to 4.2% of the cells depleted of YY1 expressing the GFP protein from the HIV-1 LTR in the 2D10 cells but not in J89 cells (Fig. 2.3). However, in contrast to studies in HeLa cells, single knockdown of cMyc did not have a significant effect on transcription of mRNA from the HIV-1 LTR or on the induction of GFP protein expression from the HIV-1 LTR (Fig. 2.3). Furthermore, depletion of cMyc and YY1 together did not induce HIV-1 transcription in either cell line. As cMyc has been show to be required for Tat mediated elongation of the HIV-1 promoter (7), it is possible that cMyc’s role in elongation may account for the lack of HIV-1 transcription that was observed after depletion of YY1 and cMyc and may explain
why the significant increase in histone 3 acetylation did not correlate with an increase in expression. Depletion of cMyc, YY1 or cMyc and YY1, followed by activation with PMA, induced a significant amount of expression from the HIV-1 LTR in 2D10 and J89 cells (Fig. 2.8). Therefore, it can be concluded that cMyc is not absolutely required for activation of the HIV-1 promoter by PMA. However, because depletion of cMyc and YY1 resulted in less activation of the HIV-1 promoter than depletion of YY1 alone, the mechanism of repression that is mediated by YY1 may act through a pathway that requires cMyc. These results are particularly surprising in light of previous studies that found disruption of cMyc or YY1 was sufficient to disrupt quiescent HIV-1 proviruses. Because YY1 depletion did not activate HIV-1 transcription in both cell lines, targeting YY1 as part of a future anti-latency therapy may not broadly disrupt latency in all proviral integrants.

When markers of repressive and activated promoters were further studied at the HIV promoter using ChIP following knockdown of cMyc, YY1 or cMyc and YY1, no significant change in histone 3 occupancy of the HIV-1 LTR was observed. The lack of change in histone 3 occupancy indicates that nucleosomes at the HIV-1 promoter were not significantly disrupted following depletion of cMyc, YY1 or both factors. Although cMyc and YY1 are known to recruit HDACs to the HIV-1 LTR in HeLa cells, the ChIP results from this study indicate that they are not absolutely required to recruit HDAC1, HDAC2 or HDAC3 to the HIV-1 LTR in Jurkat cells. Contrary to previous findings, these results indicate that in T cells, other transcription factors that bind to the HIV-1 LTR may be able to compensate for the loss of cMyc, YY1 or both and maintain HDAC occupancy. Specifically, NF-κb and CBF1 have
been implicated in recruitment of HDAC1 to the HIV-1 LTR may be able to compensate for the loss cMyc and YY1 (25, 28). Furthermore, these or other proteins may contribute to the repression of HIV-1 transcription in T cells. Additionally, these finding highlight an important difference between HeLa cell line models of HIV-1 latency and T cell line models of HIV-1 latency and indicate that the epigenetic environment surrounding the HIV-1 LTR in HeLa cells may be less stable than in T cells. In light of the results in this study, the benefits and limitations of the HeLa cell model should be carefully considered before future studies are performed using HeLa cells to study HIV. Although Jurkat cells more closely resemble primary CD4+ T cells, it is still important to develop more advanced tools and model system for the future study of the epigenetics of quiescent HIV-1 proviruses.

Interestingly, targeting HDAC recruitment through depletion of YY1 in conjunction with HDAC inhibition resulted in a significant increase in GFP protein expression from the HIV-1 promoter. This finding indicates that targeting multiple restrictive mechanisms at the HIV-1 LTR may be an innovative method for disrupting HIV-1 latency. YY1 is involved in several malignancies and some new classes of chemotherapeutics have been demonstrated to decrease expression of YY1 (5). Such approaches might be used for the treatment of latent HIV-1 infection, and may be able to augment the effects of SAHA (4). However, additional development of drugs that directly target the protein interaction domains of YY1 could be pursued and could possibly be used in conjunction with compounds that are currently being studied to perturb quiescent HIV such as SAHA.
Previous studies have found that the chromatin environment surrounding the HIV-1 insertion site may affect transcription. Furthermore, in vivo infected CD4+ T cells have a range of insertion sites. To account for the effect of insertion site differences, the studies were performed in two Jurkat cells lines with distinct proviral insertion sites. The differences seen between the two cells lines may be attributable to the difference in proviral locations. However, apart from the effects of YY1 depletion, most of the results were seen in both cell lines, indicating that the proviral location may have had only modest effects. The differences in results found in this study as compared to previous studies in HeLa cells highlights the importance of conducting studies in multiple lineages of cell lines. Although Jurkat cells are derived from CD4+ T cells, there are still some important differences between these cells and in vivo HIV-1 infected cells. Therefore, our lab and others are working to develop models of HIV-1 quiescence that more accurately mimic the in vivo environment of HIV-1 quiescence.

In conclusion, the results indicate that depletion of the transcription factor YY1 using transduction of shRNAs in Jurkat cells is sufficient to disrupt the repression of the HIV-1 promoter in select cellular contexts. However, depletion of the transcription factor cMyc does not induce HIV-1 expression in Jurkat cells. Importantly, depletion of cMyc, YY1 or cMyc and YY1 is not sufficient to disrupt the binding of HDAC complexes to the HIV-1 LTR in Jurkat cells. Therefore, it can be concluded that the mechanism of maintenance of HIV-1 transcriptional repression is complex and may require a combination of therapies that target multiple levels or several factors to reverse transcriptional repression of the HIV-1 LTR.
Contributions

KM Barton performed the experiments, analyzed the data, wrote the manuscript and participated in experimental design. Dr. David Margolis was instrumental in experimental design, analysis and development of the manuscript. We thank Dr. Kara Keedy and Dr. Nancie Archin for their assistance with experiments and review of the manuscript, and members of the Karn laboratory for additional review. We also would like to thank Dr. Jonathan Karn for the gift of the 2D10 cells, Dr. David Levy for the gift of the J89 cells and Merck Research Laboratories for providing the SAHA used in the study. D.M.M. is a consultant for and has received grants from Merck Research Laboratories. All remaining authors declare no conflicts of interest. This study was supported by the National Institutes of Health’s grants DA030156 to D.M.M., RR024383 to the UNC TRaCS Institute, AI50410 to the UNC Center for AIDS Research, and an equipment grant from the James B. Pendleton Charitable Trust. The UNC Flow Cytometry Core Facility is supported in part by an NCI Center Core Support Grant (P30CA06086) to the UNC Lineberger Comprehensive Cancer Center.
Figure 2.1. Depletion of cMyc or YY1 does not affect Jurkat cell viability.
Lentiviral shRNAs specific to cMyc, YY1 or cMyc and YY1 significantly deplete the protein levels of cMyc (a), YY1 (b) or cMyc and YY1 (c) in 2D10 cells as determined by Western blot analysis. d) Depletion of cMyc, YY1 or cMyc and YY1 does not affect the viability of 2D10 cells 72 h after transduction of shRNAs.
Figure 2.2. Depletion of cMyc, YY1 or cMyc and YY1 does not significantly affect expression of HDAC1, HDAC2 or HDAC3 in 2D10 cells. a) HDAC1, HDAC2 and HDAC3 protein levels remain stable in 2D10 cells after depletion of cMyc, YY1 or cMyc and YY1. (b) HDAC1, (c) HDAC2, or (d) HDAC3 mRNA expression levels are not significantly affected by the depletion of cMyc, YY1 or cMyc and YY1 in 2D10 cells as measured by RT-qPCR.
Figure 2.3. Depletion of YY1 significantly increases HIV-1 expression in 2D10 cells

a) GFP mRNA expression from the HIV-1 LTR significantly increased after depletion of YY1 from 2D10 cells. No changes were observed in GFP mRNA expression levels following depletion of cMyc or cMyc and YY1 from 2D10 cells. b) The percentage of 2D10 cells expressing GFP protein, as determined by flow cytometry, significantly increased after depletion of YY1, but not after depletion of cMyc or cMyc and YY1. c) Expression of gag mRNA was not significantly affected by knockdown of cMyc, YY1 or both in J89 cells. d) The percentage of J89 cells expressing GFP protein, as determined using flow cytometry, did not significantly increase after knockdown of cMyc, YY1 or cMyc and YY1. * indicates a p-value of less than 0.05.
Figure 2.4. HDAC occupancy of the HIV-1 promoter is not affected by depletion of cMyc or YY1. a) HDAC1, b) HDAC2 and c) HDAC3 occupancy of the HIV-1 promoter was not significantly altered after depletion of cMyc or YY1 from 2D10 cells, as determined using a ChIP assay. d) HDAC1, e) HDAC2 and f) HDAC3 occupancy of HIV-1 LTR did not change significantly after depletion of cMyc or YY1 from J89 cells as measured by ChIP assay.
Figure 2.5. Histone 3 and acetylated histone 3 levels on the HIV-1 promoter did not significantly change following depletion of cMyc or YY1 in Jurkat cells. a and c) Histone 3 occupancy of the HIV-1 promoter in 2D10 (a) and J89 (c) cells did not significantly change following depletion of cMyc or YY1. b) The levels of histone 3 acetylation significantly increased after depletion of cMyc in 2D10 cells. However, the level of acetylated histone 3 did not change following knockdown of cMyc or YY1 in J89 cells (d) or after depletion of YY1 in 2D10 cells (b). * indicates a p of less than 0.05.
Figure 2.6. Concurrent knockdown of cMyc and YY1 did not significantly affect HDAC1, 2, or 3 recruitment to the HIV-1 promoter or the levels of histone 3. Depletion of cMyc and YY1 from 2D10 cells did not affect the levels of HDAC1 (a), HDAC2 (b), HDAC3 (c), histone 3 (d) or acetylated histone 3 (e) at the HIV-1 LTR. Similarly, no changes in HDAC occupancy or histone 3 occupancy or acetylation was observed at the HIV-1 LTR following depletion of cMyc and YY1 in J89 cells (f-j) as measured by ChIP.
Figure 2.7. Targeting YY1 and HDAC activity significantly increases expression from the HIV-1 LTR. Depletion of YY1 in conjunction with the addition of 500 nM of SAHA for 18 h resulted in a significant increase in the percentage of 2D10 cells expressing GFP as compared to cells that were transduced with the vector control and treated with SAHA as measured using flow cytometry. Depletion of cMyc or cMyc and YY1 in combination with SAHA did not significantly increase the percentage of 2D10 cells expressing GFP. * indicates a p-value of less than 0.05.
Figure 2.8. Expression from the HIV-1 LTR does not require cMyc or YY1.
Depletion of cMyc, YY1 or cMyc and YY1 followed by incubation with 10 nM PMA for 18 h resulted in a significant increase in expression from the HIV-1 LTR in 2D10 and J89 cells over treatment with DMSO only. However, no significant changes were observed in the amount of expression from the HIV-1 LTR between 2D10 or J89 cells that were depleted of cMyc, YY1 or cMyc and YY1 as compare to the cells transduced with the vector control following treatment with PMA.
REFERENCES


CHAPTER 3

HDAC3 IS A KEY THERAPEUTIC TARGET FOR THE DISRUPTION OF LATENT HIV-1 INFECTION

Overview

Selective histone deacetylase (HDAC) inhibitors have emerged as a potential anti-latency therapy for persistent human immunodeficiency virus type 1 (HIV-1) infection. A short hairpin (sh)RNA-mediated knockdown strategy was used to delineate the key HDAC(s) to be targeted by inhibitors for optimal induction of latent HIV-1 expression. Individual depletion of HDAC3 significantly induced expression from the HIV-1 promoter in the 2D10 latency cell line model. However, depletion of HDAC1 or -2 alone or in combination did not significantly activate HIV-1 induced expression. Co-depletion of HDAC2 and -3 resulted in a significant increase in expression from the HIV-1 promoter. Furthermore, concurrent knockdown of HDAC1, -2, and -3 resulted in a significant increase in expression from the HIV-1 promoter. When the residual HDAC activity was inhibited after depletion of HDAC1, -2 or -3 with small molecules the effect of depletion of HDAC3 alone was further enhanced. Thus, HDAC3 is essential for suppression of HIV-1 transcription and may be an important target for future therapeutics that seek to purge and eradicate latent HIV-1 infection.

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Introduction

During latency, several restrictive factors are associated with the HIV-1 long terminal repeat (LTR) promoter that block efficient transcriptional initiation and mRNA elongation. Among these factors are HDACs, which are a family of enzymes that regulate transcription of numerous cellular and viral genes by removing acetyl groups from the lysine residues on both histones and non-histone proteins (6, 20). Deacetylation of histone tails results in removal of important docking signals that are required for binding of activating transcription factors. The result is an overall repressive transcriptional environment. HDACs are divided into four classes based upon their amino acid sequence and domain organization (10). The class I HDACs include HDAC1, -2, -3, and -8. HDAC4, -5, -6, -7, -9, and -10 make up the class II HDACs, and HDAC11 is the sole member of class IV. Class III HDACs include sirtuins 1-7, which are nicotinamide adenine dinucleotide-dependent deacetylases that are structurally unrelated to class I, II, and IV HDACs. Class III HDACs are not sensitive to the type of HDAC inhibitors that induce HIV-1 expression.

Nonselective and class I-selective HDAC inhibitors are potent inducers of HIV-1 expression in both cell line models and ex vivo outgrowth assays using resting CD4+ T cells from HIV-1-infected individuals (3, 9, 24, 25, 30). Furthermore, the HDACi SAHA activates expression from quiescent proviruses in vivo (2). The class I HDACs, HDAC1, -2, and -3, are recruited to the HIV-1 LTR in cell line models of HIV-1 latency (4, 16, 20, 26, 28). These class I HDACs are highly expressed in the nuclei of resting CD4+ T cells and inhibitors selective for HDAC1, -2, and -3 are strong inducers of latent HIV-1 expression in resting CD4+ T cells (4). However,
inhibitors selective for the class II HDACs do not induce expression of HIV-1 (4). HDAC4, -6, or -7 have not been demonstrated to directly bind to the HIV-1 LTR (4). Highly selective inhibitors for HDAC1, -2, or -3 in isolation are not yet available, and an inhibitor selective for HDAC1 and HDAC2—but not HDAC3—does not activate latent HIV-1 (4). This data suggests that HDAC3 enzymatic inhibition is crucial for induction of expression from quiescent HIV-1 proviruses, but it is unclear if simultaneous inhibition of HDAC1 and -2 is also needed. Determining the minimum HDAC inhibition required to induce latent HIV-1 expression may focus efforts to identify and develop selective HDAC inhibitors for anti-latency therapies that would have fewer effects on cellular promoters and gene expression when compared to pan-HDAC inhibition.

In an effort to better understand the role of individual class I HDACs in regulation of HIV-1 transcription, the impact of isolated and combination shRNA-mediated depletion of HDAC1, -2, and -3 on HIV-1 expression in a T cell line model of latency was explored.
Materials and methods

2D10 cells

2D10 cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). All experiments were performed using cells that had been passaged fewer than ten times. Cell cultures were maintained at 37°C under 5% CO₂.

Flow cytometry analysis

First, the cells were washed once in PBS and then fixed in PBS containing 3.2% paraformaldehyde. Flow cytometry was performed using an Attune flow cytometer (Applied Biosystems, Carlsbad, CA). Analysis of GFP expression was performed using FlowJo software (Tree Star inc.; Ashland, OR) and the statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Results are shown as the mean of at least three independent experiments, and the error bars indicate the standard error of the mean.

Transduction of shRNAs

Three million 2D10 cells were aliquoted into fresh media 24 h prior to transduction. Then, lentiviruses containing shRNAs specific to the individual HDACs were added to the 2D10 cells, and 2 μg/ml puromycin was added to the cells 24 h after addition of lentiviruses to select for cells containing the shRNA expressing vectors. In experiments that required drugs to be added to the cells, the drugs were
added at 72 h post-transduction. At 96 h post-transduction, cell samples were collected for flow cytometry and mRNA expression analysis. Independent transductions were performed for each experiment. Values shown are the mean of at least three independent experiments, and the error bars indicate the standard error of the mean (SEM).

**HDAC inhibitors**

2D10 cells that had been depleted of HDAC1, -2, or -3 with shRNAs were incubated with the indicated HDAC inhibitors for 18 h. Merck 12 was used at a concentration of 20 μM, Merck 13 was used at a concentration of 200 nM, and SAHA was used at a concentration of 250 or 500 nM. Mrk12, Mrk13, and SAHA were generously provided by Merck Research laboratories (West Point, PA). TSA (T8552, Sigma, St Louis, MO) was used at a concentration of 25 nM and droxinostat (S1422, Selleckchem.com, Houston, TX) was used at a concentration of 2 μM.

**RNA extraction and quantitative RT-PCR**

RNA was extracted from cells using a QIAgen RNeasy Mini Kit (Valencia, CA) following the manufacturer’s protocol. DNA was removed from RNA extracts by DNase digestion (Promega; Madison, WI), and cDNA was synthesized using the SuperScript III First-Strand Synthesis for RT-PCR kit from Invitrogen. Quantitative PCR was performed on cDNA with a Bio-Rad CFX96 or CFX384 using QuantiTect Multiplex PCR Mastermix (QIAgen) and the following primer pairs and 5' FAM-labeled probes: HDAC1 5' TGAGGACGAAGACGACCCT (forward), 5'
CTCACAGGCAATTCGTTTGTC (reverse), and 5’
CAAGCGCATCTCGATCTGCTCCTC (probe) (23); HDAC2 5’
CTTTTCTGGCACAGGAGACTT (forward), 5’
CTCATTTGAAATTGACAGCATGT (reverse), and 5’
AGGGATATTGGTGCTGGAAAGGCAA (probe); and HDAC3 5’
GTTGTTATACTGTCCGAAATGGT (forward), 5’ GCTCCTCACTAATGGCCTCTTC (reverse), and 5’ AGCAGCGATGTCCAGCATAGT (probe). Expression of
GFP mRNA from the HIV-1 promoter was measured using the primers 5’
GGAGCGCACCAT CTTCTTCA(forward) and 5’ AGGGTGTCGCCCTCGAA reverse
along with the 5’ FAM labeled probe 5’ CTACAAGACCCGCGCCGAGGTG. A
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pair and 5’ HEX-
labeled probe were included with each reaction for normalization: 5’
GGCACCACCAACTGCTTAGCACC (forward), 5’ TCTTCTGGGTGGCAGTGATG (reverse), and 5’ TCGTGGGAAGGACTCATGACCACAGGT (probe) (27). Relative
mRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method. The data shown is the
mean of at least three independent experiments, and the error bars represent the
standard error of the mean.

**Cell proliferation assays**

Cellular proliferation and viability of the 2D10 cells were determined 96 hours
post transduction using the CellTiter-blue cell viability assay (Promega; Madison, WI) according to the manufacture’s instructions. The assay was read using a
Spectramax M3 microplate reader (Molecular devices, Sunnyvale, CA) at
fluorescence 560/590 nM. Viability was calculated as the percent of viability relative to the scrambled shRNA condition. At least three independent experiments were performed for each condition. The values shown in the graphs represent the mean ± the SEM.

**Statistical analysis**

GraphPad Prism software was used to analyze the data. The Student’s t test was used to compare the mean GFP protein or mRNA expression in 2D10 cells following transduction with a scrambled shRNA to cells transduced with HDAC1, -2, and -3 shRNAs. Furthermore, the Student’s t test was used to compare the mean GFP expression following incubation with HDAC inhibitors after transduction from at least three independent experiments. A p-value of less than .05 was considered statistically significant.

**Results**

**HDAC3 negatively regulates HIV-1 expression in 2D10 cells.**

To determine the individual contribution of each HDAC to the maintenance of HIV-1 latency, first isozyme-specific HDAC knockdowns in the 2D10 cell line model of HIV-1 latency was evaluated. 2D10 cells contain a single, transcriptionally silent HIV-1 genome that is integrated into the cellular DNA (18). The 2D10 cell line was clonally selected for a low level of basal HIV-1 expression. However, viral expression is inducible by exposure to appropriate stimuli, such as tumor necrosis factor alpha (TNF-α) or HDAC inhibitors (18). 2D10 cells were transduced with a scrambled
shRNA control sequence or with shRNAs targeting HDAC1, -2, or -3. The HDAC mRNA levels following knockdown with the shRNAs were compared to the expression levels from the scrambled shRNA control condition 96 h post-transduction. Following transduction, HDAC1, -2, and -3 mRNA levels were reduced by 70%, 92%, and 60%, respectively (Fig. 3.1A). The knockdowns were specific to the HDAC targeted (Fig 3.1A). Furthermore, cellular viability was not affected by transduction with shRNAs targeting HDAC1, -2, or -3 when compared to cells that had been transduced with the scrambled shRNA control 96 h post-transduction as measured by the cellTiter-blue cell viability assay (Promega, Madison, WI) (Fig. 3.1B).

Because transcription from the HIV-1 promoter is induced by HDAC inhibitors that target the class I HDACs, HDAC1, -2 and -3, it was important to determine if depletion of a single HDAC was sufficient to induce transcription from the HIV-1 LTR. Therefore, the impact of individual HDAC knockdown on HIV-1 expression in 2D10 cells was assessed by monitoring GFP protein and mRNA expression from the HIV-1 LTR at 96 h post-transduction. Depletion of HDAC3 in 2D10 cells led to a statistically significant increase in the percentage of cells expression GFP protein and in mRNA expression from the HIV-1 promoter when compared to the scrambled shRNA control cells (Fig. 3.1C). However, individual HDAC1 or -2 knockdown did not induce a significant amount of HIV-1 LTR driven GFP protein or mRNA expression in 2D10 cells. Thus, depletion of HDAC3 alone, but not HDAC1 or -2, is sufficient to induce transcription from the HIV-1 LTR.
Concurrent knockdown of HDAC1 or HDAC2 with HDAC3 does not further increase expression from the HIV-1 promoter compared to depletion of HDAC3 alone.

Previously, it was reported that inhibitors selective for HDAC1, -2, and -3 are potent inducers of HIV-1 expression (4). Therefore, to determine if dual inhibition of a pair of these HDACs resulted in improved induction of HIV-1, compared to inhibition of HDAC3 alone, the effects of combined shRNA-mediated HDAC knockdown on latent HIV-1 expression were evaluated in 2D10 cells. The targeted HDAC mRNA expression levels following combination knockdowns were similar to those obtained following individual knockdowns at 96 h post transduction (compare Figs. 3.1A and 3.2A). Cell viability was not affected at 96 h following combination HDAC depletion when compared to the scrambled shRNA control as determined using the cellTiter-blue viability assay (Promega, Madison, WI) (Fig. 3.2B). Depletion of HDAC2 and -3 resulted in a significant increase in the percent of cells expressing HIV-1 LTR driven GFP protein and in HIV-1 driven GFP mRNA expression over the cells that were transduced with the scrambled shRNA (Fig. 3.2C). However, this increase was not significantly different from the increase observed following depletion of HDAC3 alone. The combined knockdown of HDAC1 and -3 resulted in a modest, but not significant, increase in the percent of cells expressing GFP protein and HIV-1 driven mRNA expression over cells that were transduced with the scrambled control (Figs. 3.2C). Similar to single knockdown of HDAC1 or -2, no induction of GFP protein or mRNA expression was observed when HDAC1 and -2 were inhibited together. Therefore, depletion of HDAC1 or -2 in combination with
HDAC3 does not enhance the inducing effects observed following depletion of HDAC3 alone.

**Combined knockdown of HDAC1, -2, and -3 induces expression from the HIV-1 LTR.**

As it has been previously observed that induction of HIV-1 expression in 2D10 cells is induced with inhibitors that selectively target HDAC1, -2, and -3, it is possible that optimal induction of HIV-1 may require simultaneous inhibition of all three HDACs (4). Thus, the effects of combined knockdown of HDAC1, -2, and -3 on HIV-1 expression were tested. HDAC mRNA levels were reduced at 96 h post-transduction with HDAC shRNAs when compared to transduction with the scrambled shRNA control (Fig. 3.3A). Concurrent knockdown of HDAC1, -2, and -3 did not affect cellular viability at 96 h post-transduction when compared to the scrambled shRNA control using the cellTiter-blue viability assay (Promega, Madison, WI)(Fig. 3.3B). A significant increase in the percent of cells expressing GFP protein and in mRNA expression from the HIV-1 promoter was observed in cells depleted of HDAC1, -2, and -3 as compared to the scrambled shRNA control (Fig. 3.3C). However, as with the dual combination knockdowns, the amount of expression induced by all three HDACs was not significantly more than that observed following depletion of HDAC3 alone. Therefore, the significant increase observed following depletion of all three HDACs over the scrambled shRNA control condition is likely due to the effects of HDAC3 depletion.
Enzymatic inhibition of HDACs potentiates the effects of HDAC3 depletion.

As the depletion of the HDACs was not complete when using shRNAs, next sub-optimal concentrations of HDAC inhibitors were used to enzymatically inhibit the remaining HDAC activity following targeted shRNA mediated depletion. Selective inhibition of HDAC1 and -2 with 20 μM of the drug Mrk12 in combination with depletion of HDAC1 did not significantly affect the percentage of cells expressing GFP from the HIV-1 promoter (Fig. 3.4A) (4). However, when Mrk12 was added to cells that were depleted of HDAC2 or -3 a significant increase in the percentage of cells expressing GFP from the HIV-1 promoter was observed over cells treated with the scrambled shRNA control and Mrk 12 (Fig. 3.4A). Furthermore, 200 nM of Mrk13, an inhibitor selective for HDAC1, -2, and -3, again induced a significant increase the percent of cells expressing GFP from the HIV-1 promoter in cells that were depleted of HDAC2 and -3 but not HDAC1 over expression from cells that had been transduced with the scrambled shRNA control and treated with Mrk 13 (Fig. 3.4B).

Next, either a suboptimal concentration (250 nM) or a maximal concentration (500 nM) of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA or vorinostat), which is selective for HDAC1, -2, -3 -6, and -8, was used. Similar to the results found with Mrk12 and 13, the suboptimal concentration of SAHA induced a significant increase in the percentage of GFP expressing cells in cells that were depleted of HDAC2 and -3 but not HDAC1 over expression of GFP from the cells
that were transduced with a scrambled shRNA control and treated with SAHA (Fig. 3.4C). When the maximal concentration of SAHA was combined with depletion of HDAC1, -2, or -3, a significant increase in the percent of cells expressing GFP from the HIV-1 promoter from the cells depleted of HDAC3, but not HDAC1 or -2, over 2D10 cells treated with the scrambled shRNA control and SAHA was observed (Fig. 3.4C). Furthermore, inhibition with the global HDAC inhibitor TSA also significantly enhanced the effect of HDAC3 depletion (Fig. 3.4D). As depletion of HDAC2 significantly increased the percentage of cells expressing GFP from the HIV-1 promoter in conjunction with three of the conditions, a supportive role for HDAC2 in repression of HIV-1 transcription cannot be ruled out.

To determine whether further inhibition of HDAC3 without inhibition of HDAC1 or -2 was sufficient to induce transcription from the HIV-1 LTR, an HDAC inhibitor that is selective for HDAC3, but not HDAC1 or -2 was used. Droxinostat inhibits HDAC3, -6 and -8 but inhibits HDAC3 at the lowest concentration, with an IC₅₀ of 2 μM (15). Therefore, 2 μM of Droxinostat was used to inhibit HDAC3 activity after depletion of HDAC1, -2 or -3. Again, depletion of HDAC1 or -2 with inhibition of HDAC3 did not increase the percent of cells with LTR driven GFP protein expression observed over Droxinostat treatment of the cells transduced with the scrambled shRNA (Fig. 3.4E). However, inhibiting the enzymatic activity of the remaining HDAC3 enzyme with Droxinostat following HDAC3 depletion induced a significant increase in the percent of cells expressing GFP protein from the HIV-1 LTR compared to cells that were transduced with the scrambled control and treated with Droxinostat (Fig. 3.4E). Altogether, these results support the above results,
indicating that inhibition of HDAC3 is key for reactivation of latent HIV-1 and that inhibition of HDAC1 or -2 does not enhance the effect of reducing the activity of HDAC3 through depletion or enzymatic inhibition.

**Discussion**

HDAC inhibitors selective for the class I HDACs HDAC1, -2, and -3, but not those that are selective for HDAC1 and -2, are potent inducers of HIV-1 transcription (4, 25). In an effort to determine the minimal HDAC-inhibitory requirement for optimal induction of latent HIV-1, an shRNA-mediated strategy was employed to deplete individual and combinations of the class I HDACs, HDAC1, -2, and -3, in the 2D10 T cell line model of quiescent HIV-1. Isolated depletion of HDAC3 expression resulted in a statistically significant induction of LTR driven GFP protein expression in 2D10 cells (Fig. 3.1D). These findings support our previous studies on the effects of selective HDAC inhibitors and further define the minimal HDAC inhibition that is required for reactivation of latent HIV-1 (4).

To determine whether depletion of HDAC1 or -2 could possibly enhance the effects of depletion of HDAC3, shRNAs were used to deplete these HDACs in combination. Similar to the results of Huber et al., concurrent depletion of HDAC1 and -2 did not induce expression from the HIV-1 promoter (15). After depletion of HDAC2 and -3 a significant increase in the percent of cells with HIV-1 driven GFP protein expression and GFP mRNA expression were observed (Fig. 3.2C). However, the amount of induction seen after dual inhibition is not significantly more than the amount of induction following depletion of HDAC3 alone, which indicates that the
observed induction may be due to HDAC3 depletion. Furthermore, depletion of HDAC1 and -3 did not induce a significant increase in LTR-driven GFP expression compared with transduction with the scrambled shRNA control. Therefore, depletion of HDAC1 may interfere with the effect of depletion of HDAC3 alone. Another study reported similar results when analyzing expression of cyclin D1 by quantitative RT-PCR following knockdown of HDAC1, HDAC2, or HDAC1 and -2 in combination. In that study, isolated knockdown of HDAC1 reduced cyclin D1 mRNA levels by 75% and knockdown of HDAC2 led to a 50% reduction in cyclin D1. However, when HDAC1 and -2 were knocked down concurrently, no reduction in cyclin D1 expression was observed (7). Thus, concurrent depletion of two HDACs can suppress the repressive effects of either HDAC, perhaps due to the counter-regulatory effects of the acetylation of HDACs themselves (7).

We and others have previously reported that latent HIV-1 expression is induced with HDAC inhibitors that selectively target the class I HDACs (i.e. HDAC1, -2, and -3) (4, 25). Therefore, to determine whether induction of the HIV-1 promoter was improved by inhibition of all three HDACs, shRNAs were used to deplete all three HDACs at once. The depletion observed following transduction with shRNAs targeting HDAC1, -2, and -3 was similar to that observed following individual depletion and cell viability was not affected. Similar to the results found using enzymatic inhibition, simultaneous depletion of HDAC1, -2, and -3 by shRNA in 2D10 cells induced a significant amount of HIV-1 driven GFP protein and mRNA expression (Fig. 3.3C). However, the percent of cells expressing GFP was not as high as it was after depletion of HDAC3 alone, suggesting that depletion of multiple
HDACs may be less effective than depletion of HDAC3 alone or may be a artifact of the shRNA mediated knockdown strategy.

It’s worth noting that the rate of knockdown achieved by the individual HDAC shRNAs in this study ranged from 48% to 95% (Figs. 3.1A, 3.2A, and 3.3A); thus, there was not a complete elimination of any of the HDACs targeted. Because HDAC1, -2, and -3 are all known to associate with the HIV-1 LTR (4), it is possible that some combination of these HDACs may remain to regulate the viral promoter following knockdown with shRNA. Furthermore, transduction with shRNAs results in a slow depletion of protein expression. In contrast, chemical HDAC enzymatic inhibition occurs rapidly and persists until the drug is cleared and the active enzyme is reconstituted.

To address some of these concerns, 2D10 cells were depleted of HDAC1, -2, or -3 and then small molecules were used to inhibit the remaining HDAC activity. Depletion of HDAC2, or -3 followed by enzymatic inhibition of HDAC1 and -2 with the small molecule Mrk12 resulted in a significant increase in the percentage of cells with GFP protein expression from the HIV-1 LTR over cells that were treated with the scrambled shRNA and Mrk12 (Fig. 3.4A). However, no change was observed in the cells depleted of HDAC1 followed by treatment with Mrk12 over treatment of cells treated with the scrambled shRNA and Mrk 12 (Fig. 3.4A). Similar results were obtained using an inhibitor specific for HDAC1, -2, and -3 (Mrk 13) and a suboptimal concentration of the broad HDAC inhibitor SAHA (250 nm) (Fig. 3.4B-C). These results demonstrate that inhibiting the remaining HDAC activity after depletion of
HDAC2 or -3 enhances the effects of depletion alone. Furthermore, use of a maximal concentration of SAHA (500 nM) or the broad HDACi TSA augmented the effect of depletion of HDAC3 alone (Fig. 3.4C-D). Because the maximal concentration of SAHA or the broad acting HDACi TSA are high, the contributory effect of HDAC2 that was observed in the previous conditions may have been masked. However, the effects of HDAC3 depletion were sufficient to enhance the activity of all of the small molecules that were tested in this study.

To date, no specific inhibitors of HDAC3 have been described. However, Huber et al. recently demonstrated that Droxinostat, which is a selective inhibitor of HDAC3, -6, and -8 at low concentrations, activated transcription from a cell model of HIV-1 latency (15). The concentrations of Droxinostat used in that study, 60 µM, were sufficient to inhibit HDAC1 as well. Therefore, following that study, it was still unknown whether inhibition of HDAC1 in addition to HDAC3 was required for activation of transcription from the HIV-1 LTR. In this study, treating cells depleted of HDAC3 with 2 µM of Droxinostat resulted in a significant increase in GFP expression from the HIV-1 LTR over cells transduced with the scrambled shRNA (Fig. 3.4E). In this study, a significant increase in GFP protein expression from the HIV-1 LTR was detected with 2 µM of Droxinostat, which is the IC₅₀ of this drug for HDAC3 and is 2.5 fold below the IC₅₀ value for HDAC8 and 30 fold below the IC₅₀ for HDAC1 (15). However, as with previous studies, it is difficult to rule out a contributory effect of HDAC6 or -8 when treating with Droxinostat. Altogether, these results support the conclusion that HDAC3 is the primary target of class I HDAC inhibitors that reactivate transcription of latent HIV.
Microarray studies have demonstrated that increased acetylation at the promoter of genes is associated with active transcription. Furthermore, acetylated histone tails recruit bromodomain containing proteins such as BRG1 of the SWI/SNF complex and TFIID, which promote transcription from the promoter (1). HDAC1, -2, and -3 have been demonstrated to physically associate with the HIV-1 LTR (17), and inhibition of these HDACs with small molecule HDAC inhibitors is associated with increased acetylation of histones at the HIV-1 LTR (19). Therefore, it is interesting that depletion of HDAC3, but not HDAC1 or -2, activates transcription from the HIV-1 promoter. However, HDAC3 has been demonstrated to have some key differences from HDAC1 and -2 that may account for its unique role.

HDAC1 and -2 are 85% similar to each other; however, HDAC3 is only 53% and 52% similar to HDAC1 and -2, respectively. Furthermore, HDAC3 contains a unique amino acid sequence at its carboxy terminus (29). In addition to its nuclear localization signal, HDAC3 contains cytoplasmic and cell membrane-targeting regions that are not found in HDAC1 and -2 (29). HDAC2 and -3 are located in both the cytoplasm and nucleus in resting and activated CD4+ T cells from HIV-1 infected aviremic patients (17). In contrast, HDAC1 is only found in the nucleus (17). In addition to affecting localization, these sequence differences may also account for HDAC3’s unique complex association. HDAC3 is associated with the NCoR and SMRT complexes, while HDAC1 and -2 are associated with the CoREST, NuRD, REST, and Sin complexes (11, 14). NCoR has been demonstrated to bind to the HIV-1 LTR in monocyte-derived macrophages (12). Furthermore, NCoR is found at the HIV-1 LTR in a Jurkat cell line model of HIV-1 latency (J89 cells)(Barton and
Margolis, unpublished data). The association of NCoR with the HIV-1 promoter indicates that HDAC3 may be mediating its effects through the NCoR complex. Of the HDACs that are found at the HIV-1 promoter, HDAC1 and -3 have the most highly expressed mRNAs and are expressed five and eight times more than that of HDAC2, respectively (17). Therefore, the minimal role of HDAC2 in reactivation of latent HIV-1 could be associated with the minimal amount of expression relative to the other two HDACs. Additionally, studies looking at histone deacetylation patterns have found that HDAC3 preferentially deacetylates histone 4 lysine 5 > lysine 8 > lysine 12 > lysine 16 (13). The substrate specificity of HDAC1 and -2 are not fully elucidated. Considering the many differences between HDAC1, -2, and -3, it is not surprising that the effect of HDAC3 depletion on the HIV-1 LTR is distinct from that of HDAC1 and -2. Therefore, the differences in effects observed following individual depletion of these HDACs may be due to the differences in complex association, sequence diversity, expression, or substrate preference.

In addition to the differences in complex associations and sequence diversity, HDAC3 has been demonstrated to have non-histone targets that may explain its role in activation of transcription from the HIV-1 LTR. HDAC3 specifically deacetylates the RelA component of the NF-κb transcription factor, which then results in disassociation of RelA from promoters, association with IκBα and export from the nucleus (5). Localization of the RelA/p50 heterodimer of NF-κb to the HIV-1 promoter and displacement of the NF-κb p50/p50 homodimer activates transcription from the HIV-1 promoter (21). Furthermore, several compounds such as TNFα and PMA have been demonstrated to activate transcription from the HIV-1 promoter.
through promoting relocation of the NF-κb RelA/p50 heterodimer to the nucleus (8, 22). Therefore, in addition to its role as a histone deacetylase, HDAC3 depletion may further affect HIV-1 transcription by preventing deacetylation of RelA and dissociation of the activating NF-κb heterodimer from the HIV-1 LTR.

The next step of this project was to determine the minimal HDAC inhibition required to induce latent HIV-1 expression. Selective HDAC inhibitors targeting HDAC1 and -2 were not sufficient to induce latent HIV-1 expression (4). Because small molecules that target HDAC3 alone have only recently been developed, previous studies have not been able to determine the minimal HDAC inhibition that is required for induction of transcription from latent HIV-1 proviruses. In this study, selective targeting of HDAC3 was demonstrated to be sufficient to induce expression from the HIV-1 promoter.

A future important research goal is to validate the centrality of HDAC3 in the induction of HIV-1 transcription from patient cells. As part of this study, depletion of HDAC1, -2, and 3 from patient cells was attempted. Transduction of peripheral blood mononuclear cells with shRNAs did not result in depletion of the targeted HDACs. However, nucleofection of siRNAs into resting primary T cells using the Amaxa Nuclefect (Lonza, Switzerland) did result in a significant and specific depletion of the targeted HDAC (Keedy and Margolis, unpublished data). Furthermore, nucleofection did not significantly affect cell viability as compared to cells transduced with a control siRNA. A modest induction of HIV-1 outgrowth in a co-culture assay was observed in resting memory T cells following individual depletion of each
HDAC2 or -3 (Keedy and Margolis, unpublished data). However, perturbation of the resting state in these memory T cells was also observed following the nucleofection procedure, as indicated by an increase in CD69 expression 24 h post nucleofection. Therefore, the results are difficult to interpret because it is unclear whether viral expression is due to the unique or combined effects of the nucleofection procedure or of HDAC depletion. Therefore, the identification or development of more selective class I HDAC inhibitors is required to determine the extent of expression that is induced by complete and selective enzymatic inhibition of HDAC3.

Selective HDAC inhibitors that target a limited number of the class I HDACs have potential as anti-latency therapies with fewer host-toxicities and non-HIV specific effects than pan-HDAC inhibitors. It was previously demonstrated that inhibitors that selectively target HDAC1, -2, and -3—but not HDAC1 and -2 alone—are potent inducers of latent HIV-1 (4). In this study, whether a more selective induction of HIV-1 could be achieved using a shRNA-mediated strategy of HDAC knockdown was evaluated. The results indicate that depletion of HDAC3 significantly induces expression from the HIV-1 promoter. Thus, potently and selectively targeting HDAC3 alone may be sufficient to induce transcription from quiescent proviruses.

Contributions

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Figure 3.1. Depletion of HDAC3 significantly increases expression from the HIV-1 promoter. A. Expression of the indicated HDAC mRNAs following shRNA mediated depletion of HDAC1, -2, or -3 in 2D10 cells. Depletion of HDAC1, -2, or -3 was significant and specific for the targeted HDAC. B. Cell proliferation was not significantly affected following depletion of HDAC1, -2 or -3 in 2D10 cells. C. The percentage of 2D10 cells expressing GFP protein from the HIV-1 promoter following depletion significantly increased following depletion of HDAC3 in comparison to control cells transduced with the scrambled shRNA, but not following depletion of HDAC1 or -2. This same effect was seen when GFP mRNA expression was directly measured. Expression was not significantly altered from baseline following transduction with non-specific shRNA.
Figure 3.2. Co-depletion of HDAC2 and HDAC3 significantly increases expression from the HIV-1 promoter. A. Expression of the indicated HDAC mRNAs following shRNA mediated depletion of HDAC1 and -2, HDAC1 and -3, or HDAC2 and -3. The depletion was significant and specific for the targeted HDACs. B. Cell proliferation and viability of 2D10 cells that were transduced with shRNAs targeting HDAC1 and -2, HDAC1 and -3, or HDAC2 and -3. No significant changes in cell proliferation and viability were observed following co-depletion of these HDACs as compared to cells that were transduced with the scrambled shRNA control. C. Expression of GFP from the HIV-1 promoter following depletion of HDAC2 and -3 increased compared to the control cells transduced with a scrambled shRNA. However, depletion of HDAC1 and -2 or HDAC1 and -3 did not have a significant effect on GFP expression. This same result was seen when GFP mRNA expression was directly measured. Expression was not significantly altered from baseline following transduction with non-specific shRNA.
Figure 3.3. Depletion of HDAC1, -2, and -3 significantly increases expression from the HIV-1 promoter. A. Fold change of HDAC1, -2, and -3 mRNA expression as compared to 2D10 cells transduced with the scrambled shRNA control. A significant reduction in the mRNA expression of all three HDACs was observed. B. Cell viability and proliferation as a percentage of the 2D10 cells transduced with the scrambled shRNA control. Depletion of HDAC1, -2, and -3 did not significantly affect cell viability and proliferation. C. A significant increase in the percentage of cells expressing GFP protein from the HIV-1 promoter was observed following depletion of HDAC1, -2, and -3. Furthermore, a significant increase in expression of GFP mRNA from the HIV-1 promoter was observed following depletion of HDAC1, -2, and -3.
Figure 3.4. Chemical inhibition of HDACs following depletion of HDAC3 significantly increases expression from the HIV-1 promoter. A. Chemical inhibition of HDAC1 and -2 using Mrk 12 (20 μM) does not result in a significant increase in the percentage of 2D10 cells expressing GFP following depletion of HDAC1 or -2. However, similar to depletion of HDAC3 alone, a significant increase in the percent of cells expressing GFP was when Mrk 12 was added to cells depleted of HDAC3. B. Chemical inhibition of HDAC1, -2, and -3 with Mrk13 (200 nM) resulted in a significant increase in the percent of GFP positive 2D10 cells in cells depleted of HDAC2 or -3, but not HDAC1. C. A minimal (250 nM) dose of SAHA resulted in a significant increase in the percent of GFP positive cells in cells that were depleted of HDAC2 and a maximal dose (500 nM) resulted in a significant increase in the percent cells expressing GFP in cells depleted of HDAC2 or -3. However, neither dose resulted in a significant increase in GFP expression in 2D10 cells that were depleted of HDAC1. D. Chemical inhibition of HDACs with the non-selective HDAC inhibitor TSA (25 nM) resulted in a significant increase in the percent of GFP expressing 2D10 cells in cells depleted of HDAC3, but not HDAC1 or HDAC2. E. Chemical inhibition of HDAC3, HDAC6 and HDAC8 with the selective HDAC inhibitor Droxinostat resulted in a significant increase in the percent of cells expressing GFP in 2D10 cells that had been depleted of HDAC3 but not HDAC1 or HDAC2.
REFERENCES


CHAPTER 4
DISCUSSION

Introduction

The HIV-1 epidemic has caused significant social and economic difficulties for communities and nations around the world. Over the last thirty years, substantial advances have been made in understanding the virus, which has led to the development of very effective ART regimens. However, a “cure” has not been developed thus far. The primary impediment to complete clearance of the virus is the existence of a long-lived population of cells harboring replication competent but transcriptionally silent virus. One of the most promising strategies to address the problem of latent virus is induction of viral transcription and replication followed by clearance of the virus. This thesis primarily focused on identifying cellular therapeutic targets to induce transcription from quiescent HIV-1 promoters.

Findings and Implications

Initially this project aimed to determine whether targeting cMyc, YY1 or both could induce expression from the HIV-1 promoter in a Jurkat cell line model of quiescent HIV-1 and to determine whether the factors cMyc and YY1 are important for recruitment of HDAC2 and HDAC3 to the HIV-1 promoter. To determine whether cMyc and YY1 are important for maintenance of the quiescent proviral state, transduction of shRNAs was used to deplete cMyc and YY1 individually or in combination and then transcription was measured from the HIV-1 promoter using qPCR and flow cytometry. Depletion of cMyc or YY1 following transduction with the
shRNAs was measure by qPCR to determine the levels of mRNA and by Western blot to measure the amount of protein. A significant and specific reduction in the targeted product was observed following transduction of the 2D10 or J89 cells with shRNAs. Because cells release stress related factors when viability is reduced that can activate HIV-1 transcription, it was important to determine whether depletion of cMyc, YY1 or both had an effect on the viability of 2D10 and J89 cells. When cMyc, YY1 or both were depleted from J89 or 2D10 cells no significant change in viability was observed, indicating that changes in expression from the HIV-1 promoter were due to depletion of the cMyc or YY1 and not to cellular stress.

Previous studies in HeLa cell line models of HIV-1 latency demonstrated that cMyc and YY1 are important factors for the maintenance of proviral quiescence in those models (5-9). To further extend these findings to Jurkat cells, expression from the HIV-1 promoter was measured following depletion of cMyc, YY1 or both. When YY1 was depleted from 2D10 or J89 cells, a significant increase in mRNA and protein expression was observed by qPCR and Western blot, respectively. However, depletion of cMyc or YY1 and cMyc did not significantly affect expression in either cell line. This finding illustrates the necessity of demonstrating that identified mechanisms are relevant in multiple systems.

Both cMyc and YY1 have been shown to interact with HDAC-containing complexes and to be important for occupancy of HDAC1 at the HIV-1 LTR. Therefore, because the class I HDACs are very similar in sequence and because HDAC2 is found in complex with HDAC1, the hypothesis that cMyc and YY1 may be
important for occupancy of HDAC2 and HDAC3 at the HIV-1 promoter was
developed. To determine whether depletion of cMyc or YY1 affected occupancy of
HDAC2 and HDAC3 at the HIV-1 LTR in Jurkat cells, ChIP was used to measure
occupancy of the class I HDACs in cells that had been depleted of cMyc or YY1. It
was found that depletion of cMyc or YY1 did not significantly affect occupancy of
HDAC2 or HDAC3 at the HIV-1 LTR in 2D10 or J89 cells. Because many
transcription factors that bind to the HIV-1 promoter recruit HDACs, it was
hypothesized that redundant recruitment mechanisms may compensate for the loss
of a single factor. Therefore, the occupancy of HDAC2 and HDAC3 at the HIV-1 LTR
was measured in J89 and 2D10 cells that had been depleted of both cMyc and YY1.
The levels of HDAC2 and HDAC3 did not significantly change following depletion of
both factors. These results indicate that in addition to cMyc and YY1, other factors
contribute to recruitment of HDAC2- and HDAC3-containing complexes.
Furthermore, additional or separate factors may need to be targeted to induce
transcription from the HIV-1 promoter. Alternatively, because shRNAs only reduce
and do not completely eliminate protein, more advanced technologies that can
completely eliminate cMyc or YY1 may have potential for therapeutic treatment of
HIV. Therefore, although cMyc and YY1 are involved in recruitment of HDACs to the
HIV-1 LTR, depletion of cMyc or YY1 is not sufficient to significantly alter HDAC
occupancy of the HIV-1 LTR. Therefore, the redundant mechanisms that exist at the
HIV-1 promoter may be sufficient to maintain HDAC occupancy and repression of
HIV-1 transcription in the absence of cMyc or YY1.
Future directions

The goal of this study was to find a selective way to target the HIV-1 LTR while minimizing the effect on cellular genes. The results from this study indicate that cMyc and YY1 may not be ideal candidates for inducing transcription from quiescent proviruses. To this end, targeting the other transcription factors such as NF-κb or CBF-1 either alone or in conjunction with cMyc or YY1 may be required to disrupt HDAC occupancy at the HIV-1 promoter and induce transcription (10, 11). However, many of the transcription factors found at the HIV-1 promoter are ubiquitous and affect many cellular promoters (11). Therefore, it will be important to assess the effect of these potential approaches on cellular genes and whether it is less disruptive than globally targeting HDACs.

Findings and implications

The next objective was to determine which individual or a combination of HDACs is important for maintenance of quiescent HIV. To address this question, a shRNA mediated strategy was used to deplete HDAC1, HDAC2 or HDAC3 individually or in combination. Depletion of the individual HDACs did not affect the viability of 2D10 cells or the expression of the non-targeted HDACs. Furthermore, the shRNA-mediated depletion of HDAC1, HDAC2 or HDAC3 resulted in a significant decrease in mRNA expression of the targeted HDAC. Depletion of HDAC1 or HDAC2 did not significantly increase mRNA or GFP expression from the HIV-1 promoter. However, depletion of HDAC3 did significantly induce protein and mRNA expression from the HIV-1 promoter, which indicates that HDAC3 activity
may be the key target of HDAC inhibitors that reactivate expression from the HIV-1 promoter. To further determine whether co-depletion of the HDACs would enhance the effects observed following single depletion, shRNAs were used to deplete combinations of the class I HDACs. Depletion of HDAC1 and HDAC3 or HDAC1 and HDAC2 did not affect expression from the HIV-1 LTR. However, depletion of HDAC2 and HDAC3 did result in a significant increase in protein and mRNA expression from the HIV-1 promoter. The amount of expression from the HIV-1 LTR following co-depletion of HDAC2 and HDAC3 was not significantly greater than that observed following depletion of HDAC3 alone, indicating that the effects observed may be due to depletion of HDAC3. It is interesting that the same did not hold true when HDAC1 and HDAC3 were depleted, which indicates that depletion of HDAC1 may interfere with reactivation that is observed following depletion of HDAC3. These results indicate that more selective HDAC inhibitors may be sufficient to induce transcription from quiescent proviruses.

Because the shRNA strategy does not completely eliminate the targeted enzyme(s) from the cells, low levels of small molecules were used to inhibit the residual HDAC activity following depletion of HDAC1, HDAC2 or HDAC3. Merck 12 is an HDAC inhibitor that is selective for HDAC1 and HDAC2 (3). When depletion of HDAC1 was combined with inhibition of HDAC1 and HDAC2 using Merck 12, no significant increase in expression from the HIV-1 promoter over treatment with Merck 12 only was observed (3). However, depletion of HDAC2 or HDAC3 coupled with inhibition using Merck 12 resulted in a significant increase in expression from the HIV-1 promoter when compared to treatment of with Merck 12 only. Next, Merck
13 was evaluated, which is an inhibitor of HDAC1, HDAC2 and HDAC3, in conjunction with depletion of the individual HDACs (3). Treatment with Merck 13 enhanced the induction of expression from the HIV-1 LTR in cells that had been depleted of HDAC2 or HDAC3, but not those depleted of HDAC1, over cells treated with Merck 13 and the scrambled shRNA.

Suberoylanilide hydroxamic acid (SAHA) has recently been demonstrated to activate viral transcription \textit{in vivo} in a clinical trial (1). SAHA is an HDAC inhibitor that is selective for HDAC1, HDAC2, HDAC3 and the class II HDAC, HDAC6 (2). A low level of SAHA (250 nM) and a high level of SAHA (250 nM) were both used in combination with depletion of HDAC1, HDAC2, or HDAC3. Following depletion of HDAC2 or HDAC3, cultures that were exposed to 250 nM of SAHA had a significant increase in the percent of cells expressing GFP under the control of the HIV-1 LTR, but not in cells depleted of HDAC1. Furthermore, when the cells depleted of HDAC3 were exposed to the high dose of SAHA (500 nM), a significant increase in the number of cells expressing GFP was observed. However, this increase was not observed in cells that were depleted of HDAC1 or HDAC2. To further understand the effect of interfering with HDAC activity, a low concentration of the global HDAC inhibitor trichostatin A (TSA, 50 nM) was also evaluated in cells depleted of HDAC1, HDAC2 or HDAC3 and a significant increase in protein expression from the HIV-1 LTR in cells depleted of HDAC3, but not HDAC1 or HDAC2 was observed (13). These results further confirm the findings from the depletion experiments.
HDAC inhibitors that are selective for HDAC3 are relatively new and have not been fully characterized. However, Droxinostat, which is selective for HDAC3, but not HDAC1 or HDAC2, is commercially available. Droxinostat has also been shown to inhibit HDAC6 and HDAC8. Similar to the previous drug experiments, Droxinostat was used to inhibit residual HDAC3 activity in cells depleted of HDAC1, HDAC2 or HDAC3 (12). A significant increase in the percent of cells expressing GFP in cells that were depleted of HDAC3, but not HDAC1 or HDAC2. These results provide supporting evidence that depletion and enzymatic inhibition of HDAC3 result in expression from the HIV-1 LTR in 2D10 cells.

Several of these inhibitors also induced a significant increase in the percent of cells expressing GFP in cells depleted of HDAC2, which indicates that HDAC2 may also play a role in suppression of the HIV-1 LTR, however, it can not be definitively concluded that HDAC2 activity is relevant because expression was only observed in a subset of the HDAC2 experiments.

Overall, this study indicates that targeting HDAC3 alone is sufficient to relieve HDAC mediated repression of the HIV-1 promoter and that novel small molecules that are selective for HDAC3 may be a promising therapies for treatment of latent HIV-1 infection.
Future directions

The next step of this study, is to extend these findings to patient cells. Collaborations with Dr. Alan Kozikowski at the University of Illinois and the Dr. Ed Holson at the Broad Institute have been initiated to further characterize and test small molecules that are selective for inhibition of HDAC3 (4). Experiments to test three molecules from the Kozikowsky lab including 11, 14c and 14d are currently being conducted in cell line models of latent HIV-1 (4). Preliminary studies indicate that the compounds are able to activate transcription from the HIV-1 promoter at concentrations upwards of 5 uM (4). However, at these concentrations the cell viability is significantly affected. The compound from the Broad institute, which is currently unpublished, induces GFP expression in a significant number of 2D10 cells and has very little toxicity. Additional experiments in patient cells are currently underway.

The 2D10 cell line is a clonal cell line that contains a single integration site, which is not representative of the in vivo condition where the latent population consists of cells with HIV-1 proviruses integrated into variable locations. To make our findings more relevant to the in vivo state, it will also be prudent to extend these findings in additional cell lines and to primary cells from aviremic patients.

Initial studies using SAHA, which is selective for HDAC1, HDAC2, HDAC3 and HDAC6, have not observed significant adverse events associated with treatment. However, determining which targets of SAHA are important for its in vivo activity are needed to facilitate the development of future therapeutics.
Concluding Remarks

The persistence of a replication competent viral reservoir in HIV-infected patients is the primary obstacle to a resolution of the HIV epidemic. Substantial efforts have led to significant advances in our understanding of how HIV-1 is able to persist in the presence of ART, and parallel efforts have identified many pathways that can be targeted to reactivate the virus. The findings in this dissertation have further characterized the role of cMyc and YY1 in the maintenance of HIV-1 transcriptional repression. Furthermore, these studies indicate that HDAC3 may be the critical enzymatic target of HDAC inhibitors that are capable of activating transcription from the HIV-1 promoter. If these findings hold true in patient cells, it will be exciting to determine whether more specific HDAC inhibitors are as effective at activating latent HIV-1 \textit{in vivo}. 
REFERENCES


