LYSINE ACETYLATION AND SMALL MOLECULE EPIGENETIC INHIBITION REVEAL NOVEL MECHANISMS CONTROLLING CELLULAR SUSCEPTIBILITY TO HIV-1 INFECTION

BY

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DEDICATION

To my best friend,

It's only fitting that I'm writing to you on the brink of finishing my Ph.D. It wasn't a hard decision when asked who to thank or who to dedicate this work to. You are the reason I've even come this far. It started with Uncle Tungsten, our love for chemistry, and surprisingly dangerous science experiments in your backyard. Your parents were incredibly patient with us... I remember that you always talked about going for your doctorate - in biochemistry, meteorology, or maybe even psychology. Regardless of the subject, you would have been a rock star. You always made science look incredibly easy and I'm certain your intelligence is part of what pushed me to achieve in my own work.

When I think back to my Case admissions essay, I remember writing something along the lines of, "I told myself I would work twice as hard now for the both of us". Well, how did I do? I hope I've lived up to those words. Graduate school has been a wild and stressful journey but I think you would be proud of where I stand now.

So --- here we are. I wrote about you to get in and now I'm writing to you to get the heck out. It would be easier and far more genuine if I could thank you in person today. I know you would have been in the front row at my final defense armed with clever questions and remarks. Unfortunately, life is cruel and it can upend you when you least expect. This adventure has not been the same without you but I am grateful I still see parts of you sprinkled throughout it. At every obstacle I would hear you saying, "to quit complaining and just do it". This is as much your success as it is mine. Thank you for being there for so many years of my life. You made me a better person and influenced the scientist I am now. You were a brother, mentor, ally, and my best friend. I owe you a lot more than you'll ever know.

R.I.P Randy A. Passaro
LIST OF ABBREVIATIONS

Ab: Antibody
AGM: African Green Monkey
AIDS: Acquired Immune Deficiency Syndrome
BSA: Bovine Serum Albumin
CA: HIV Capsid
cART: Combination Anti-Retroviral Therapy
CPZ: Chimpanzee
DNA: Deoxyribonucleic Acid
DMEM: Dubelco's Modified Eagle Medium
Env: HIV-1 Envelope
FBS: Fetal Bovine Serum
Gag: HIV Group-Specific Antigen
gp160: HIV Glycoprotein 160
gp41: HIV Glycoprotein 41
HAT: Histone Acetyltransferase
HDAC: Histone Deacetylase
HIV: Human Immunodeficiency Virus
HMT: Histone Methyltransferase
HTLV: Human T-Lymphotropic Virus
IN: HIV Integrase/Integration
kb: Kilobase
Kda: Kilodalton
LAV: Lymphadenopathy-Associated Virus
LTR: Long Terminal Repeat
LV: Lentivirus
MA: HIV Matrix
MHC-II: Major Histocompatibility Complex 2
NC: Nucleocapsid
Nef: HIV Negative Factor
NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI: Nucleoside Reverse Transcriptase Inhibitor
nt: Nucleotide
mg: Milligram
mM: Millimolar
ng: Nanogram
NLS: Nuclear Localization Signal
nM: Nanomolar
PAGE: Poly-acrylamide Gel Electrophoresis
PIC: Pre-Integration Complex
Pol: Polymerase
PBMCs: Peripheral Blood Mononuclear Cells
PBS: Phosphate Buffered Saline
PPT: Polypurine Tract
PR: Protease
qPCR: Quantitative Polymerase Chain Reaction
Rev: HIV Regulator of Expression of Virion Proteins
RNA: Ribonucleic Acid
RNase H: Ribonuclease H
RRE: Rev Responsive Element
RT: HIV Reverse Transcriptase/Transcription
RTC: Reverse Transcription Complex
SAHA: Suberoyl Anilide Hydroxamic Acid
SDS: Sodium Dodecyl Sulfate
SIV: Simian Immunodeficiency Virus
SMM: Sooty Mangabee
TAR: Trans-Activation Response Element
Tat: Trans-Activator of Transcription
tRNA: Transfer RNA
µg: Microgram
µl: Microliter
µM: Micromolar
Vif: HIV Viral Infectivity Factor
Vpr: HIV Viral Protein R
Vpu: HIV Viral Protein Unique
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Lysine Acetylation and Small Molecule Epigenetic Inhibition Reveal Novel Mechanisms Controlling Cellular Susceptibility to HIV-1 Infection

Abstract
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Research into the HIV "shock-and-kill" approach has become increasingly popular in recent years following identification of agents that can reactivate latent proviruses. These latency research agents (LRAs) include compounds such as disulfiram, protein kinase C agonists, and histone deacetylase (HDAC) inhibitors. Here we report that treatment with the clinically approved HDAC inhibitor vorinostat significantly increases productive viral infection in both single-cycle and replication-competent assays, raising potential concern for its use as a LRA. This effect was also observed with other broad-spectrum HDAC inhibitors and the cytoplasmic HDAC6-specific inhibitor tubacin. Vorinostat enhances post-entry infection efficiency by increasing the kinetics and efficiency of reverse transcription and nuclear import of HIV DNA. Furthermore, these HDAC inhibitors induce acetylated microtubule networks in CD4+ T cells, which likely enhance infection by facilitating viral transit from the periphery to the nucleus.
Our work with HDAC inhibitors also led us to hypothesize that treatment with opposing histone acetyltransferase (HAT) inhibitors will decrease HIV productive infection. Indeed, the HAT inhibitors garcinol and curcumin significantly reduced LTR-driven EGFP expression in primary unstimulated CD4+ T cells in a dose- and time-dependent manner using both single cycle and replication-competent viruses. We believe these findings demonstrate a novel proof-of-concept principle that the susceptibility of cells to HIV can be modulated by directly targeting cellular processes and that this may be of benefit to increase the barrier to sexual transmission at mucosal sites.

Finally, we proposed that the regulation of HIV infection might extend beyond the "axis" of acetylation and assessed a library of small molecule inhibitors in conjunction with our combination reporter virus system. Preliminary analysis revealed numerous targets involved in cell signaling and epigenetic regulation. These results not only hint to the importance of signaling during viral replication but also highlight that epigenetic modifications may have previously unknown effects outside the scope of viral latency and reactivation.
CHAPTER ONE:

INTRODUCTION
1.1 History of HIV-1 and the AIDS Pandemic

Human Immunodeficiency Virus (HIV), the causative pathogen of Acquired Immune Deficiency Syndrome (AIDS), became a significant threat to the global society in the early 1980s. At its onset in the United States, the illness presented itself through a series of opportunistic bacterial infections and malignant cancers in the homosexual population [1, 2]. More specifically, these individuals succumbed to persistent fevers, weakness, oral thrush, severe diarrhea, and significant weight loss. The rapid decline of patients with seemingly unknown source triggered dramatic concern in the homosexual population and their treating physicians. During this time, the plague was described as "Gay Related Immune Deficiency", or GRID, though cases within Haitian populations, hemophiliacs, intravenous drug users, and heterosexual partners challenged the boundaries of the illness [3-5]. This narrow-focused label was officially disputed and recoined AIDS by the Centers for Disease Control in 1982 [6].

By late 1982, evidence for transfusion-mediated infections pointed to transmission between bodily fluids and/or blood products [7]. Without a sufficient testing surrogate, blood banks were quickly viewed as unsafe and potential sources for outbreaks. On the immunological level, analysis of infected blood samples revealed rapid depletion of CD4+ T helper cells. (While healthy adult cell counts can vary between 500-1,200 cells/mm³, an individual with AIDS can steadily decline below 200 cells/mm³, significantly increasing their
vulnerability to opportunistic pathogens) [8]. This revelation supported the ongoing hypothesis that a virus was directly responsible for AIDS, as this timeline coincided with the isolation of a novel retrovirus, Human T-lymphotropic Virus (HTLV) [9]. Consequently, a race for the unknown virus emerged between investigators Robert Gallo (credited with discovery of HTLV), Luc Montagnier, and Francoise Barre-Sinoussi. The rivalry between laboratories ultimately led to successful isolation of a virus termed HTLV-III/LAV (Gallo and Montagnier respectively) in 1983, which was appropriately renamed HIV in 1986 [10, 11].

Since the beginning of the pandemic, an estimated 39 million people with AIDS have died worldwide. 35 million people currently remain infected, with roughly 2 million new cases and an additional 1.5 million deaths each year. Though a significant portion of HIV/AIDS history has stemmed out of the United States, Sub-Saharan Africa continues to bear the largest burden of illness with over two thirds of all infections on the planet [12].

### 1.2 Diversity of HIV

HIV is classified within the *Lentivirus* genus, a part of the major family of enveloped viruses *Retroviridae*. Lentivirus (LV) infections are chronic, persistent, and carry long asymptomatic phases in a variety of mammalian species (most commonly ungulates, felines, or primates). Primate LVs specifically possess the ability to bind and infect cells by use of CD4 cell surface receptor (see 1.4.1 for
Among primate species, lentiviruses have diverged significantly over the course of evolution to produce a heterogeneous population of viruses with varying pathogenicity. For example, the Simian Immunodeficiency Viruses (SIVs) infect a wide range of non-human primate (NHP) species and are believed to be the origin of the HIVs [13]. Analysis of several NHPs revealed that infection of select species such as African Green Monkeys (AGM) by SIV<sub>AGM</sub> results in a non-pathogenic disease state presenting markedly less immune activation and disease progression [14]. Conversely, SIV infection in Rhesus Macaque species is pathogenic and mirrors the AIDS progression seen in humans [15]. Dissecting the underlying differences between SIVs may shed light on related HIV pathogenesis mechanisms.

Within the human population, genetic variability of lentiviruses exists in the form of HIV-1 and HIV-2. Phylogenetically, HIV-1 is more closely linked to SIV<sub>cpz</sub> while HIV-2 is related to SIV<sub>smm</sub>. Both viruses share basic gene structure with the exception of accessory proteins vpr/vpx. HIV-1 and HIV-2 follow the same transmission routes, albeit with significant differences in their infectivity and time to progression of AIDS. HIV-2 incidence is markedly lower and as such the social vernacular most often refers to that of HIV-1.

A greater understanding of HIV-1 at the nucleotide level has allowed for its sub classification into four groups, M, N, O, and P, with each originating from a distinct cross-species transmission event. Minor groups N, O, and P encompass less than 5% of the total number of infections and are localized to
individuals from West Africa [16]. The pandemic group M viruses account for the majority of infections worldwide and are fractionated again by subtype or clade: A,B,C,D,E,F,G,H,I,J, and K (In addition, viral recombination has led to accumulation of numerous circulating recombinant forms {CRFs}). Dramatic geographic prevalence exists across subtypes as seen in Figure 1.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Geographic Region</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Central &amp; East Africa, Eastern Europe</td>
<td>12%</td>
</tr>
<tr>
<td>B</td>
<td>Americas, Europe, Southeast Asia</td>
<td>10%</td>
</tr>
<tr>
<td>C</td>
<td>Sub-Saharan Africa, India</td>
<td>52%</td>
</tr>
<tr>
<td>D</td>
<td>North Africa</td>
<td>3.6%</td>
</tr>
<tr>
<td>F</td>
<td>South Asia</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>G</td>
<td>West &amp; Central Africa</td>
<td>5%</td>
</tr>
<tr>
<td>H, J, K</td>
<td>Middle East &amp; Africa</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>CRFs</td>
<td>----------------------------------</td>
<td>14%</td>
</tr>
</tbody>
</table>

*Global subtype frequency data from 2007

**Figure 1: Group M Virus Distribution.** Group M viruses encompass more than 90% of total worldwide HIV infections. Within group M, considerable subtype variation exists across geographic regions. Subtype or clade B viruses are highly endemic across the Americas. Similarly, the majority of infections in African regions are a product of clade C viruses. This is reflected in the global frequency, as the incidence of HIV/AIDS in Africa comprises nearly two-thirds of all worldwide events. Circulating Recombinant Forms (CRFs) of HIV are progeny of multi-clade recombination events and as such, exist with a high degree of heterogeneity.[16]
Phylogenetic differences between group M clades can be mostly attributed to the vast diversity of viruses generated at both an individual and a population level. In an individual infected with a single virus particle, heterogeneity of resultant progeny viruses is rampant for several reasons: 1) HIV reverse transcriptase is a highly error prone enzyme when "scanning" viral RNA [17]. It is estimated to introduce a mutation 1/1700 nucleotides; 2) Host restriction factors such as APOBEC3G directly act on viral sequences to generate hypermutations (in this case, hypermutation more often results in nonfunctional viruses); 3) Anti-retroviral drugs and immune system pressure drive evolutionary selection causing resistant mutants to arise over time. This is especially problematic during antiretroviral treatment interruptions or lack of adherence [18]; 4) viral recombination can occur by template switching across the diploid genome during reverse transcription, or by co-infection of two genetically distinct viruses within the same cell [19]; 5) Mutations in the envelope region can give rise to viral tropism switch, in which incoming viruses recognize the additional co-receptor CXCR4, as opposed to CCR5 [20, 21]. Taken together, these mechanisms set the stage for insurmountable genetic variation on the individual level. It should be noted that significant accumulation of mutations impose detrimental defects or fitness costs to a large proportion of progeny virions. This helps to explain why only a small number of total viruses are infectious and transmissible.
Functional viral variants are disseminated at the population level, generating greater complexity in HIV-1 diversity. Conversely, the inefficiency of mucosal HIV-1 transmission, approximately 1/200 - 1/2000 exposures, coupled with reports that a single founder virus is linked to the transmission event, act as a bottleneck to limit the gross expansion of the HIV-1 genetic repertoire [22, 23]. Additionally, viruses harboring tropism to CXCR4 co-receptor have shown poor transmissibility at mucosal sites. Studies analyzing transmitted/founder strains of HIV-1 have suggested that features such as envelope structure and the degree of glycosylation may be responsible for these differences, along with the density of co-receptor on targeted mucosal cells.

1.3 Structure of HIV

1.3.1 - Genomic Organization

HIV-1 and all other retroviruses share several underlying characteristics including a single stranded positive sense RNA genome. Flanking the ends of the ~9.8 kilobase (kb) HIV genome are 640 basepair (bp) untranslated regions of RNA, designated Long Terminal Repeats (LTRs, Figure 2). Each HIV LTR plays a critical role during viral replication. First, the U5 sub region of the 5' LTR contains the primer-binding site (PBS) essential for initiation of reverse transcription [24]. Downstream of successful RT events, both LTRs are required for insertion of viral DNA (integration) into the host genome. Subsequently, the
integrated 5' LTR includes transcription factor binding elements and acts as the promoter for viral transcription [25, 26]. At the 3' end of integrated DNA, HIV Nef protein translation terminates within the 3' LTR while the remaining downstream region provide polyadenylation sites for the nascent mRNAs. HIV LTRs contain dimerization signals critical for assembly of progeny virions (along with the RNA packaging signal psi downstream of the 5' LTR U5 element) [27].

Along with critical the LTR regions, retroviruses share expression of three structural genes: \textit{gag}, \textit{pol}, and \textit{env}. The HIV-1 \textit{gag} gene encodes for a 55 kilodalton (kDa) polyprotein that is packaged into newly forming particles and undergoes proteolytic cleavage during the final viral maturation step [28]. However, prior to gag cleavage events, full-length polyprotein directs the virion assembly at the cell membrane and subsequently multimerizes to shape the budding particle [29, 30]. Released virions containing functional protease (PR) enzyme will cleave gag and gag-pol polypeptides into the structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC). This final processing of gag polyprotein is critical for HIV-1 maturation and drastically influences the virion's downstream infectivity [31, 32].
Figure 2: HIV-1 Genomic Organization. The HIV genome encodes for 9 genes and a total of 15 proteins. Flanking both ends of the viral genome are 5' and 3' long terminal repeats (LTRs) which act as promoter and polyadenylation sites, respectively. Gag, pol, and env encode for structural components of the virion as well as the enzymatic machinery responsible for reverse transcription, integration, and maturation/polyprotein cleavage. HIV also contains two regulatory genes tat and rev, which are spliced from separate reading frames following transcription. Accessory genes vif, vpr, vpu, and nef, though "non-essential" for replication, play a significant role in regulating overall infection efficiency.

The distal region of the HIV-1 gag gene contains two small cis-acting motifs that give rise to an even larger polyprotein, gag-pol. In short, the slip site motif and nearby secondary RNA structure generate a -1 nucleotide (nt) ribosomal frame shift ~5% of the time, allowing for transcription of the downstream pol gene [33]. HIV pol encodes for the enzymatic machinery absolutely required for replication, and includes the protease (PR), reverse transcriptase (RT), RT subunit Ribonuclease H (RNase H), and integrase (IN) enzymes. As is seen with gag polyprotein, the gag-pol polyprotein multimerizes in newly forming virions and is also cleaved during maturation.
The final shared gene, env, encodes for an additional structural component of the HIV virion, glycoprotein 160 (gp160). This precursor protein is initially expressed in full length and subsequently processed by host cell furin proteases to yield two glycoproteins, gp41 and gp 120 [34, 35]. Both gp41 and gp120 will form complexes on the surface of released HIV particles and mediate host cell attachment and viral fusion (See section 1.4.1).

In addition to the structural genes gag, pol, and env, HIV encodes for several regulatory and accessory genes. The HIV regulatory genes tat and rev are products of split exons and are once again required for successful viral replication. Accessory genes vif, vpr, vpu, and nef are classified as non-structural. Despite accessory genes being considered "non-essential" for viral replication, their conservation over the course of HIV evolution suggest an important role in vivo [36]. Furthermore, the diminished infection efficiency associated with vpr and vpu knockouts point to their significance in regulating cellular susceptibility to HIV [37-39].

1.3.2 - Protein Function

a) Matrix (MA / p17) - Matrix structural protein, derived from 55 kDa gag polyprotein, functions as a stabilizing lattice within the virion [40]. The majority of MA is bound directly to the virion lipid bilayer through electrostatic interactions with negatively charged phospholipids. Furthermore, this interaction is mediated by the post-translational addition of a myristoyl group on the N-terminal domain
of the protein [29, 41, 42]. Following viral fusion with the target cell, a fraction of MA protein bound to the HIV pre-integration complex (PIC) facilitates nuclear import by means of embedded nuclear localization signals (NLS).

b) Capsid (CA / p24) - Capsid protein forms the conical coat that shields the diploid HIV genome. Within virions, capsid cones exist with significant heterogeneity due to a flexible linker region in their secondary structure. Recent studies of CA function have also determined significant roles during uncoating, reverse transcription, and nuclear import pathway selection [43-45].

c) Nucleocapsid (NC / p7) - Beneath the capsid cone, nucleocapsid acts as an additional layer of protection for the viral genome. NC proteins anneal with the single stranded RNA, blocking the digestion of nucleic acids by host nuclease enzymes. Additionally, during the process of reverse transcription, NC facilitates the priming of tRNAs and the strand transfer of minus strand strong stop cDNA [46, 47].

d) SP1, SP2, and p6 - Proteolytic cleavage of the gag polyprotein results in three additional small peptides dubbed spacer 1 (SP1), spacer 2 (SP2), and p6. Though little is known regarding these peptides, deletions of the SP1 domain result in deleterious effects on particle assembly [48, 49]. P6 has been shown to play an important role in viral budding via interactions with the ESCRT-III and Alix machinery.

e) Protease (PR) - As discussed previously, the successful cleavage of gag and gag-pol polyproteins requires the presence of functional PR enzyme. The
mechanism behind initial PR cleavage remains poorly understood but point to the requirement for dimerization between subunits to gain functionality [50, 51]. Successful processing of PR results in the sequential cleavage of the remaining polyproteins. Due to the requirement for this enzyme during HIV maturation, pharmacological inhibition of PR has been a primary drug target in clinical settings.

f) Reverse Transcriptase (RT) - HIV reverse transcriptase is a heterodimeric enzyme possessing both RNA-dependent and DNA-dependent polymerase activity. Following viral fusion and uncoating, RT facilitates synthesis of double stranded viral DNA within the Reverse Transcription Complex (RTC). Reverse transcription has recently been causally linked to uncoating and trafficking in the cytoplasm [44, 45, 52].

g) Ribonuclease H (RNase H) - RNase H exists as a domain within the heterodimeric RT enzyme. Primary function of RNase H includes degradation of the viral RNA template following synthesis of the DNA/RNA hybrid during reverse transcription.

h) Integrase (IN) - Integrase enzyme facilitates the insertion of double stranded proviral DNA into the host genome of the infected target cell. Following reverse transcription, viral IN complexes with viral DNA, MA, Vpr, and host proteins to form the Pre-Integration Complex (PIC). This PIC is then shuttled into the nucleus in preparation for integration. Importantly, IN enzyme contains
endonuclease, exonuclease, and ligase activities which each play a critical role in this stage of the replication cycle.

i) Gp41/gp120 - As mentioned previously, translation of conserved env gene and successful cleavage of the full-length gp160 polyprotein by furins yields two glycoproteins, gp41 and gp120. Each glycoprotein interacts non-covalently to form a "spike" of three gp120 and three gp41 molecules on the surface of virions. Fully assembled spikes or trimers bind to cell surface CD4 and co-receptor to initiate viral fusion.

j) Transactivator of Transcription (Tat) - Tat protein is a major regulator of HIV transcription. Being expressed as an early gene product, Tat is shuttled back into the nucleus to interact at the 5' LTR region of integrated HIV DNA. Following initiation of viral transcription, pausing of RNA polymerase II enzyme halts transcript production [53]. Elongation of the short nascent RNA requires the interaction between Tat protein and the embedded TAR RNA element.[54] Subsequent recruitment of Tat-associated kinase (also known as p-TEFb, Cyclin T1/CDK9) to Tat/TAR restores RNAPII processivity, which is mediated by hyperphosphorylation of its c-terminal domain [55]. If basal LTR promoter activity is too low to allow for sufficient Tat production, transcription is terminated and a latent proviral state will predominate.

k) Regulator of expression of virion proteins (Rev) - Rev acts as a second regulatory element during viral replication downstream of proviral transcription. Successful transcription of integrated HIV DNA results in a variety of mRNA
species as a result of extensive alternative splicing. The larger, late gene products containing \( nv \) coding region, specifically a 350 nt segment called the Rev responsive element (RRE), require Rev protein for chaperoned nuclear export [56]. Rev bound mRNAs form a complex with CRM1 and RNA helicase DDX3 to exit the nucleus in a manner dependent on GTP hydrolysis [57-59].

l) Viral infectivity factor (Vif) - Vif accessory protein has evolved a critical function to maintain infectious particle formation. During viral assembly, host cell APOBEC3G proteins can become encapsulated into budding virions resulting in dramatic hypermutation during downstream reverse transcription. However, Vif production in infected cells prompts formation of the Vif-ElonginB/C-Cullin5 ubiquitin ligase complex to degrade APOBEC3G and prevent its incorporation into virions [60-62].

m) Viral protein R (Vpr) - HIV-1 Vpr is a highly conserved protein in the lentivirus family and acts to potently block G2 to M cell cycle transition [63, 64]. The mechanism of action behind the cell cycle block involves critical interactions between Vpr, structure-specific endonuclease regulator SLX4, and the DDB1/Cul4-associated factor DCAF1 [65]. Cell cycle arrest has been shown to elevate HIV-1 gene expression, as viral LTRs are most active during this stage [66]. Vpr is also found in pre-integration complexes suggesting a role in nuclear import of HIV DNA [67]. Surprisingly, knockout of Vpr is well tolerated in CD4+ T cell infection as viral replication can still occur. This suggests a non-essential support role in this particular cell type. Conversely, a study just published this
year by Collins et al. reports that Vpr may play a more critical role in permitting infection of monocyte/macrophage lineages [68]. Their findings implicate Vpr in preventing lysosomal targeting of virus particles.

A unique property of Vpr is its ability to tolerate the addition of fusion proteins to either its N or C termini. This finding has stemmed the development of quantitative assays to detect HIV entry into cells [69-71]. It is important to note that during virion assembly, Vpr is packaged within capsid cones suggesting its detection within cells may act as a surrogate for successful uncoating following viral fusion [72].

n) Viral protein U (Vpu) - To replicate efficiently, viruses must subvert a number of host restriction factors that can directly derail the steps of replication. One such antiviral host factor BST-2/Tetherin prevents release of assembled viral particles from the host cell. To counteract this mechanism, HIV-1 group M viruses have adapted their own accessory protein Vpu to prevent tetherin function [73]. Interestingly, Vpu from HIV-1 group O and P have not yet acquired this property [74, 75].

In addition to antagonism of Tetherin, Vpu can also prevent newly synthesized CD4 protein from being trafficked to the lipid membrane [76]. Downmodulation of chemokine receptor CCR7 has also been recently shown to occur by a Vpu-mediated mechanism [77]. Loss of chemoattractance by CCR7 will decrease the homing of T-cells to peripheral lymphoid organs and can impair the resultant immune response.
o) Negative factor (Nef) - The final HIV-1 accessory protein Nef has a wide variety of functions. Most notably it is known for its ability to downregulate CD4 surface protein [78]. Numerous groups have also identified a role for Nef in downmodulating major histocompatibility complex II (MHC-II) expression on the surface of cells [79]. Lack of MHC-II will stunt antigen presentation and decrease the adaptive immune response to a pathogen or antigen. To prevent killing by cytotoxic T cells, Nef can also induce the Fas/Fas-Ligand pathway triggering apoptosis of bystander cells [80]. Nef additionally downregulates T cell receptor (TCR) and inhibits TCR-mediated activation [81].

1.3.3 - Virion Architecture

Mature HIV-1 particles vary greatly in size, with an average 120 nm diameter. (Figure 3) Each virion carries two copies of single stranded genomic RNA surrounded by a protective layer of nucleocapsid protein. Along with genomic RNA and nucleocapsid, reverse transcriptase, integrase, and several accessory proteins are packaged within capsid cones inside the particle.
Figure 3: Structure of the HIV-1 Virion. Spherical, enveloped HIV particles are roughly 120 nm in diameter and express 8-16 envelope trimers on their lipid surface. Successful maturation of budding particles cleaves the highly ordered gag and gag-pol polyproteins, allowing for rearrangement to occur forming matrix (MA), capsid (CA), and nucleocapsid (NC) layers. Beneath the capsid cone, enzymatic machinery and accessory proteins surround the NC wrapped RNAs.

The conical shape formed by capsid is only achieved by the incorporation of 12 CA pentamers at crucial sites in the largely hexamer oriented CA lattice [82]. An additional layer of matrix protein, which is anchored into the lipid bilayer by post-translational myristoyl groups, then surrounds the capsid cone. On the outer region of the virion, ~8-16 envelope trimers rest on the surface.
This relatively low number of surface spikes may have evolved to subvert antibody binding and neutralization by the host immune response.

### 1.4 HIV Replication Cycle

Generation of progeny viruses is only successful following completion of a number of finely orchestrated cellular steps including viral attachment and membrane fusion, capsid uncoating and reverse transcription, nuclear import, genomic integration, transcription, translation, assembly, and maturation. Over the past three decades, each step in the replication cycle has received significant attention with the goal of designing strategies to subvert infection (direct antiretrovirals will be discussed in section 1.7.1). An overview of viral replication is visualized in Figure 4. Each step in this process will be discussed in detail below:

### 1.4.1 Viral Attachment and Entry

HIV replication begins with the binding of viral gp120 to cell surface CD4 receptor [83]. This binding event triggers a rearrangement in gp120 protein structure to allow for secondary binding to chemokine receptor, either CCR5 or CXCR4 [84, 85]. The specificity for which co-receptor is used along with the cell type primarily affected first led to the naming classification of M-tropic (binding
CCR5 and entering macrophage cell types) versus T-tropic (binding CXCR4 and entering T cells.) It is important to note the M- versus T- distinction is somewhat a misnomer on the basis that M-tropic viruses can also infect T cells. Furthermore, increasing evidence for "non-M-tropic" CCR5 utilizing viruses that poorly infect macrophages complicate this dated bi-modal naming scheme.

Following the initial steps of HIV attachment to CD4 and co-receptor, a second confirmation change occurs in the envelope triggering the insertion of the gp41 N-terminal region (fusion peptide) into the target cell membrane. Upstream of the transmembrane domain of gp41, coiled-coil heptad repeat sequences (HR1 and HR2) compress onto one another to form a highly stable structure known as the six helix bundle [86]. Successful bundle formation pulls the attached virion and host membrane together allowing for mixing of phospholipid layers and subsequent delivery of viral components into the cytoplasmic space.

1.4.2 - Capsid Uncoating, Trafficking, and Reverse Transcription

Following membrane fusion, viral components including capsid cores are released into the cytoplasm. The subsequent trafficking of viral components does not occur simply by diffusion but rather a finely tuned cytoskeletal network.
Figure 4: The Viral Replication Cycle. Successful replication of HIV-1 requires a number of well-coordinated cellular events beginning with host cell attachment and entry. Uncoating and reverse transcription of viral RNA occur during cytoplasmic trafficking along the cytoskeleton. Following (next page)
genomic integration, transcription, and protein synthesis, assembled viruses are released and undergo a final step of maturation to liberate structural and enzymatic machinery inside the particle.

Indeed, many pathogens including HIV require the use of these networks for transport [87]. After entry, HIV first encounters a densely packed network of cortical actin filaments. Crosslinking between filaments creates a barrier that is thought to be a first line of defense from incoming pathogens. However, viruses such as HIV may have prevented strategies to subvert this process. Several reports suggest that critical CD4 and co-receptor signaling events may restructure the cytoplasm by altering cytoskeletal protein expression [88]. For example, Yoder et al. provide evidence for cofilin-mediated rearrangement of actin to permit entry [89].

Beyond the short distance actin transport, McDonald et al. has demonstrated that HIV traffics along microtubule filaments to reach centromeres and associated nuclear space [90]. Others report a critical role for microtubule motor proteins dynein/kinesin, as well as additional interactions between HIV CA and adaptor protein FEZ1 [91-93]. More recently, Sabo et al. provided first evidence that HIV directly induces the formation of stable, detyrosinated, and acetylated microtubules to promote viral replication [94, 95]. These post-translationally modified microtubules are thought to be more stable, resistant to depolymerization, and may provide efficient highways for trafficking of viral complexes within a cell. This relationship between HIV, actin, and microtubule networks seems negatively regulated in part by the cytoskeletal proteins ezrin,
moesin, and end-binding protein 1 [96, 97]. Though these findings shed new light on the importance of the cytoskeleton in HIV infection, the exact relationships between cell signaling, cytoskeleton reorganization, and microtubule stability during viral replication still needs to be explored.

Prior to entry into the nucleus, higher order capsid structure must be dissociated to allow for access to genomic material, enzymes, and accessory proteins. There are several proposed models to explain how this may occur [44]. The first model proposes that the loss of capsid concentration in the cytoplasm (in contrast to the tightly packed virion space) induces the collapse of the ordered structure. This idea is supported by the abundant amounts of free CA in virus particles suggesting a "saturated" supply for core formation. A second model suggests that capsid cores remain largely intact until the initiation of nuclear import, in efforts to promote the close interaction between reverse transcriptase enzyme and the RNA template.

The final model, and one which seems increasingly favorable, proposes that capsid uncoating occurs kinetically throughout the course of cytoplasmic trafficking along the cytoskeleton. This model has been supported by multiple findings. Firstly, the identification of various sized HIV complexes within the cytoplasm suggests a rate of capsid dissociation over time. Additionally, introducing point mutations that render capsid structures less stable impair the efficiency of viral replication [98]. More recently, Lukic et al. identified a relationship between dynein motor transport, microtubule stability, and the rate
of HIV-1 uncoating [92]. This study was one of several to establish novel assays that seek to measure uncoating directly [99-101]. It is likely that additional host proteins influence the kinetics of this process. Given the identification that cytosolic DNA sensors cGAS and IFI16 can sense viral nucleic acids, it is probable that uncoating may have adapted as a mechanism to protect viral material from innate immune sensing [102, 103].

Reverse transcription is also closely coupled with cytoplasmic trafficking and uncoating. The conversion of single stranded genomic RNA to double stranded DNA is mediated by the viral reverse transcriptase (RT). This enzyme has several key features including RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and RNase H activities. The RNase H function is a crucial component as it degrades RNA templates within a DNA-RNA complex. RT is first initiated by priming of the RNA genome with host cell tRNA in a region of the 5' LTR known as the primer binding site (PBS, Figure 5). RNA dependent DNA polymerase drives the generation of DNA towards the direction of the 5' end. As new DNA is synthesized, RNase H degrades the RNA template strand into smaller fragments. This short region of newly synthesized DNA ranging from PBS to 5' cap is commonly referred to as minus strand strong stop DNA. Due to the homology in the R regions of both 5' and 3' LTRs, the (-) ssDNA undergoes a first strand displacement, continuing DNA polymerase activity from the 3' end.
Along the RNA template exists a region called the polypurine tract. This purine-dense sequence will not undergo RNase H digestion and as such remain bound in a DNA-RNA complex. The PPT is critical as a priming site for plus strand DNA synthesis by the DDDP. Successful generation of (+) ssDNA will result in a second strand displacement event, where (+)ssDNA meets the 3' end of previously formed (-)ssDNA. Second strand transfer allows for completion of double stranded DNA synthesis. During this process, the viral nucleic acid and enzymatic machinery is referred to as the reverse transcription complex (RTC). Upon completion of RT, the complex is dubbed the pre-integration complex (PIC) and undergoes nuclear import. The exact transition between a RTC and PIC is not thoroughly defined, nor are the extent of proteomic changes between each complex.

1.4.3 - Nuclear Import and Genomic Integration

Fully reverse transcribed HIV DNA reaches the nucleus through interaction between the PIC and nuclear pore complexes (NPCs). NPCs span the nuclear envelope and consist of a 5.2 nanometer channel made up of nucleoporin proteins. Over 30 proteins make up each channel, which contains distinct domains such as the outer ring, spokes, and inner basket. In addition to the pore itself, cytoplasmic cargo relies on the use of chaperone proteins known as
**Figure 5: Process of HIV Reverse Transcription.** HIV reverse transcription is initiated by priming of a tRNA molecule to the PBS present in the 5' viral LTR. RDDP activity of reverse transcriptase mediates synthesis of minus strand strong stop DNA. Following strand displacement to the 3' LTR, minus strand synthesis continues while RNase H degrades the template RNA. Plus strand strong stop synthesis is primed at the undigested polypurine regions and towards the tRNA along the cDNA template. Following second strand displacement, plus strand strong stop DNA synthesis continues to product a full double stranded HIV genome suitable for host integration.

importins to traverse the nucleus. The Ran GTPase mediates the binding and release between importins and their cargo. More specifically, the directionality of nuclear translocation is controlled by concentration of Ran between the nucleus and cytoplasm, as well as its co-factors RanGEF and RanGAP that mediate the exchange of GTP.

Several HIV proteins have been implicated in regulating nuclear import (Figure 6A). Namely, embedded nuclear localization signals have been identified in MA, IN, NC and Vpr. This confers ability to bind to the karyopherin/importin family, which act as chaperones through the nuclear pore itself. A non-classical pathway for import has also been suggested by which HIV proteins can bind directly to nuclear pore proteins (Nups) and bypass the need for importin adaptors. In addition, several distinct pathways have been reported during HIV infection including those that use Nup153, Nup358, or TNPO3 to mediate import. Unexpectedly, the selectively for each pathway revolves around HIV CA interactions and its amino acid structure [104]. For instance, it has been
reported that CA directly binds to the cyclophilin domains of both Nup153 and 358 [105]. Additionally, CA binds cyclophilin A (CypA) in the cytoplasm. Upon inhibition of CypA by cyclosporine, HIV PICs show less reliance on Nup153 and Nup358 during import. Conversely, CypA binding capsid mutants G89V and P90A subvert this effect. The CA mutant N74D CA has been shown to prevent binding to a negative import regulator CPSF6 [106]. Additional mutants R132K and L136M have been identified in elite controller populations (those possessing HLA-B27 alleles) that do not use TNPO3, Nup153, or Nup358 for import [107]. The apparent complexity of nuclear import during viral replication does not go unnoticed.

The number of proteins regulating nuclear import extends far beyond those mentioned in this short review. Furthermore, the discoveries that CA is involved in uncoating and nuclear import are very recent, and in fact, may be closely linked. Evidence for this exists in the finding that MX2 restriction factor antagonizes HIV uncoating yet also possesses the ability to bind CypA [108, 109].

Prior to nuclear import, 3' ends of double stranded HIV DNA must first be processed by dimerized IN enzyme. This reaction cleaves GT dinucleotides and generates what is referred to as 3' hydroxyl ends (Figure 6B). Following nuclear import, IN catalyzes similar strand processing on genomic DNA with the aid of LEDGF/p75 chromatin binding protein to bridge the two nucleic acid species. The interaction with LEDGF is thought to be critical to integration, as
depletion markedly reduces both overall integration and integration within active transcription units [110, 111]. Non-homologous 5' ends of viral DNA are further removed by HIV IN and re-synthesized by host machinery to complement the genomic overhangs. DNA ligase enzymes complete this process to form fully integrated proviral DNA.

Proviruses entering the nucleus can undergo additional unintegrated fates through the process of autointegration (Figure 6B) [112]. Through homologous recombination within conserved LTR regions, viral DNA can attack and self-ligate yielding 1-LTR circle products. To a lesser extent, autointegration can occur onto pre-existing LTR circle products resulting in truncated or extended genomes. Most significantly, however, viral DNA can be ligated to form 2-LTR circles as a product of non-homologous end joining. This product exists at high abundance and is used as a marker for the overall level of nuclear import of HIV DNA.[113] Furthermore, the abundance of 2-LTR circles has been shown to dramatically increase during ART (in the presence of an integrase inhibitor) [114]. Though viral transcription requires integrated copies of proviral DNA, it has been shown to an extent that 2-LTR circles can successfully integrate upon removal of Integrase inhibitor treatment [115]. This raises new concern for scheduled ART interruption, as cells in pre-integration latency could potentially serve as a source to reseed the viral reservoir.
Figure 6: Nuclear Import and Integration. The process of A) nuclear import requires the cooperation between viral preintegration complexes (PICs) and nuclear pore complexes (NPCs). This interaction is first mediated through viral nuclear localization signals (NLS) and Importin family proteins. HIV cargo is shuttled through nuclear channels consisting of a variety of host proteins, which have been separately identified as regulators of HIV nuclear import. The selectivity for proteins is in part attributed to viral CA protein and its interaction with cytoplasmic cyclophilin A (CypA). B) Integration of double stranded DNA requires cleavage of viral and genomic DNA strands. Successful (next page)
integration culminates with strand ligation and synthesis of complimentary DNA spanning strand gaps. "Dead-end" integration products can also result in formation of 1-LTR and 2-LTR circles within the nucleus.

1.4.4 - Viral Transcription and Nuclear Export

After genomic integration, HIV-1 proviruses undergo active transcription or remain transcriptionally silent (referred to as post-integration latency, to be discussed in section 1.6). The regulation of transcription is controlled by a number of mechanisms proximate to the 5' LTR promoter region [116]. HIV promoters contain abundant transcription factor-binding elements (notable in the list are a TATA box, SP1 binding sites, and NF-kB/NFAT enhancer motifs). Recruitment of transcription machinery to integrated proviruses occurs at the LTR but is assisted by a critical player: viral Tat. Surprisingly, Tat transactivation was found not to occur on proviral DNA but rather a downstream secondary RNA structure that is generated shortly after initiation of transcription [54].

The transactivation-responsive region (TAR) of RNA interacts directly with Tat, culminating in the recruitment of the positive-transcriptional elongation factor b complex (P-TEFb) [117]. The P-TEFb complex, consisting of cyclin T1 and CDK9, mediate the phosphorylation of stalled RNA polymerase II. Multiple phosphorylation events on the c-terminal domain relieve the DSIF/NELF pausing complex [118]. Loss of negative elongation factors dramatically increases RNA polymerase II processivity, allowing for transcription of the nascent RNA to continue. The importance of Tat during HIV transcription should not be
understated. By itself, HIV promoters are marginally active and basal transcription is low. It is believed that low-level transcription must accumulate sufficient Tat to trigger transactivation and boost promoter activity.

Tat RNA transcripts are only one of many generated during HIV transcription. Full length HIV RNA contain multiple splice donor and acceptor sites (as seen in Figure 7) that act in consort with cellular spliceosome machinery to yield three major RNA species: Fully spliced 1.8kb RNA transcripts, partially spliced 4kb transcripts, and full length unspliced transcripts [119]. Fully spliced transcripts primarily corresponding to Tat and Rev are produced first in the process and can freely export the nucleus for translation. The early translation of Tat and its re-entry into the nucleus promotes high-level transcription.

Alongside Tat, nuclear re-entry of Rev is also required to assist longer RNA transcripts in export [56]. Both partially spliced and unspliced HIV RNAs do not freely leave the nucleus and instead rely on Rev protein binding to an RNA region referred to as the Rev-responsive element (RRE). The RRE is present in the gp120 region of env and serves as a docking site for multiple rev proteins to bind. Initial binding of Rev to RRE induces conformational changes in secondary structure and promotes oligomerization of additional Rev proteins. Rev bound RNAs can directly interact with nuclear receptor Exportin1/CRM1 and exit the nucleus in a Ran GTPase mediated fashion [59]. Once exported, hydrolysis of GTP destabilizes Rev interactions and releases bound RNAs.
Figure 7: HIV Alternative Splicing. Following transcription, HIV mRNAs undergo extensive alternative splicing events to generate unspliced genomic RNA, partially spliced 4 kb RNAs, and short 1.8 kb transcripts. Splice donor and acceptor sites regulating this process are highlighted above. Fully spliced 1.8 kb transcripts corresponding to tat, rev, nef, and vpr are the first to be exported from nuclear space. The export of partially spliced and genomic RNAs requires chaperoning by HIV rev protein, which re-enters the nucleus and binds the rev-responsive element (RRE) located within the env region of RNAs.

1.4.5 - Virion Assembly, Budding, and Maturation

Export of partially and completely unspliced RNAs from the nucleus allow for translation of gag and gag-pol polyproteins. Both polyproteins are directed towards the plasma membrane by the post-translationally modified MA domain embedded in gag [120]. Myristoylated MA interacts with negatively charged
phospholipids to anchor polyproteins to the lipid bilayer. Interactions between NC domains in gag seem to play an important role in the oligomerization of multiple polyproteins [121]. Furthermore, interactions between packaging signal psi (Ψ) and NC ensure incorporation of full-length diploid genomes [122].

Coinciding with transport of Gag and Gag-pol, envelope protein gp160 undergoes extensive glycosylation modifications and cleavage by cellular furin proteases to liberate gp120 and gp41 proteins [34, 123]. Both envelope proteins are delivered to the plasma membrane where their localization is associated with lipid raft structures.

The targeting of the Gag, Gag-pol, and genomic RNA to areas of envelope expression are not entirely understood. Evidence for gp41 cytoplasmic motifs interacting with MA has been reported despite an unknown mechanism [124]. Host factors such as mannose-6-phosphate receptor binding protein 1 can bind both gp41 and MA and have been shown to be critical for virus production in macrophages. This evidence has been debated in other cell types and models of infection [125, 126].

HIV Gag hijacks the cellular ESCRT (endosomal sorting complexes required for transport) pathway to assist in virion budding [127]. Late domain motifs present in the p6 region of Gag first bind TSG101 and ALIX present in the ESCRT complex. The accumulation of TSG101 and ALIX at the budding particle leads to recruitment of CHMP family proteins and the VPS4 ATPase. Assembly of
a helical CHMP structure at the membrane coupled with VPS4 mediated ATP hydrolysis mediate the final release of virus particles.

Newly released HIV particles are immature (Figure 4) and require a series of 12 protease cleavage events in Gag/Gag-pol to liberate structural and enzymatic proteins within the virion. This process is highly specific and dependent upon a small segment of amino acids recognized by Protease [128]. This specificity seems to be largely dependent on the secondary structure, allowing for a variety of sites to undergo cleavage. Failure to complete maturation is detrimental to the particle and results in a marked decrease in infectivity.

1.5 Host and Virus Arms Race

The human immune system has encountered viral infections over the course of evolution [129, 130]. As a result, both the virus and the host have adapted the means to either promote or prevent infection. From the host perspective, an antiviral protein or restriction factor that has gained function to directly subvert pathogens must have several defining characteristics: 1) The protein must directly reduce viral infection; 2) a potent restriction factor will have likely caused a counteraction by the virus to produce its own offensive protein; 3) Restriction factors will show evidence for positive selection, an evolutionary pressure by which an accumulation of non-synonymous mutations is larger than
that of synonymous mutations. More often, acquisition of non-synonymous mutations reflects adaptations for host proteins to subvert viral offensives. Key examples of HIV restriction factors include:

a) **SAMHD1** - Identification of the SAMHD1 as a viral restriction factor spawned from the findings that an HIV-2 protein, Vpx, dramatically improved HIV-1 replication in myeloid lineage cells [131, 132]. Though susceptible to viral fusion, these cells control downstream productive infection to a higher extent than CD4+ T cells. The protein responsible was found to be dNTP phosphohydrolase SAMHD1. Physiologically, SAMHD1 minimizes the reservoir of dNTP pools in non-dividing cells. As a result, accessibility to dNTPs during viral reverse transcription is limited and viral replication is diminished. There is increasing evidence that the expression of SAMHD1 is interferon inducible and may be linked to the HIV immune response [133]. Though absent in HIV-1, HIV-2 Vpx has gained function to recruit the E3 ubiquitin ligase complex which promotes proteasomal degradation of SAMHD1. More interestingly, non-human lentiviral Vpr can antagonize SAMHD1 suggesting an ongoing evolution between Vpr, Vpx, and SAMHD1 [134].

b) **Tetherin** - BST-2/Tetherin acts a late stage restriction factor that limit the release of progeny virions [73]. It's discovery as a restriction factor stemmed from the observation that Vpu (-) viruses were permissive in HEK293 and COS-7 cell lines yet replication deficient in HeLa and primary CD4+ T cells. Further investigation uncovered an interferon-inducible transmembrane glycoprotein
dubbed BST-2/Tetherin. Tetherin localizes to the plasma membrane of infected cells and anchors transmembrane domains into the lipid bilayer of budding particles. Resulting Tetherin bound viruses remained tethered to the membrane and are inhibited in protease-mediated maturation. Several lines of evidence exist for internalization and degradation of tetherin bound particles by a mechanism involving interactions with BCA2 proteins [135].

To counteract Tetherin function, HIV has adapted tetherin antagonism by way of Vpu. Vpu triggers downregulation of surface Tetherin and relocalization to the trans-golgi network due to interactions between transmembrane domains [136]. Subsequent interactions in the Vpu c-terminal domain are required for Tetherin sequestration [137]. It should be noted that while Vpu/Tetherin interactions have been extensively studied for HIV-1, there have been a number of unique adaptations in retroviruses for anti-Tetherin activity [138]. SIV infections in rhesus macaques, African green monkey, and sootey mangabey utilize Nef protein for Tetherin antagonism (as they lack Vpu). Potent Vpu anti-tetherin function observed in HIV-1 is limited to group M viruses [75]. Additionally, HIV-1 and HIV-2 Nef proteins possess anti-Tetherin activity to rhesus macaques and sooty mangabey tetherins. The unusual diversity in vpu and Nef functions to human and non-human primate Tetherins demonstrate the strength of the selective pressures on viruses and hosts. It is believed that SIV chimpanzee, which encodes both Vpu and Nef, primarily utilized Nef to antagonize Tetherin. Following SIV shift to humans, the Nef interacting domain
was absent in human tetherin driving the current adaptation for Vpu function in human HIV-1 infection.

c) APOBEC3G - Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G, or APOBEC3G, was identified as an antiviral restriction factor that is packaged into newly assembled particles by interaction with viral Nucleocapsid protein [139]. APOBEC3G enzymes act to convert deoxycytidine to deoxyuridine on single stranded DNA. This is highly detrimental during HIV replication, as the hypermutation of HIV DNA during reverse transcription can induce premature stop codons or "lethal" mutations in the proviral genome. Additionally, APOBEC3G can directly interfere with reverse transcription and integration by preventing initial tRNA binding on viral RNA or by inhibiting the removal of tRNA on the 3' LTR prior to integration [140, 141]. Studies have shown as few as seven molecules of APOBEC3G were sufficient to inhibit subsequent HIV-1 infection [142].

To prevent incorporation into budding virions, HIV Vif has adapted the ability to bind APOBEC3G and recruit the cullin-5 E3 ubiquitin ligase complex.[60] Formation of the complex targets APOBEC3G for proteasomal degradation and maintains infectivity of progeny virus particles [62]. Despite extensive investigation into this mechanism, the interactions between APOBEC3G and Vif may not be entirely mapped. More recent studies by Valera et al. have identified interactions between the histone deacetylase 6 (HDAC6) enzyme and APOBEC3G. Formation of the HDAC6/APOBEC3G complex competed with Vif-
mediated APOBEC3G degradation [143]. Furthermore, HDAC6 interactions may promote Vif degradation directly. The authors suggest the extent of HDAC6 expression in an infected cell influences the amount of Vif incorporated into virions.

1.6 Establishment and Maintenance of HIV latency

Despite success of ART over the last several decades, latent HIV reservoirs remain the major obstacle to completely eliminating virus in patients. Individuals undergoing long-term viral suppression will persistently harbor a small population of long-lived memory cells that are susceptible to rebound following removal of therapy. Designing novel therapeutic agents to eliminate the latent reservoir remain of great interest and as such, the effort to understand molecular mechanisms underlying HIV latency continues to increase.

A significant early discovery was the finding that HIV-1 does not integrate into cellular DNA randomly. These previous findings have shown that HIV-1 preferentially integrates into actively transcribing genes. This mechanism of action is largely ascribed to the interaction between viral Integrase, nuclear LEDGF, and chromatin remodeling factors. Indeed, depletion of LEDGF results in markedly decreased integration efficiency with an appreciable loss of integration into active transcription units [110, 111].
Once integrated, there is the matter of transcription fate. Namely, a number of factors occurring at the molecular level will dictate active transcription versus establishment of viral latency [116]. For instance, the integration of HIV-1 DNA in a convergent manner, denoting the opposing direction of the host gene's orientation, will inhibit proviral gene expression. This transcriptional interference reduces recruitment of transcription factors to the viral promoter. A study published by Siliciano et al. concluded from in vitro cell models of HIV infection that latent proviruses have a preferential orientation in the same direction as the host gene [144]. More recently, longitudinal analyses of HIV integration sites in vivo have also identified an almost exclusive "same" orientation preference in the highlighted genes of interest [145]. Taken together, the directionality of HIV integration plays a major role not only in the ability for active transcription but for the persistence of the latently infected cell over time.

The limited availability of transcription and elongation factors in resting cells also directly influences HIV silencing. This is consistent with diminished active viral transcription occurring in the resting cell population, where cell-signaling cascades are largely inactive. Key activators in the transcription machinery such as NF-kappa B and STATs require these phosphorylation-dependent signals to initiate their activity and downstream nuclear translocation [146, 147]. Similarly, transcriptional repressors such as YY1 protein binding upstream in the 5' LTR or DSIF and NELF arresting RNAPII activity result in inactive transcription [148]. In short, these examples highlight that temporal
regulation of transcriptional activators and repressors act as molecular switches to determine the fate of HIV transcription.

The epigenome also plays a significant role in the regulation of viral latency. Most importantly, the equilibrium of histone acetylation and deacetylation has been reported to affect HIV transcription. The classes of enzymes responsible, histone deacetylases (HDACs) and histone acetyltransferases (HATs), control global cellular transcription in part by chemically modifying lysine residues of histone tails.

The first of the two enzyme families, HDACs, is a widely diverse group divided into four classes: 1) Class I HDACs, which are primarily nuclear acting; 2) Class IIa/IIb HDACs, which either shuttle between nucleus and cytoplasm or are found predominantly in the cytoplasm; 3) class III "sirtuins", a somewhat unique class in its own with multiple post-translational modifying functions; and 4) class IV HDACs, namely HDAC11, which possesses similarities to both Class I and II enzymes. In addition to deacetylation of histones, HDACs have a number of substrates including alpha tubulin, cortactin, HSP90, PTEN phosphatase, and NF-κB transcription factor [149].

Within the context of HIV, the recruitment of HDACs and NF-κB complexes to the viral LTR can repress transcription by deacetylation of proviral-associated nucleosomes nuc-0 and nuc-1 [150]. The increased positive charge on histones promotes high-affinity binding between DNA and nucleosomes, condensing the chromatin and impairing binding of RNA polymerase II. This interaction has
been shown to dramatically influence the degree of latency as pharmacological
treatment with HDAC inhibitors (HDACi) can prompt proviral reactivation [151].
As will be discussed later, the notion of HDACi mediated reactivation has an
important place in novel HIV eradication strategies.

Conversely, the histone acetyltransferase family of enzymes transfers
acetyl groups from acetyl CoA to conserved lysine residues on histone tails,
discouraging high-affinity binding of nucleosomes to DNA [152]. Histone
acetylation is generally associated with more exposed chromatin and elevated
gene expression. HATs are less understood than their HDAC counterparts and
are divided into two classes: Class A HATs, located primarily in the nucleus, and
Class B HATs, located in the cytoplasm, where they function to modify newly
generated histone proteins. These classes are further characterized by their
structural domains into GCN5-related N-acetyltransferases (GNATs), MOZ
Ybf/Sas3, Sas2, Tip60 related (MYST), p300/CREB-binding protein (CBP) HATs,
transcription factor associated HATs, and nuclear hormone-related HATs. Both
GNAT and MYST have distinct sequence motifs and as a result, act on specific
histone substrates.

HIV Tat interacts with HATs hGCN5, p300 and PCAF, resulting in
recruitment to the LTR promoter and increased proviral transcription [153, 154].
Subsequently, overexpression of both p300 and CBP has proportionate effects on
transcription activity. Tat itself can also become acetylated which has been
shown to promote transactivation of the promoter [155]. An additional study
has also highlighted the importance of Tat acetylation during mRNA splicing [156]. The results from these studies stress the critical role of HDACs and HATs during HIV replication. Notably, HDAC and HAT enzymes represent cellular machinery that can be manipulated to modulate the extent of active versus latent HIV transcription.

Beyond acetylation-based control of transcription, there has been increasing evidence for the involvement of methylation during viral latency. Increased DNA methylation has been shown to increase recruitment of HDACs to the HIV LTR subsequently promoting silencing [157]. This has been attributed to the discovery of CpG islands flanking the transcription start site (as identified in latent cell line models of HIV infection). Apart from histone, several groups have identified that increased levels of histone lysine methyltransferases (HKMTs), in particular EZH2, are present at high levels in silenced proviruses [158]. This discovery has sparked interest in elucidating the role of the related polycomb repressive complex 2 during HIV replication [159, 160].

Finally, insufficient levels of viral Tat protein will prevent high-level HIV transcription from occurring within an infected cell [161]. All of these factors can contribute to the balance of productive versus latent infection, where the latter is estimated to occur in approximately 1:1,000,000 memory T cells in vivo (though this number is currently being re-evaluated) [162, 163].
1.7 Current Therapies and Strategies for Eradication

1.7.1 - Combination Anti-Retroviral Therapy

To date there are in excess of 20 antiretroviral medications approved by the Food and Drug Administration (Figure 8). Each drug is classified by its target and mechanism of action into the following: Non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), fusion/entry inhibitors, and integrase strand-transfer inhibitors (INSTIs). The first successful medication to reach the general population was the thymidine analog zidovudine, more commonly referred to as "AZT". As a nucleoside reverse transcriptase inhibitor, AZT intercalates into cDNA and is converted from its active form containing a 5' phosphate group. The deletion of a 3' hydroxyl group on AZT prevents the subsequent nucleotide addition and terminates viral reverse transcription. Despite 100 times greater selectivity for HIV reverse transcriptase, AZT can still target host cell DNA synthesis by inhibiting DNA polymerase [164]. As a result, the side effects of AZT and other NRTIs at high doses are unpleasant where at the highest severity include cardiac and muscle disorders.
<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Name</th>
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<td>non-ARV</td>
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**Figure 8: FDA Approved Anti-retroviral Drugs.**

*Used in combination therapies*
Beyond AZT and other NRTIs, non-competitive inhibitors directly targeting viral enzymes were more desirable. In the late 1980s, the first non-nucleoside reverse transcriptase inhibitor was developed [165]. The drug was designed to target an allosteric region of the enzyme and prevent activity. Though effective in blocking reverse transcription, administration of NNRTIs as monotherapy results in rapid acquisition of drug resistance by the virus pool [166]. NNRTIs are often administered as combination therapy with additional ART medications such as Protease inhibitors, which act to directly inhibit viral maturation, or Integrase inhibitors that prevent strand transfer of viral DNA to host chromosomes.

In addition to targeting reverse transcription, integration, and maturation, newer research has spawned drugs that act at the level of entry. The first, enfuvirtide/T20, mimics the heptad repeat in gp41 preventing the conformational change required for pore formation. The second inhibitor maraviroc antagonizes CCR5 and prevents gp120 binding.

1.7.2 - Approaches to an HIV Vaccine

The quest to achieve protection by an HIV vaccine has been an arduous one. For starters, the use of attenuated HIV as a vaccine itself faces hurdles because of its high mutagenicity and ability to integrate into the host genome. Trials to evaluate subunit vaccines carrying gp120 or gp160 protein have yielded lackluster results despite stronger pre-clinical data [167, 168]. More often than not, neutralization across multiple clades did not occur and protection was only
seen in a small subset of laboratory-adapted viruses. More recently, the motivation for a vaccine was reignited by the minor success of the Thailand RV144 trial [169, 170]. RV144 is a combination boost-prime regimen with initial vaccination by a canarypox vector carrying Gag, Gag-pol, and Env antigens. This was followed by a boost using the AIDSVAX gp120 subunit vaccine. Testing with RV144 occurred between 2003-2006 and excitingly yielded ~31% protection from HIV when compared to placebo vaccination. Though mildly protective, the trial showed protection could indeed be acquired in vivo.

Alongside conventional approaches to elicit B-cell or T-cell mediated immunity against HIV, new directions are being considered for vaccine development. Most notably, the advent of gene-editing methods such as the CRISPR/Cas9 and TALEN systems sparked pre-clinical studies targeting HIV co-receptor CCR5 [171, 172]. Successful knockout of CCR5 genes would protect individuals from the majority of infections by R5-tropic viruses. Parallel studies are also being conducted in attempt to target integrated proviruses for deletion with the CRISPR/Cas9 system.

The most recent vaccine approach, dubbed vectored immunoprophylaxis (VIP), has been receiving substantial attention based on promising preliminary data [173, 174]. VIP involves delivery of engineered protein constructs that are constitutively expressed in the recipient. Gardner et al. have demonstrated just recently that injection of adeno-associated vectors encoding a CD4 immunoglobulin linked to CCR5 mimetic protects rhesus macaques from
challenge with simian human immunodeficiency virus (SHIV) [175]. These pre-clinical studies are promising approaches to combat viral infection by inducing protection through engineered delivery of a protein or antibody.

1.7.3 - Purging the Latent Reservoir

Despite the efforts to design an effective prophylactic vaccine, the persistence of long-lived latently infected cells in patients represents a significant hurdle in our attempts to eradicate the virus completely. Though highly effective, global access to anti-retroviral therapies is a significant challenge. Cost and burden of life long drug treatment is also high. As a result, designing novel strategies to combat HIV infection are of significant interest. As was previously mentioned, the persistence of long-lived latently infected cells in patients represents a significant hurdle in our attempts to eradicate the virus. However, the findings that the epigenome plays a critical role in modulating HIV transcription has led to the notion of using pharmacological intervention targeting nuclear and epigenetic machinery with the hopes of reactivating latent proviruses. This strategy, generically referred to as "shock-and-kill" or "kick-and-kill" is relying on the hypothesis that latently reactivated cells can be recognized as infected and eliminated by phagocytic or cytotoxic immune cells (Figure 9A) [176]. Research over the past several years has yielded several compounds that show efficacy in reactivating viral reservoirs including histone deacetylase inhibitors (HDACi), protein kinase c agonists (PKCa), bromodomain
inhibitors (BETi), and toll-like receptor agonists (TLRa). Several of these compounds have begun pre-clinical testing in small cohorts of patients (Figure 9B). Most notably, the broad-spectrum histone deacetylase inhibitors have received considerable attention despite producing mixed results when translated into patient studies [177, 178]. It is now thought that combinations of latency reversing agents (LRAs) may be a possibility to ensure sufficient reactivation of latent reservoirs [179]. However, several questions remain unanswered: To what extent will the latent reservoir be diminished by treatment? Can therapy ensure reactivated cells are sufficiently eliminated by host cell responses?
Figure 9: The HIV Shock and kill strategy.  

**A.** A novel therapeutic approach to eradicate latent HIV in patients, dubbed "shock and kill" involves pharmacological stimulation of integrated proviruses to induce viral gene expression. Reactivated cells expressing viral proteins can be recognized and eliminated by the immune system while under HAART to prevent reseeding of reservoirs.  

**B.** Several classes of latency reversing agents (LRAs) are currently being investigated in small cohorts of patients with hopes of identifying a safe and highly potent compound.
1.7.4 - Pre-exposure Prophylaxis (PrEP)

In addition to "cure" driven research to combat HIV latency, a significant effort is being put forth to identify strategies to reduce the initial acquisition and transmission of HIV. This has stemmed from the increasing cost of ART drugs and their inaccessibility to regions of sub-Saharan Africa that are primarily affected with HIV/AIDS. Additionally, development of an effective pre-exposure prophylactic would reduce infection spread, effectively lowering the social and economical burden of the disease while improving global quality of life. Current studies on PrEP development involve oral and topical applications of antiretroviral drug such Tenofovir and Emtricitabine [180, 181]. This combination has been recently FDA approved for use as PrEP in partners of HIV infected individuals and has shown remarkable efficacy in preventing transmission between discordant couples [182-185]. Additional drugs being investigated for PrEP administration include co-receptor antagonist Maraviroc and CCR5 ligand RANTES.
CHAPTER TWO:

THE HISTONE DEACETYLASE INHIBITOR VORINOSTAT (SAHA) INCREASES THE SUSCEPTIBILITY OF UNINFECTED CD4+ T CELLS TO HIV BY INCREASING THE KINETICS AND EFFICIENCY OF POST-ENTRY VIRAL EVENTS

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2.1 Abstract

Latently infected cells remain a primary barrier to eradication of HIV-1. Over the past decade, a better understanding of the molecular mechanisms by which latency is established and maintained has led to the discovery of a number of compounds that selectively reactivate latent proviruses without inducing polyclonal T cell activation. Recently, the histone deacetylase (HDAC) inhibitor vorinostat has been demonstrated to induce HIV transcription from latently infected cells when administered to patients. While vorinostat would be given in the context of antiretroviral therapy, infection of new cells by induced virus remains a clinical concern. Here, we demonstrate that vorinostat significantly increases the susceptibility of CD4+ T cells to infection by HIV in a dose- and time-dependent manner that is independent of receptor and coreceptor usage. Vorinostat does not enhance viral fusion with cells, but rather enhances the kinetics and efficiency of post-entry viral events including reverse transcription, nuclear import, and integration and enhances viral production in a spreading infection assay. Selective inhibition of the cytoplasmic class IIb histone deacetylase (HDAC)-6 with tubacin recapitulated the effect of vorinostat. These findings reveal a previously unknown, cytoplasmic effect of HDAC inhibitors promoting productive infection of CD4+ T cells that is distinct from their well-characterized effects on nuclear histone acetylation and LTR transcription. Our results indicate that careful monitoring of patients and ART intensification are warranted during vorinostat treatment and indicate that HDAC inhibitors that
selectively target nuclear class I HDACs could reactivate latent HIV without increasing the susceptibility of uninfected cells to HIV.

2.2 Significance of Work

HDAC inhibitors, particularly vorinostat, are currently being investigated clinically as a part of a ‘shock and kill’ strategy to purge latent reservoirs of HIV. We demonstrate here that vorinostat increases the susceptibility of uninfected CD4+ T cells to infection with HIV, raising clinical concerns that vorinostat may reseed the viral reservoirs it is meant to purge, particularly during conditions of suboptimal drug exposure. We demonstrate that vorinostat acts following viral fusion and enhances the kinetics and efficiency of reverse transcription, nuclear import, and integration. The effect of vorinostat was recapitulated using the cytoplasmic HDAC6 inhibitor tubacin, revealing a novel and previously unknown cytoplasmic mechanism of HDAC inhibitors on HIV replication that is distinct from their well-characterized effects of LTR-driven gene expression. Moreover, our results suggest that treatment of patients with class I-specific HDAC inhibitors could induce latent viruses without increasing the susceptibility of uninfected cells to HIV.
2.3 Introduction

Human immunodeficiency virus type 1 (HIV-1) establishes a pool of latently infected, resting memory CD4+ T cells during primary infection that persist during treatment with antiretroviral therapy (ART) [186-188] and can resume active viral replication within weeks following treatment interruption [189-192]. Latently infected cells are the primary barrier to eradication of HIV infection and are a principal reason that lifelong treatment with ART is typically required in the vast majority of patients to prevent disease progression. The latent reservoir is very stable with a half-life of 44 months [193, 194] and can be replenished by homeostatic proliferation of latently infected cells [195] or possibly during intermittent viremia[196], although this latter mechanism remains controversial. The reservoir is not significantly diminished by intensified ART regimens [197, 198].

Interest in eradicating HIV infection has been rekindled due to the unprecedented success of the 'Berlin patient,' an HIV-infected patient treated for acute myelogenous leukemia with allogeneic stem cell transplantation consisting of cells from a ccr5Δ32 homozygous donor that do not express functional CCR5[199]. Despite not receiving antiretroviral therapy for years, no HIV RNA or DNA has been detected in plasma, peripheral blood mononuclear cells, gastrointestinal tissues or mucosal target cell populations from this patient [200]. However, two factors make replicating the success of the Berlin patient difficult.
First, ablative chemo- and radiation therapy in conjunction with stem cell transplantation is an expensive medical procedure with significant risks to the patient. Second, $ccr5\Delta32$ homozygous patients are present at a frequency of only $\sim1\%$ of the Caucasian population and are considerably more rare in other racial and ethnic groups; therefore finding appropriately matched donors will be extremely challenging.

An alternative approach being investigated is to purge latent reservoirs by specifically inducing HIV transcription without inducing polyclonal activation of non-infected CD4+ T cells. Once the virus has been reactivated, the immune system, viral cytopathic effects, or cytotoxic drugs could theoretically eliminate infected cells. This strategy, referred to as ‘shock and kill’ or ‘kick and kill’ would be performed in the presence of ART to block infection of new target cells. Advances in our understanding of the molecular mechanisms underlying HIV latency have led to the identification of several drugs that might be efficacious at triggering HIV transcription in latently infected cells.

HIV latency is established by a combination of events that reduce initiation and prevent elongation of transcripts from the viral long terminal repeat (LTR) promoter and is subsequently reinforced by epigenetic modifications of the promoter region, as has been recently reviewed [201, 202]. The N-terminal tails of histones are subject to multiple forms of post-translational modifications, including acetylation, methylation, and phosphorylation. Histone acetyltransferases (HATs) act to acetylate histone tails; histone deacetylases
(HDACs) remove acetyl groups. One of the epigenetic mechanisms limiting HIV transcriptional initiation is the recruitment of HDACs to the LTR (17-20) via interactions with cellular proteins including LSF and YY-1, NF-kB p50 homodimers, and CBF-1 [150, 203, 204]. HDAC1 recruitment results in hypoacetylation of nuc-1, a nucleosome interacting with the HIV proviral DNA immediately downstream of the transcriptional start site, leading to transcriptional repression. In contrast, Tat-mediated recruitment of HATs, including CBP/p300 and PCAF, enhances expression from the viral LTR [153, 154, 205] via recruitment of the chromatin remodeling complex BAF and remodeling of nuc-1 [206].

The relationship between histone acetylation and HIV transcription regulation raised the possibility that HDAC inhibitors could reactivate latent proviruses. Indeed, HDAC inhibitors were subsequently found to disrupt HIV latency in cell lines and primary cell models in vitro [177, 207-210]. More recently, in vivo administration of the HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid, SAHA) was well tolerated and induced a 4.8-fold increase in HIV RNA expression in resting CD4+ T cells in patients on combination antiretroviral therapy (cART)[178], demonstrating that targeted reactivation of latent HIV is feasible in patients. The safety and efficacy of vorinostat in patients on stable ART is being investigated in ongoing clinical trials.

One of the key concerns with the ‘shock and kill’ strategy is that uninfected CD4+ T cells will become infected by HIV as it is purged from latent
reservoirs, potentially due to noncompliance with cART, drug resistance, or in sanctuary sites with suboptimal drug concentrations. In light of this concern, we investigated the effect of vorinostat on the susceptibility of uninfected cells to HIV. Here, we report that in addition to its well-characterized effects stimulating HIV transcription in latently infected cells, vorinostat dramatically increased the vulnerability of uninfected primary CD4+ T cells to HIV in a dose- and time-dependent manner and promoted viral replication in a spreading infection assay. This effect was independent of receptor and coreceptor usage. Vorinostat did not affect viral fusion with target cells, but increased the kinetics of post-entry events including reverse transcription and integration. The enhanced kinetics of infection correlated with increased efficiency of viral events leading to higher levels nuclear import as reflected by 2-LTR circles. Selective inhibition of the cytoplasmic class IIb HDAC6 with tubacin recapitulated these features of vorinostat-mediated enhancement of HIV infection. These findings reveal that HDAC inhibitors have a previously unknown, cytoplasmic effect promoting productive infection of CD4+ T cells that is distinct from their effects promoting histone acetylation and LTR activity. From a clinical perspective, these results raise a cautionary note for the use of vorinostat to purge latent reservoirs and suggest that ART intensification or careful monitoring of new infection events may be warranted. Furthermore, they indicate that treatment with class I-specific HDAC inhibitors could be effective at reactivating latent viruses without increasing the susceptibility of uninfected cells to HIV.
2.4 Materials and Methods

2.4.1 - Production of viruses

Combination reporter viruses were produced as previously detailed[71]. Briefly, 293 T cells were transfected with 10 mg pNL4-3-deltaE-EGFP (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-delta-E-EGFP (Cat #11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano), 7.5 mg bla-Vpr plasmid, and 6.0 ug of HIV Envs REJO.D12.1972 [211] (CCR5-tropic) or JOTO.TA1.2247 [212] (CXCR4-tropic) using calcium phosphate methods. Virus was harvested 72 hours after transfection, filtered, and concentrated by ultracentrifugation through a 20% sucrose cushion according to published protocols [213]. Viral concentrations were determined by p24 ELISA (Cell Biolabs) and empirical virus titers determined on purified CD4+ T cells to ensure the dose used was within the linear range of the assay VSV-G viruses were produced in a similar way. Briefly, 293 T cells were transfected with 10mg pNL4-3-d2EGFP-Nef, 6.5mg pCMVdelta8.91, and 3.5mg pMDG. Virus was harvested 72 hours after transfection, filtered, and concentrated by ultracentrifugation at 28K for 1.5 hours. Virus was titered by qPCR (Lentivirus qPCR Titer Kit, Abcam) and by GFP expression in Jurkat cells.
2.4.2 - Cells

CD4+ T cells were obtained by leukopheresis from ALLCELLS, LLC. All subjects were healthy control donors negative for HBV, HCV, and HIV. PBMCs were isolated by ficoll gradient separation while CD4+ T cells were purified by adding RosetteSep CD4+ T cell enrichment kit antibodies (STEMCELL Technologies) and autologous red blood cells. Cells were cryopreserved and treated with benzonase prior to infection. For experiments using polarized primary CD4+ T cells, naïve CD4+ T cells were isolated from frozen leukapheresis samples using a RoboSep instrument (STEMCELL Technologies). Cells were stimulated using Dynal CD3/28 beads in the presence of cytokines IL-6, IL-23, IL-1b, IL-2, TGF-b, anti-IL-4 antibody, and anti-IFN-g antibody for 6 days. Beads were removed using Dynal magnet and cells were resuspended in RPMI with 10% FBS supplemented with IL-2 and IL-23. This study was approved by the Case Western Reserve University Institutional Review Board.

2.4.3 - Infection experiments

Unstimulated primary CD4+ T cells: 10^6 CD4+ T cells were infected in parallel with 125 ng p24 equivalent of viruses bearing R5 tropic (REJO.D12.1972) or X4-tropic HIV Envs. Unless stated otherwise, cells were incubated for 4h with 2 mM vorinostat or 2 mM tubacin prior to addition of virus. Cells were spinoculated with virus at 1200g at 24°C for two hours unless otherwise stated and incubated at 37°C for 1 hour. The first plate of the parallel infections–
measuring fusion—was then washed with CO2-independent media (Gibco), resuspended in media containing CCF2-AM (Invitrogen) for 1 hour at room temperature, washed, and incubated overnight at room temperature in CO2-independent media containing probenicid. Cells were then prepared for flow cytometric analysis as described below. The second plate of parallel infections—to measure LTR-driven EGFP expression—was incubated for 72 hours at 37°C prior to preparation for flow cytometry. Jurkat cells or polarized primary CD4+ T cells were infected in parallel with VSV-G or HIV env viruses at an MOI of 1. Cells were incubated for 4h with 1 mM vorinostat prior to addition of virus. Cells were spinoculated with virus at room temperature for 1.5 hours. At 72 hours after infection, cells were harvested, washed, and flow cytometry analysis was performed.

2.4.4 - Viral kinetics experiments

Experiments were performed on $10^6$ cells spinoculated as described above. Cells were incubated 4 hours prior to infection with 2 mM vorinostat, spinoculated with virus, and AMD3100 (20 mM), enfuvirtide/T20 (20 mM), raltegravir (10 mM), and efavirenz (100 nM) added at varying time points before or after spinoculation. Inhibition kinetics graphs represent averaged infection levels normalized to no inhibitor controls. Error bars are excluded to prevent figure congestion.
2.4.5 - Flow cytometry

Cells were stained with anti-human CCR7 IgM (Becton Dickinson) and live/dead fixable yellow viability dye (Invitrogen) at 37°C for 30 min, washed, and incubated with anti-human CD3 BV650 (Biolegend), CD4 APC (eBioscience), CD45RO ECD (Beckman Coulter), CD27 PE-Cy7 (eBioscience), and anti-IgM PE (Invitrogen) for 4°C at 30 min. All cells were washed with PBS/BSA and resuspended in 1% paraformaldehyde prior to analysis. At least 50,000 events were collected per sample. All infection conditions were performed in triplicate. FlowJo version 9.6 (Tree Star, Inc.) was used for analysis of flow cytometry experiments.

2.4.6 - Determination of 2-LTR circle products

Primary CD4+ T cells and polarized activated T cells were spinoculated in parallel in the presence or absence of 2 µM vorinostat with CXCR4-tropic HIV-1. Following spinoculation, cells were harvested at indicated time points, washed with PBS, and DNA extracted. All extracts were incubated at 55°C with proteinase K prior to purification (Thermo Fisher Scientific GeneJet PCR purification kit). 2-LTR circle products were amplified using the following primer set: 2-LTR circle forward: 5’-CAAGCAGAAGACGGCATACCAGATTAAGGGAA CCCACTGC-3’; 2-LTR circle reverse: 5’-CCTCTCTATGGGCAGTGGTGATTC CACAGATCAAGGATATCTTTGTC -3’ (Integrated DNA Technologies). Purified DNA was amplified in a qPCR machine using 12.5 µM primers specified above in
addition to barcoded sequencing adapters to allow multiplexing of samples. PCR was stopped during the log phase of amplification and barcoded products were pooled, column purified, and run on a gel to select amplicons and remove residual primer. Fragments were gel purified and DNA concentration determined on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). 2-LTR products were sequenced using Ion Torrent PGM NGS following manufacturers protocol. Transcript abundances were determined using Genomics Workbench 5 (CLC bio). Number of reads between experiments were normalized to sequencing chip size.

2.4.7 - Replication competent experiments

10x10^6 primary CD4+ T cells were treated with 100 IU/mL rIL-2 and infected with 2700 ng p24 equivalent of replication competent virus bearing X4 tropic envelope (HIV-NLG-Nef). Cells were incubated for 4h with 2 uM vorinostat prior to addition of virus and spinoculated as described above. Following incubation at 37°C for one hour, cells were supplemented with additional media to a final concentration of 1x10^6 cells/mL. At indicated time points, supernatant was preserved at -80°C and cells were washed and stained with anti-human CD3 BV650 (Biolegend), CD4 APC (eBioscience), and live/dead near-IR viability dye (Invitrogen). All cells were washed with PBS/BSA and resuspended in 1% paraformaldehyde prior to analysis on a BD Fortessa flow cytometer. Infection conditions were performed in triplicate. Viral concentrations in supernatants
were quantified by p24 ELISA (Cell Biolabs) and normalized to cell viability as
determined by flow cytometry. ELISA experiments performed in duplicate using
two independent supernatant collections.

2.4.8 - Microscopy

Primary CD4+ T cells were allowed to adhere to poly-l-lysine treated
coverslips for 5 minutes, rinsed with 1x PBS, and treated in the presence or absence of 2 µM HDAC inhibitors for 4 hours. Cells were subsequently fixed in 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 containing 10% normal donkey serum (SBTx buffer). Samples were incubated with anti-acetyl tubulin IgG (Sigma), AlexaFluor 647-conjugated phalloidin (Invitrogen), and bis-Benzimide 33258 (Hoescht 33258, Sigma) for 30 minutes followed with secondary Cy-3 anti IgG (Jackson ImmunoResearch) for an additional 30 minutes. Coverslips were washed 3X with PBS and mounted onto glass coverslips using Fluoro-gel (Electron Microscopy Services). Images were acquired on a DeltaVision RT epifluorescence microscopy system equipped with an automated stage. Images were captured in a z-series and deconvoluted using the Softworx deconvolution program (Applied Precision).
2.4.9 - Western Blotting

10 x 10^6 populations of primary CD4+ T cells were incubated for 4 hours in the presence or absence of 2 µM HDAC inhibitors and subsequently lysed using cold RIPA buffer containing HALT protease inhibitor cocktail. Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes with Tris/Glycine/SDS buffer containing 20% methanol. Membranes were probed against acetylated tubulin (1:3000, Sigma), total alpha tubulin (abcam), and developed using standard ECL reagents. Membranes were exposed using the ImageQuant LAS 4000 system and related software.

2.4.10 - Statistics

Data in figures represent mean values and standard error of the mean unless stated otherwise. All differences with a p value of <0.05 were considered statistically significant, correcting for multiple comparisons when appropriate. Statistical analyses were performed using the paired T-test using GraphPad Prism v5.0d unless otherwise specified.
2.5 Results

2.5.1 - Virus and Cell Models

The histone deacetylase inhibitor vorinostat is currently being investigated as a treatment strategy to purge latent reservoirs in HIV-infected patients (30) due to its efficacy in activating latent proviruses ex vivo (26). Although vorinostat will be administered in the presence of ART to block infection of uninfected cells, seeding of new reservoirs remains a clinical concern. To determine if vorinostat affects the susceptibility of uninfected CD4+ T cells to HIV, we employed a novel flow cytometric combination reporter virus system [71] that can measure viral fusion and LTR-driven EGFP expression using a single reporter virus construct (Figure 10A). Fusion of viral and cellular membranes is identified by virion-associated b-lactamase–Vpr (bla-Vpr)–mediated cleavage of the b-lactamase substrate CCF2 [69], which alters the fluorescence characteristics of the cell (Figure 10B). In cells successfully completing post-entry events including uncoating, reverse transcription, integration, and LTR-driven gene expression, EGFP protein accumulates and can be detected. Spontaneous EGFP expression requires sufficient Tat and Rev levels to promote LTR transcription and export of incompletely and unspliced mRNAs, respectively. Since the NL4-3 core used to produce the combination reporter viruses contains an intact nef gene, CD4 downregulation is also observed in EGFP+ cells (Figure 10B, bottom).
Figure 10: Generation of Combination Reporter Viruses.  A. Combination reporter viruses are produced by cotransfection of 293T cells with plasmids encoding an egfp-containing HIV core lacking an envelope gene (pNL4-3-DE-EGFP), β-lactamase–vpr, and an HIV env with known coreceptor tropism. Virions package bla–Vpr protein and the egfp–HIV core. B. Fusion between virions and CD4+ T cells is detectable by flow cytometry as bla–Vpr–mediated CCF2 cleavage alters cellular fluorescence. Cells undergoing reverse transcription, uncoating, nuclear import, integration and LTR-dependent gene expression are identified by Nef-mediated CD4 downregulation and EGFP accumulation. C. In combination with the memory markers CCR7 and CD45RO, naïve (TN), central memory (TCM), effector memory (TEM), and terminal effector (TEE) subsets can be identified. Cells undergoing fusion or LTR-driven EGFP expression are shown in blue.

By incorporating conjugated antibodies directed against CCR7 and CD45RO, naïve and memory subsets of primary CD4+ T cells undergoing fusion or LTR-driven EGFP expression can be precisely determined (Figure 10C). Using this combination reporter virus system and traditional GFP reporter viruses, we examined the effects of vorinostat on three different cell models: (1) unstimulated primary CD4+ T cells, (2) polarized, activated CD4+ T cells, and (3) Jurkat cells. Unstimulated primary CD4+ T cells are predominantly resting cells, typically with greater than 96% lacking expression of CD25 or CD69.
2.5.2 - HDAC Inhibitor treatment Enhances HIV Infection of CD4+ T Cells in a Dose- and Time-Dependent Manner

To investigate the effects of vorinostat on CD4+ T cell susceptibility to HIV, we infected purified, unstimulated primary CD4+ T cells with combination reporter viruses bearing a patient-derived CXCR4-tropic Env, JOTO.TA1.2247[212]. Addition of vorinostat to CD4+ T cells 4 hours prior to infection significantly increased EGFP+ cell frequencies compared to untreated controls at doses as low as 500 nM (Figure 11A). Similar results were seen with the pan-HDAC inhibitors panobinostat and romidepsin, indicating that the enhancement of HIV infection was related to the HDAC-inhibitory activity of vorinostat (Figure 11B). Unstimulated CD4+ T cells are primarily in a resting state; to determine whether vorinostat had similar effects on activated cells, we infected polarized, activated primary CD4+ T cells and Jurkat cells. Similar to its effects on unstimulated cells, 4 hour pretreatment with vorinostat significantly increased the percentage of EGFP+ activated primary cells and Jurkat cells compared to untreated controls (p=0.003 and p=0.03, respectively, Figure 11C).

An X4-tropic HIV Env was initially selected for examination because CXCR4 is expressed on a far higher percentage of primary CD4+ T cells than HIV. However, R5-tropic viruses are more common in vivo, particularly among patients in early stages of infection. To determine whether vorinostat-mediated
Figure 11: Vorinostat enhances HIV infection of CD4+ T cells to HIV in a dose- and time-dependent manner. A. 4h pretreatment of unstimulated primary CD4+ T cells with vorinostat enhanced LTR-driven EGFP expression by combination reporter viruses pseudotyped with a patient-derived CXCR4-tropic Env in a dose-dependent manner. B. Infection of activated, polarized CD4+ T cells and Jurkat cells with reporter viruses bearing CXCR4-tropic Env is also enhanced by 4h pretreatment with vorinostat. C. Pretreatment of cells with vorinostat increases their susceptibility to reporter viruses pseudotyped with VSV-G or CCR5-tropic HIV Env, suggesting enhanced infection is independent of receptor or coreceptor usage. D. HIV infection of unstimulated primary CD4+ T cells was enhanced by 4h pretreatment with vorinostat compared to addition 24 or 48h after infection. *p<0.05, **p<0.01. Exact p-values are provided in the text.

enhancement of T cell vulnerability was dependent on receptor or coreceptor expression, we infected Jurkat cells with viruses pseudotyped with the CD4-independent VSV-G envelope or with a patient-derived R5-tropic Env, REJO.D12.1972 [211]. As was observed for X4-tropic virus, vorinostat significantly
increased the percentage of EGFP+ cells following infection with VSV-G and R5-tropic pseudotyped viruses (p=0.01 and p=0.04, respectively; Figure 11D), suggesting that the enhancement of CD4+ T cell vulnerability to infection is receptor- and coreceptor-independent.

Since vorinostat would be expected to promote transcription from viral LTRs that might otherwise become silenced soon after infection, these findings were not entirely unexpected. However, pre-treatment with 2 mM vorinostat significantly enhanced LTR-driven EGFP expression compared to treatment 24 or 48 hours after infection (p=0.02 and p=0.006, respectively), conditions typically used to activate silent proviruses (Figure 11E). Raltegravir reduces EGFP+ cell frequencies by >95% when added as late as 30 hours post-infection; therefore, the observation that a 4 hour pre-incubation with vorinostat was significantly more effective at increasing EGFP+ cell frequency compared to addition 24 hours after infection was surprising and suggested that vorinostat might have additional, unknown activities at a stage of the viral life cycle proximal to integration into the host chromosome.

2.5.3 - Vorinostat Does Not Enhance Viral Fusion

To investigate how vorinostat promotes susceptibility to infection, we measured its effect on fusion using combination reporter viruses pseudotyped with either R5- and X4-tropic Envs. 4h pre-incubation with vorinostat had no effect on fusion (Figure 12A). However, our infection protocol involved a 2h
spinoculation step, which has recently been reported to induce remodeling of the cortical actin barrier and promote viral infection [214]. We reasoned that spinoculation might mask effects of vorinostat on fusion and therefore repeated the experiment in the absence of spinoculation. A 10-15-fold higher viral inoculum was used in these studies, as fusion is significantly diminished in the absence of spinoculation in the combination reporter virus assay [71]. Again, no enhancement of viral fusion was observed for reporter viruses pseudotyped with either R5- or X4-tropic Envs (Figure 12B); in fact, there was a slight decrease in fusion of X4-tropic HIV in the presence of vorinostat, consistent with an observation that CXCR4 receptor levels can be decreased by HDAC inhibitors [215, 216].

To gain further insight into how vorinostat influences CD4+ T cell vulnerability to HIV, we infected unstimulated primary CD4+ T cells with an X4-tropic combination reporter virus and examined both fusion and LTR-driven EGFP expression in naïve and memory subsets. Consistent with the total CD4+ T cell analysis, vorinostat did not increase HIV fusion within any of the CD4+ T cell subsets but did enhance reporter gene expression (Figure 12C).
Figure 12: Vorinostat does not increase viral fusion with CD4+ T cells.
A. Unstimulated primary CD4+ T cells were infected with X4- or R5-tropic combination reporter viruses, spinoculated at 1200g for 2 hours, and fusion levels determined by bla-Vpr–mediated CCF2 cleavage. 4h vorinostat pretreatment did not affect fusion levels of either X4- or R5-tropic HIV. B. Vorinostat pretreatment did not increase fusion levels in the absence of spinoculation for either R5- or X4-tropic HIV. 10–15–fold higher concentrations of HIV were used to compensate for the reduction in fusion in the absence of spinoculation. C. Pretreatment with vorinostat did not affect the percentages of CD4+ T cell in T_N, T_CM, T_EM, or T_TE subsets fusing with HIV but enhanced the likelihood of infection following fusion. Representative data from one of five patients is shown. Numbers reflect the percentage of cells undergoing fusion or LTR-driven EGFP expression in each CD4+ T cell subset.

Interestingly, the effect of vorinostat appeared most pronounced in CCR7+CD45RO- naïve (T_N) and CCR7+CD45RO+ central memory (T_CM) subsets that are less activated than the CCR7-CD45RO- effector memory (T_EM) and CCR7-CD45RO+ terminal effector (T_TE) subsets. Together, these data suggest that vorinostat increases productive infection by modifying the efficiency of post-entry steps of the viral life cycle and may have the greatest effects on
unstimulated naïve and central memory CD4+ T cells which appear to be resting since they do not express the classical phenotypic surface markers CD25 and CD69.

2.5.4 - The Kinetics of Postentry Viral Events

To further investigate the post-entry events influenced by vorinostat, we performed experiments where the CXCR4 antagonist AMD3100, fusion inhibitor T20, reverse transcriptase inhibitor efavirenz, and integrase inhibitor raltegravir were added to unstimulated primary CD4+ cells prior to or at varying time points following infection with HIV, either in the absence or presence of vorinostat. As expected, the kinetics of HIV binding to CXCR4 or fusion of the viral and host cell membranes were unaffected by the presence of vorinostat (Figure 13A, B). In contrast, cells pre-treated with vorinostat became insensitive to efavirenz more rapidly than control cells (t½ = 35.4 h vs. t½ = 41.8 h), indicating that the kinetics of reverse transcription were accelerated in the presence of vorinostat (Figure 13C). Similarly, cells became refractory to raltegravir more rapidly when pretreated with vorinostat (t½ = 35.9 h vs. t½ = 47.2 h) reflecting enhanced integration kinetics (Figure 13D).
Figure 13: Vorinostat enhances the kinetics of post-entry viral events including reverse transcription and integration. Uninfected CD4+ T cells were infected with X4-tropic reporter viruses in the presence or absence of 4h pretreatment with vorinostat. Prior to infection or at varying time points after infections, the (A) coreceptor antagonist AMD3100, (B) fusion inhibitor T20, (C) reverse transcriptase inhibitor efavirenz, or (D) integrase inhibitor raltegravir were added. Infection values were normalized to uninfected controls. Graphs represent averaged levels among three replicates. Error bars are excluded to prevent figure congestion.

2.5.5 - Vorinostat Enhances Nuclear Import Efficiency

Since the kinetics of viral post-entry events have been correlated with the efficiency of viral infection [217], we reasoned that the enhanced kinetics of entry in the presence of vorinostat would translate into more efficient progression through post-entry stages of the viral life cycle. To directly test this hypothesis we measured the production of 2-LTR circles, a marker of nuclear
import of viral DNA, in unstimulated or activated primary CD4+ T cells infected with HIV using qPCR followed by deep sequencing on an Ion Torrent personal genome machine to confirm specificity of the products. Both unstimulated primary CD4+ T cells and polarized, activated CD4+ T cells demonstrated increased levels of 2-LTR circles in the presence of vorinostat (Figure 14). Treatment with vorinostat resulted in a ~3-fold increase in the number of sequence reads by 72 hours. In both cell types, the accumulation of 2-LTR circles was not observed until at least 24 hours post infection, consistent with the kinetics data regarding the timing of reverse transcription. Furthermore, polarized, activated CD4+ T cells yielded greater overall sequence reads, in agreement with previous findings that activated cells are increasingly susceptible to HIV infection. Together with our kinetics data, these data suggest that vorinostat increases the efficiency of viral post-entry events including reverse transcription, nuclear import, and integration.

Figure 14: The efficiency of post-entry viral events including reverse transcription and nuclear import is improved by vorinostat. (A) Primary unstimulated and (B) polarized, activated CD4 T cells were infected (next page)
with X4-tropic reporter viruses and spinoculated. At various time points following infection, the cells were harvested and DNA extracted for qPCR of 2-LTR circles followed by deep sequencing to confirm specificity. Product abundances were determined using Genomics Workbench 5. Times are as indicated in hours. Data normalized by input DNA and sequencing chip size.

2.5.6 - Enhancement of Replication Competent Infection

As the combination reporter virus is a single-cycle assay due to the replacement of the viral env gene with egfp, we also examined the effects of vorinostat in a spreading infection assay (Figure 15A). Briefly, 10x10⁶ primary CD4+ T cells were stimulated with 100 IU/mL IL-2 and infected with a replication competent HIV reporter virus (HIV-NLG-Nef), spinoculated, and incubated at 37°C for up to 13 days. On d1, d3, d5, d7, d9, d11, and d13, cells were removed to determine EGFP expression and supernatant harvested and stored at -80° for p24 ELISA. As expected, treatment with vorinostat significantly increased EGFP expression of replication-competent HIV beyond a single round of infection, evident by a steady rise in EGFP+ cells from days 7-13 when compared to untreated controls (Figure 15B). This finding was further supported by viral p24 protein concentration in cell supernatants. Treatment with vorinostat dramatically increased the level of p24 produced by day 13, indicating that vorinostat is acting to both augment LTR driven EGFP expression and amplify virion release throughout multiple rounds of HIV replication. Together, these data demonstrate that in addition to its effects promoting reverse transcription, nuclear import, and integration in a single-cycle assay, vorinostat also enhances productive infection and spread of replication-competent HIV.
Figure 15: Vorinostat enhances productive infection and replication of HIV in a spreading infection. Primary CD4+ T cells were stimulated with 100 IU/mL IL-2, infected with X4-tropic replication-competent virus and spinoculated. Cells and supernatants were harvested at days 1,3,5,7,9,11, and 13 post-infection. (A) Pretreatment with vorinostat increased the percentage of CD4+ T cells infected by replication-competent HIV, measured by EGFP accumulation using flow cytometry. (B) p24 produced from primary CD4+ T cells as measured by ELISA. Pretreatment with vorinostat dramatically increased the levels of p24 throughout multiple rounds of infection, evident by a steady increase by 13 days post-infection. Data normalized to ng p24 per 1x10^6 viable cells.

2.5.7 - Specific Inhibition of HDAC6

In humans, HDACs are divided into four classes based upon their homology to yeast proteins [149]. Class I HDACs, including HDAC1, 2, 3 and 8, are predominantly located in the nucleus and are the primary mediators of histone deacetylation. Class IIa HDACs shuttle between the nucleus and the cytoplasm of cells and include HDAC4, 5, 7, and 9. Class IIb HDACs are predominantly cytoplasmic and include HDAC6 and 10. Class III HDACs bear homology to the yeast Sir2 protein and include NAD^+-dependent sirtuin family members SIRT1-7, while class IV HDACs consist of HDAC11, differentiated from
other classes based upon conserved catalytic elements shared by both class I and class II HDACs. Vorinostat inhibits the activity of HDAC1-9, albeit with reduced potency against HDAC8. Our observation that vorinostat increased the kinetics of reverse transcription in CD4+ T cells suggested that inhibition of cytoplasmic HDACs might contribute to increased susceptibility to HIV. Of these, the class IIb HDAC6 was of particular interest because specific inhibitors have previously been reported to enhance HIV infection; however these studies reported enhancement of viral fusion [218, 219], which we did not observe with vorinostat treatment.

Figure 16: Specific inhibition of HDAC6 by tubacin recapitulates the effect of vorinostat on uninfected CD4+ T cells. (A) 4h pretreatment of CD4+ T cells with 2 mM tubacin did not affect fusion of R5- or X4-tropic combination reporter viruses in the presence or absence of spinoculation. 10-15-fold higher viral concentrations were used in the absence of spinoculation to compensate for lower fusion levels. (B) Pretreatment of CD4+ T cells with 2 mM tubacin increased the percentage of CD4+ T cells infected by HIV for R5- and X4-tropic viruses.
HDAC6 has multiple cytoplasmic targets, including α-tubulin, the cortical actin binding protein cortactin, and heat shock protein 90 [149]. To test whether inhibition of HDAC6 might contribute to the vorinostat-mediated enhancement of HIV infection, we pretreated unstimulated primary CD4+ T cells with 2 µM of the HDAC6-selective inhibitor tubacin [220] for 4h prior to infection with HIV. In contrast to previously published results (41, 42) we did not observe any effect of HDAC6 inhibition on HIV fusion with target cells (Figure 16A). The lack of an effect on fusion was observed with both R5- and X4-tropic HIV in the presence or absence of spinoculation, and was consistent with our previous observations with vorinostat. Tubacin pretreatment increased EGFP+ CD4+ T cell frequency compared to untreated controls (R5-tropic HIV: 1.64 ± 0.35-fold increase, p=0.057, X4-tropic HIV: 1.89 ± 0.41-fold increase, p=0.011, Figure 16B), indicating enhanced HIV infection as was seen with vorinostat. Together, our results indicate that inhibitors targeting cytoplasmic HDACs promote HIV infection by enhancing efficiency of post-entry events in the viral life cycle, a previously unknown mechanism distinct from reactivation of latent proviruses by histone modifications in the nucleus.

2.5.8 - HDACi Treatment Upregulates Acetylated Microtubule Networks

Our findings that inhibition of HDAC6 by the small molecule inhibitor tubacin introduced the notion that non-histone targets may be responsible for enhanced infection efficiencies observed with HDACi treatment. A primary
cytoplasmic target of HDAC6 is alpha tubulin, regulating its acetylation and deacetylation. To confirm the effects of broad spectrum vorinostat and specific inhibitor tubacin, unstimulated primary CD4+ T cells were treated with 2 µM vorinostat or tubacin for 4 hours and probed for acetylated alpha tubulin by western blot and wide field fluorescence microscopy. Indeed, treatment with both inhibitors led to dramatic up-regulation of microtubule associated acetylated tubulin (Figure 17A) total acetylated alpha tubulin (Figure 17B). Together with our results from Figure 16 and the notion that HIV requires microtubule networks for intracellular transport[90], our findings suggest that the HDACi-mediated up regulation of acetylated tubulin may be linked to the increased susceptibility of CD4+ T cells to infection by HIV.
Figure 17: HDACi induce the formation of acetylated microtubules. A. 4 hour treatment with inhibitors SAHA or tubacin dramatically upregulated the formation of acetylated microtubule networks in primary unstimulated CD4+ T Cells. B. Total levels of acetylated alpha tubulin (free and microtubule associated) are increased by treatment with histone deacetylase inhibitors.
2.6 Discussion

Latently infected resting CD4+ T cells are a primary barrier to the eradication of HIV infection due to their long half-life, persistence in the presence of antiretroviral therapy, and ability to renew the reservoir during periods of intermittent viremia or by homeostatic proliferation. Over the past decade, significant progress has been made in identifying compounds that are capable of reactivating latent HIV without inducing polyclonal T cell activation [177, 210, 221-224]. The HDAC inhibitor vorinostat has shown efficacy both in vitro and in vivo in inducing HIV transcription in latently infected CD4+ T cells [177, 178] which in combination with antiviral immune responses, antiretroviral drugs, or cytopathic effects, may eradicate latent reservoirs. This strategy, known as ‘shock and kill’, is currently being investigated in HIV-infected patients. A major concern with this strategy is that uninfected CD4+ T cells will become infected during periods of viral activation from latency.

In this study we investigated the effect of vorinostat on uninfected cells using unstimulated and activated polarized primary CD4+ T cells and Jurkat T cell lines. Vorinostat was found to promote HIV infection in a dose- and time-dependent manner that was independent of receptor and coreceptor usage. Vorinostat did not affect HIV fusion, but rather increased the kinetics of post-entry events of the HIV life cycle including reverse transcription and integration. This was coupled with enhanced efficiency of 2-LTR circle formation, a measure
of nuclear import. Furthermore, tubacin-mediated inhibition of HDAC6, a class IIb HDAC believed to be predominantly cytoplasmic, recapitulated the effects of vorinostat by increasing the vulnerability of CD4+ T cells to infection by HIV.

Our findings raise concerns for the clinical use of vorinostat as it could reseed the viral reservoirs intended to be purged. Although ‘shock-and-kill’ strategies to eliminate latent HIV will be performed in the presence of cART, suboptimal viral inhibition could occur in the context of patient non-compliance, viral resistance, or sanctuary sites with poor drug penetration. While translating \textit{in vitro} findings to \textit{in vivo} effects is not straightforward, it is worth mentioning that the doses expected following a single 400 mg dose of vorinostat in patients (335 nM) are close to the level where a significant enhancement of viral infection was observed in this study (500 nM). Importantly, \textit{in vivo} administration of vorinostat did not increase residual viremia in patients on cART, raising doubts as to whether HDAC inhibitors alone will be sufficient to purge latent reservoirs. However, if vorinostat is eventually employed in therapeutic strategies to eliminate viral reservoirs, either alone or in combination with other latency reversing agents, intensification of ART may help reduce the likelihood of reseeding viral reservoirs.

In addition to these important clinical implications, these results reveal a previously unknown mechanism of HDACs on HIV infection that is distinct from their ability to activate viral transcription in latently infected cells. Several lines of evidence support the conclusion that these effects of HDACs are separate. First,
vorinostat significantly increased LTR-driven EGFP expression when administered 4h prior to infection compared to 24h after infection. This is unlikely to be a result of enhanced LTR transcription since raltegravir time-of-addition studies revealed that >95% of virus had not integrated at either time point. Second, the kinetics of reverse transcription—thought to be primarily a cytoplasmic process—were enhanced by vorinostat. Third, nuclear import of viral DNA—as estimated by 2-LTR circle formation—and the kinetics of integration itself were also enhanced. Both of these processes lie upstream of LTR transcription in the viral life cycle. Fourth, tubacin, a specific inhibitor of the cytoplasmic class IIb HDAC6, promoted HIV infection of CD4+ T cells at 2 mM, whereas it has no effects on histone acetylation at doses up to 20 mM [220, 225]. Finally, previous studies with the HDAC inhibitor valproic acid (VPA) did not demonstrate increased HIV infection of uninfected cells despite pronounced effects on reactivation of latent HIV (29). Intriguingly, VPA is a potent inhibitor of nuclear class I HDACs but has minimal effects upon class IIb HDACs including HDAC6 [226, 227]. Together, these data strongly suggest that vorinostat promotes de novo infection of uninfected CD4+ T cells through a novel mechanism distinct from nuclear histone acetylation. From a drug development standpoint, these findings also imply that an HDAC inhibitor that selectively targets nuclear class I HDACs could reactivate latent HIV without increasing the vulnerability of cells to infection.

The observation that tubacin can recapitulate the vorinostat-mediated enhancement of HIV infection strongly implicates HDAC6 as a cellular factor
inhibiting viral infection. Previous studies have found that inhibition of HDAC6 enhances HIV infection by increasing fusion in cell-cell and virus-cell fusion models [218, 219], results that we were unable to replicate here. Differences in the viral or cell models used may account for these differences. Multiple cellular proteins are deacetylated by HDAC6, including α-tubulin, cortactin, and heat shock protein 90. α-tubulin acetylated at the e-amino group of Lys40 is preferentially found in stable microtubules, which are both essential components of the cytoskeletal architecture and conduits for trafficking of large macromolecules through the cell via the microtubule motors dynein and kinesin. HIV has been reported to hijack the microtubule network through interactions between dynein and the viral capsid protein p24, facilitating viral trafficking movement to the perinuclear region or microtubule organizing center (MTOC) [90]. Decreased microtubule stability, such as that induced by overexpression of the ezrin-radixin-moesin (ERM) family members ezrin and moesin, is associated with reduced infection of cells by murine leukemia viruses and HIV at a step prior to reverse transcription [96, 97]. Conversely, inhibition of HDAC6-mediated α-tubulin deacetylation by vorinostat or tubacin may favor stable microtubule tracts, facilitating HIV reverse transcription and trafficking of HIV to the nucleus [90] and promoting HIV infection. Finally, a recent paper by Sabo and colleagues has demonstrated that HIV promotes the formation of acetylated and detyrosinated stable microtubule tracts during early infection [95]. Blocking the formation of these tracts using an end binding protein EB1 dominant negative
carboxy terminal fragment also reduced HIV infectivity. It is tempting to speculate that inhibition of cytoplasmic HDAC6 further stabilizes these stable microtubule networks and facilitates HIV reverse transcription and transport to the nucleus, enhancing viral infection. A better understanding of the role of HDACs in regulating cytoplasmic proteins and cytoskeletal architecture will provide insight into critical early events in the viral life cycle and provide strategies for the development of agents that specifically activate HIV transcription without increasing susceptibility of cells to de novo HIV infection.
CHAPTER THREE:

SENSITIVITY OF HIV-1 INFECTION TO HISTONE ACETYLTRANSFERASE INHIBITORS GARCINOL AND CURCUMIN

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3.1 - Abstract

Research into the HIV "shock-and-kill" approach has become increasingly popular in recent years following identification of agents that can reactivate latent proviruses. These latency reversing agents (LRAs) include compounds such as disulfurum, PKC agonists, and histone deacetylase (HDAC) inhibitors. We recently reported treatment with the clinically approved HDAC inhibitor vorinostat significantly increases productive viral infection in both single-cycle and replication-competent assays, raising potential concern for its use as a LRA. This effect was also observed with broad-spectrum HDAC inhibitors and surprisingly the cytoplasmic HDAC6 inhibitor tubacin. Our results with tubacin strengthened our hypothesis that vorinostat and other HDAC inhibitors enhance post-entry infection efficiency by increasing the nuclear import of HIV DNA.

This initial set of findings was intriguing and led us to hypothesize that treatment with small molecule inhibitors targeting opposing histone acetyltransferase (HAT) enzymes will decrease HIV productive infection. In agreement with this, HAT inhibitors garcinol and curcumin significantly reduced LTR-driven EGFP expression in primary unstimulated CD4+ T cells in a dose- and time-dependent manner, albeit curcumin to a greater extent. These compounds were also assessed in a two-week replication competent assay, where curcumin treatment dramatically suppressed EGFP expression throughout multiple rounds of infection. We believe our preliminary results demonstrate a novel proof-of-
concept to reduce initial acquisition of HIV and increase the bottleneck of transmission by directly targeting cellular processes independent of viral heterogeneity and/or resistance mutations. Additional work is being conducted to pinpoint the mechanism of these inhibitors, and determine whether they act to 1) alter the level of HIV integration, 2) promote a stronger latent environment during initial infection, or 3) act downstream of transcription.
3.3 - Introduction

Combatting HIV-1 infection is a two-sided coin. The first, or "cure" driven side, is primarily focused on overcoming the obstacle of HIV latency. This field was reignited in recent years largely due to the "berlin patient", who received a heterologous stem cell transplant from a CCR5Δ32 mutant donor[199, 200]. The virus remains undetectable despite extensive sampling and ultra-sensitive techniques, lending to the notion of eradication. However, combination chemoablation and transplant remain a high-risk and unfeasible method to implement on the large scale. The low frequency of natural CCR5Δ32 mutations complicates this issue and transplant efforts using wildtype-CCR5 donor cells have been met with failure. In response, direct manipulation of the CCR5 gene with various editing tools (CRISPR, TALENS, etc) is being pursued as an alternative approach. These tools may also hold promise to target integrated HIV DNA directly.

Pharmacological reactivation of the latent reservoir is another interest to the cure side. As was described in section 1.7.3, this shock-and-kill approach relies on rewiring the epigenetic landscape of infected cells using a "latency reversing agent", in efforts to activate the LTR promoter and drive transcription. Cells expressing viral proteins will (in theory) be targeted for elimination by innate or adaptive immune responses. We previously reported that one class of LRAs, the histone deacetylase inhibitors, should be approached with caution as
we observed an *in vitro* enhancement of HIV-1 replication that is independent of known epigenetic mechanisms[228]. In fact, it will be crucial to evaluate all LRAs for their potential adverse immunological effects.

Prophylaxis is the second side of the coin to combat infection. 2 million new infections occur each year globally despite highly effective anti-retroviral drugs[12]. In the U.S., the CDC reports an unchanging number of new cases annually. As such, stopping HIV transmission is of significant importance. This is being addressed in several ways: Development of direct-acting anti-retroviral drugs is ongoing for use as post-exposure prophylaxis (PEP). This has been the gold standard for preventing transmission and for improving quality of life of infected individuals. More recently, the use of anti-retroviral therapy was approved for use as *pre-exposure* prophylaxis (PrEP)[181]. This is proving to be highly effective as initial studies report a 92% lower risk of acquisition with daily adherence[180, 184]. Alongside oral PrEP, there are ongoing trials to evaluate the use of ART as a vaginal or rectal microbicide[229-234]. Delivered as a gel, foam, or cream, tissue-specific microbicides presents the opportunity to locally protect against acquisition while reducing side effects from systemic drug delivery. The first example demonstrating this approach was CAPRISA 004, where application of 1% tenofovir gel reduced HIV risk by 39% (alongside 51% reduction in HSV-2 risk)[229].

The use of direct-acting antiretroviral drugs as PrEP/microbicides still raises concern that breakthrough infections will be highly resistant to therapy.
These events would occur in cases where drug-resistant strains predominate in the donor individual that are then passed to the recipient. It would therefore be advantageous to design agents that confer non-specific protection (lessening the viral selection pressure). For example, several groups have demonstrated that carbohydrate binding agents griffithsin, cyanovirin-N, and Galanthus nivalis agglutinin can effectively inhibit HIV-1 or SHIV infection by interfering with envelope mediated fusion[235, 236]. Another approach involves targeting liver X receptor, as activation of LXR restores ATP-binding cassette transporter A1 (ABCA1) expression normally inhibited by Nef[237-239]. Restoration of ABCA1 disrupts cholesterol efflux to the cell surface and dramatically inhibits HIV infection. The in vivo efficacy of LXR stimulation was recently evaluated in a humanized mouse system and yielded 63% protection from virus challenge. Despite incomplete protection, animals that succumbed to infection (37%) had significantly reduced viral loads when compared to control.

This concept of targeting cellular machinery to prevent HIV infection is highly appealing. As an obligate intracellular pathogen, HIV requires extensive use of host proteins and networks to complete its replication cycle. For example, HIV hijacks the microtubule network for transport to the nucleus[90, 93, 240]. Several groups have shown that this cytoskeletal transport is linked to uncoating and reverse transcription, and that HIV can induce formation of stable acetylated microtubules to promote this process[94, 95]. We have recently built upon this by demonstrating that domain-specific inhibition of HDAC6 upregulates
acetylated microtubules and enhances infection 3-fold[228]. While this highlighted a potential concern for use of HDAC inhibitors as latency reversing agents, it also led us to hypothesize that inhibition of histone acetyltransferases may have an opposing effect. We primarily evaluated two compounds: garcinol, a polyisoprenylated benzophenone derived from the Garcinia indica plant; and curcumin, a diarylheptanoid isolated from the cooking spice turmeric[241, 242]. Both garcinol and curcumin showed dose-dependent effects to reduce LTR-driven EGFP expression in our combination reporter virus assay. We found this effect to be independent of viral fusion and independent of early replication events. Instead, our data suggests these compounds may directly act to prevent active transcription or alter the efficiency of splicing. Taken together, this study represents an additional proof of concept that targeting cellular machinery can reduce infection to HIV infection.
3.4 - Methods and Materials

3.4.1 - Acetyltransferase Inhibitors

Histone acetyltransferase inhibitors garcinol, curcumin, and anacardic acid were acquired from Cayman Chemicals, Inc. Small molecule inhibitors were diluted in RPMI 1640 with 10% FBS and 1% PenStrep. Dilutions were performed immediately prior to experimental setup to minimize freeze-thaw cycling and to prevent precipitation from solution. Unless otherwise noted, 10 µM each compound was used in each experiment.

3.4.2 - Combination Reporter Virus Infection

1 x 10^6 unstimulated primary CD4+ T cells were seeded per well in a 96-well format and incubated for 4 hours at 37°C with media alone or media supplemented with the indicated acetyltransferase inhibitor. Identical plates were prepared for viral fusion and productive infection analysis. Following incubation with small molecule inhibitors, cells were infected with HIV-1 reporter virus strain NL4-3-deltaE-EGFP bearing CXCR4-tropic envelope JOTO.TA1.2247 or CCR5-tropic envelope REJO.D12.1972. Plates were spinoculated for two hours at 1200g and 25°C. Following centrifugation, cells were incubated at 37°C for 1 hour. The fusion plate was washed, loaded with CCF2-AM, and incubated overnight at room temperature as previously described. Productive infection
plates were incubated at 37°C for a total of 72 hours prior to staining and analysis.

3.4.3 - Replication-competent infection

10x10^6 primary CD4+ T cells were treated with 100 IU/mL rIL-2 or caspase inhibitors Z-VAD-FMK/Z-IETD-FMK (20 µM) and infected with 2700 ng p24 equivalent of replication competent virus bearing X4 tropic envelope (HIV-NLG-Nef). Cells were incubated for 4h with 10 µM garcinol or curcumin prior to addition of virus and spinoculated as described above. Following incubation at 37°C for one hour, cells were supplemented with additional media to a final concentration of 1x10^6 cells/mL. At indicated time points, cells were washed and stained with anti-human CD3 BV650 (Biolegend), CD4 APC (eBioscience), and live/dead near-IR viability dye (Invitrogen). All cells were washed with PBS/BSA and resuspended in 1% paraformaldehyde prior to analysis on a BD Fortessa flow cytometer. Infection conditions were performed in triplicate.

3.4.4 - Analysis of Acetylated Tubulin

10 x 10^6 populations of primary CD4+ T cells were incubated for 4 hours in the presence or absence of HDAC inhibitors (2 µM) and acetyltransferase inhibitors (10 µM) and subsequently lysed using cold RIPA buffer containing HALT protease inhibitor cocktail. Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes with Tris/Glycine/SDS buffer
containing 20% methanol. Membranes were probed against acetylated tubulin (1:3000, Sigma), total alpha tubulin (abcam), and developed using standard ECL reagents. Membranes were exposed using the ImageQuant LAS 4000 system and coupled software. Tubulin abundances were determined by pixel density using Image J software.

3.4.5 - Viral Kinetics Experiments

Experiments were performed on 10⁶ cells spinoculated as described above. Cells were incubated 4 hours prior to infection with 10 µM garcinol or curcumin, spinoculated with virus, and raltegravir (10 mM), or efavirenz (100 nM) was added at indicated time points before after spinoculation. Inhibition kinetics graphs represent averaged infection levels normalized to no inhibitor controls. Error bars represent SEM.

3.4.6 - HIV Integration Sequencing

(Adapted from Bushman 3’ LTR integration protocol) 5 x 10⁶ primary CD4+ T cells were seeded per well in 96 well plate format and incubated in the presence or absence of 10 µM acetyltransferase inhibitors for 4 hours. Cells were subsequently infected with 125 ng p24 equivalent CXCR4-tropic reporter virus at 1200g for 2 hours. (Importantly, viral stocks were pre-treated with 100 U/ml benzonase nuclease and 10x buffer (containing 500 mM Tris-HCL, pH 8.0, 10 mg/ml MgCL₂, 1 mg/ml BSA) at 37° C for 30 minutes to remove residual
plasmid DNA carried over from transfection. Following centrifugation, cells were isolated every 12 hours, washed twice with 1X PBS, and frozen at -20° C in RIPA buffer. Genomic DNA was isolated from all samples using the DNeasy Blood and Tissue kit (Qiagen) following manufacturers protocol.

DNA was digested using MseI and BglII fast digest endonucleases (ThermoFisher) for 5 minutes at 37° C. Reaction was inactivated at 70° C for 10 minutes after. Digested DNA was ligated to phosphorylated MseI linker primer (Plus strand - 5’-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3’, minus strand - 5’-TAGTCCCTTAAGCGGAG-3’) at room temperature using quick ligation kit (ThermoFisher). Reaction was inactivated at 70° C for 10 minutes after. Ligated product was PCR amplified with primers corresponding to HIV 3' LTR and MseI linker regions (Forward - 5’-AGTGCTTCAAGTAGTGTGCC-3'; Reverse - 5’-GTAATACGACTCACTATAGGGC-3'). A subsequent nested round of PCR was performed with sequencer primers and barcoded adaptors (Forward GEX - 5’-CAAGCAGAAGACGGCATACGAGAGTCTGTTGTGTGCC-3'; Reverse TrP - 5’- CCTCTCTATGGGCAGTCGGTGAT - 3'). Final PCR products were pooled, concentrated, and run on a 1.5% agarose gel. Bands sized between 200-400 were excised and gel purified. In parallel, equal concentrations of DNA were used for amplification of control GAPDH gene. Integrants and GAPDH control were pooled and sequenced using the Ion Torrent PGM sequencer according to manufacturer recommendations. HIV integrants
were quantified using CLC Genomics Workbench 5 and normalized compared to control gene amplicons.

### 3.4.7 - Reactivation of Latent HIV

Latently infected Jurkat 2D10 cells harboring H13L tat mutations were used as described previously. Cells were maintained in RPMI medium supplemented with L-glutamine, 10% FBS, and 1% Penicillin/Streptomycin. Cells were treated with HAT inhibitors overnight and subsequently stimulated with mock, 500 mM SAHA, 10 ng/ml TNF alpha, or 1 µg/ml ionomycin as a negative control for 24 hours. Cells were plated at 10^6 per well in a 96 well plate and analyzed for EGFP expression by FACS.

### 3.4.8 - Proteomic Approach to Characterize Acetylation in Response to Acetyltransferase Inhibition

Primary unstimulated CD4+ cells were incubated in the presence or absence of 10 µM garcinol, curcumin, and anacardic acid for 4 hours. Following treatment, cells were lysed in 2% SDS containing protease and phosphatase inhibitors. Lysates were cleared using previously established FASP methods and digested with trypsin and lys-C. An unenriched fraction was reserved for proteomic analysis.

Following digest, 246 µg of each sample was incubated with 50 µl agarose conjugated acetyl-lysine antibody (ImmuneChem) that had been washed with
buffer (50mM MOPS, 10mM Na2HPO4, 1M NaCl pH 7.2) and blocked with 1.5% BSA. Samples were placed on an orbital rocker overnight at 4° C. The following day, samples were washed 4x with buffer, briefly vortexed, and centrifuged at 2000g for 2 minutes. After final wash and aspiration of buffer, acetylated peptides were eluted in 40 µl 1% TFA and supernatants were reserved following centrifugation. Two subsequent rounds of elution were performed for a total volume of 120 µl per sample. Eluates were dried in speedvac and prepared for mass spectrometry. Spectral counts were used to infer relative changes in protein abundances.

3.5 - Results

3.5.1 - Histone Acetyltransferase Inhibitors Garcinol and Curcumin Reduce Susceptibility to HIV Productive Infection

Unlike the large breadth of histone deacetylase inhibitors, there are very few inhibitors that target the histone deacetylase class of enzymes. We selected two compounds, garcinol and curcumin, for their known effects on p300/CBP and PCAF (as was previously discussed in section 1.6, HIV tat interacts with HATs p300 and PCAF, resulting in recruitment to the LTR promoter and increased proviral transcription). Both naturally occurring compounds were originally identified in plant species and later found to have acetyltransferase inhibition activity. Additionally, we chose to examine curcumin because it been previously
reported to have effects on HIV-1 infection (though these studies are weakly convincing and provide little to no mechanistic detail).

To test the potential the effects of both garcinol and curcumin on HIV-1 infection, we first relied on our previously established combination reporter virus assay. In brief, we infected primary unstimulated CD4+ T cells with CXCR4-tropic reporter viruses following a 4-hour pretreatment with each inhibitor. Initial experiments showed that treatment with both garcinol and curcumin yielded significant reduction in HIV productive infection (LTR-driven EGFP expression, figure 18A).

Figure 18: Garcinol and Curcumin Reduce Cellular Susceptibility to HIV Productive Infection. Primary unstimulated CD4+ T cells were infected or absence of 10 μM histone acetyltransferase inhibitors garcinol and curcumin. Both garcinol and curcumin significantly reduce HIV productive infection, as seen in A. representative plots, and B. compiled data from multiple healthy donors.
(P< 0.0004 and < .0001, respectively). Experiments were performed on a minimum of three patients in triplicate. Error bars represent SEM.

When experiments were repeated across multiple donors we observed a minimum 50% to 70% reduction in productive infection with garcinol and curcumin, respectively (figure 18B). This indicated that both compounds have antagonistic effects on HIV infection within our system. We next sought to determine whether these effects were sensitive to inhibitor concentration or specific time-of-addition. As expected, we observed both dose- (figure 19A) and time- (figure 19B) dependent effects of garcinol and curcumin, where curcumin exhibited more potent effects at 5 and 10 µM. Interestingly, both compounds exhibited highest efficacy when added 4 hours prior to addition of virus, as opposed to 24 or 48 hours post infection.

Figure 19: Garcinol and curcumin exhibit potent dose and time dependent effects. A. HAT inhibitors garcinol and curcumin were evaluated across a range of concentrations (0.5625-10 µM; N.D.= No drug), revealing dramatic dose dependent effects of both compounds. B. Time of addition
experiments reveal that HAT inhibitors exhibit highest effects prior to addition of virus, as opposed to 24 and 48 hour post infection. For these experiments 10 µM of each inhibitor was used for maximal effect.

Seeing as our combination reporter assay can only assess a single round of viral replication, we also chose to examine the effects of garcinol and curcumin on a replication-competent HIV infection (Figure 20). For these experiments, primary CD4+ T cells were incubated with either 100 IU/mL IL-2 or a combination of peptide caspase inhibitors in efforts to reduce death of primary cells during prolonged culture. Cells were subsequently infected with a replication competent CXCR4-tropic reporter virus (HIV-NLG-Nef) in the presence or absence of HAT inhibitor. Following infection, cells were incubated at 37°C for up to 14 days. At the indicated times, cells were isolated to determine EGFP expression by FACS.
Primary CD4s (100 IU/ml rIL-2)

- Untreated + IL-2
- Garcinol + IL-2

Primary CD4s (Caspase Inhibitor Cocktail)

- Untreated + CI
- Garcinol + CI

Figure 20: HAT inhibition delays and reduces infection of replication-competent HIV. Primary CD4+ T cells were cultured with 100 IU/mL rIL-2 or 20 μM caspase inhibitors Z-VAD-FMK/Z-IETD-FMK and subsequently spinoculated with CXCR4-tropic replication competent HIV. Cells were harvested at days 1, 3, 5, 7, 9, 11, and 13 post-infection and analyzed for EGFP production by FACS. Pretreatment with HATi garcinol delayed productive infection in both culture conditions and eventually reached untreated levels by d13. Conversely, curcumin treated cells were significantly less susceptible to replication-competent virus over the course of the two-week experiment. Experiments performed in triplicate. Error bars represent SEM.

As expected, treatment with both garcinol and curcumin significantly reduced EGFP expression of replication-competent HIV (Figure 20). The initial dampening of EGFP in garcinol treated cells was reversed by day 13, suggesting a loss of drug potency in prolonged culture or the ability of viral replication to overcome the action of garcinol. However, curcumin-treated cells were strikingly
resistant to a replication-competent infection regardless of IL-2 or caspase inhibitor culture conditions.

It is important to note the temporal differences in EGFP accumulation between the IL-2 and caspase inhibitor populations. We feel that the two "peaks" observed in IL-2 cultured cells represents only two rounds of viral replication over the course of this assay. This is not all too surprising given the slower kinetics of viral replication in primary cells compared to activated cells or cell lines. Interestingly, cells cultured with caspase inhibitors exhibited an exponential rise in EGFP positivity past day 5, suggesting that caspase inhibition allowed for survival of the first-round infected cells or allowed for a greater number of replication cycles to occur by day 13. Regardless, these data demonstrate that both garcinol and curcumin can both delay and significantly reduce EGFP production in a replication competent virus system (albeit curcumin to a much higher extent).

3.5.2 - Histone Acetyltransferase Inhibitor Treatment Does Not Impact Early Viral Replication Events

To investigate the potential molecular mechanisms of garcinol and curcumin, we next examined the levels of fusion with both CCR5 and CXCR4-tropic reporter viruses. In both cases, pretreatment with HAT inhibitors did not significantly affect HIV fusion (Figure 21A). In parallel with these experiments,
we analyzed HIV co-receptor expression on T cells which showed no significant alteration in CCR5 or CXCR4 density (Figure 21B). Taken together, these results demonstrate that garcinol and curcumin treatment regulate HIV infection at a post-fusion step independent of viral fusion or tropism.

**Figure 21:** HAT inhibitor treatment does not affect viral fusion with CD4+ T cells. **A.** Primary unstimulated CD4+ T cells were infected with either CCR5 or CXCR4-tropic reporter viruses in the presence or absence of HAT inhibitors and analyzed for βlam-mediated cleavage of CCF2 fluorescent substrate. Treatment with garcinol or curcumin did not significantly alter viral fusion. **B.** T cells treated with HAT inhibitors have unchanged surface expression of CCR5 and CXCR4 co-receptors. Experiments performed in triplicate. Error bars represent SEM.

In our previous studies of histone deacetylases (HDACs), we uncovered that treatment with HDAC inhibitors dramatically enhanced HIV productive infection. As a consequence, we were interested in whether HAT inhibitor
treatment could reverse HDAC inhibitor mediated enhancement of infection. In agreement with this, treatment with garcinol antagonized both butyrate and SAHA mediated enhancement of LTR-driven EGFP expression (Figure 22A). In some instances, HATi treatment reduced infection below untreated controls.

As was previously discussed, our investigation of HDAC inhibitors revealed that cytoplasmic inhibition of HDAC6 recapitulated the effect seen with other broad-spectrum inhibitors. Specifically, our studies demonstrated that inhibition of the tubulin deacetylase domain in HDAC6 promoted microtubule acetylation and subsequently increased HIV productive infection. Importantly, HDAC6 is not the sole regulator of cellular microtubule acetylation. In recent years, several groups identified that the primary enzyme responsible for promoting tubulin acetylation is alpha-tubulin acetyltransferase 1 (αTAT1) [243]. Like HDAC inhibitors, we reasoned that HAT inhibitors may also have multiple non-histone targets and that these compounds may act directly on cytoplasmic lysine acetyltransferases such as αTAT1. Specifically, we were interested in whether treatment with HAT inhibitors may be reducing HIV infection in part by inhibiting microtubule acetylation. We were also interested as to whether this is directly related to their antagonistic effect on HDAC inhibitors. Western blot analysis revealed that neither garcinol nor curcumin reduce HDACi mediated enhancement of microtubule acetylation (Figure 22B). However, we feel this result does not exclude potential interactions between HAT inhibitors and microtubule acetylation in the context of active viral replication.
Figure 22: HATi antagonist HDACi-mediated enhancement of infection independent of tubulin acetylation. A. Treatment of CD4+ T cells with the HAT inhibitor garcinol antagonizes butyrate and SAHA-mediated enhancement of HIV infection. B. HATi treatment does not reverse HDACi-mediated upregulation of microtubule acetylation. Protein abundances were quantified by densitometry using ImageJ analysis.

In our previous analysis of vorinostat, we also observed that enhanced microtubule acetylation was coupled with an enhancement in the kinetics of post-entry reverse transcription and integration steps. To further exclude a cytoplasmic role for the opposing HAT inhibitors in the context of HIV infection, we performed time of addition experiments by addition of efavirenz (reverse
transcription inhibitor) or raltegravir (integrase inhibitor) at indicated times following treatment with garcinol and infection with CXCR4-tropic HIV (Figure 23). One would expect a delay in kinetics if HAT inhibition had cytoplasmic effects during viral replication. In support of our analysis of microtubule acetylation, we did not observe significant changes in reverse transcription ($t_{1/2} = 40.50h$ vs $41.40h$) or integration ($t_{1/2} = 43.51h$ vs $44.01h$) kinetics following treatment with garcinol. This data indicates the effects of garcinol are downstream of these replication stages.

Figure 23: Garcinol does not alter kinetics of post-entry reverse transcription and integration. Primary unstimulated CD4+ T cells were infected with CXCR4-tropic reporter viruses in the presence or absence of garcinol. At various time point after infection, reverse transcriptase inhibitor efavirenz or integrase inhibitor raltegravir were added. EGFP was normalized to uninfected controls and reflected as percent inhibition. $R$ squared values for all curves greater than 0.88. Error bars represent SEM.
3.5.3 Analysis of HIV Integration

One early investigation into HAT inhibitors reported that HIV integrase is directly inhibited by high concentrations of curcumin (>40 µM) [244]. It was determined much later that integrase is directly acetylated by cellular histone acetyltransferases, and that acetylation enhances 3' hydroxyl processing and viral strand transfer[245]. Taken together, it is reasonable to hypothesize that HAT inhibition by garcinol or curcumin reduces productive infection by decreasing the acetylation of integrase and subsequent strand transfer efficiency.

To test this hypothesis in our system, we employed next generation sequencing to quantify HIV integrants in the presence or absence of each inhibitor (Figure 24). In brief, this method relies on sequential restriction endonuclease digests and amplification of DNA spanning the 3' LTR into a short region of the host genome. The use of barcoded adaptors allows us to multiplex conditions onto a single chip, largely improving efficiency and preventing variability between sequencing runs. The added benefit of sequencing over PCR quantification is to ensure specificity alongside chromosomal location.

Preliminary analysis of HIV integrants revealed that garcinol and curcumin treatment did not decrease the number of sequence reads (Figure 24). In fact, these data suggest that both compounds act to increase integration, as read count and number of unique integration sites were increased by garcinol and curcumin treatment (Integration sites are synonymous with number of "contigs", or consensus regions of overlapping reads obtained from each experimental
A single consensus region consisting of multiple reads can be viewed as a single integration site in the genome). This result will require replication with additional controls for confirmation. Additionally, mapping integration sites will shed light on whether HAT inhibitor treatment alters integration into active transcription units (out of the scope of this current study).

**Figure 24: Analysis of HIV integration following HAT inhibition.** Preliminary analysis of HIV integrants by next generation sequencing shows HAT inhibition treatment does not inhibit integrase and integration activity. Rather, this data supports the converse, in that garcinol and curcumin treatment increased HIV integration and number of unique integration sites (overlapping consensus sequences, or contigs). Sequencing was performed using an Ion Torrent PGM and analyzed by CLC Genomics Workbench 5.

3.3.4 - Reactivation of Latent HIV in Response to HAT Inhibition

Analysis of HIV integration revealed that HAT inhibition does not reduce levels of integrated DNA. This allowed us to focus more closely on post-integration events. All HAT inhibitors tested in this study target p300 and PCAF,
two histone acetyltransferases with known interactions with Tat at the HIV-1 LTR[153, 154, 205]. Knockdown studies of p300 and PCAF result in a reduction of Tat transactivation, suggesting that histone architecture at the viral promoter play a significant role in downstream viral transcription [246]. We chose to probe the question of HAT inhibition and HIV transcription in the context of a latently infected jurkat T cell model 2D10 [158]. This latently infected clone has been previously described and contains a largely excised integrated provirus, containing only tat, rev, vpu, env, and EGFP in place of nef. In this model, unstimulated cells express less than 10% spontaneous EGFP. This is in stark contrast to greater than 90% EGFP following treatment with an activator such as TNF-α. We hypothesized that pretreatment of 2D10 cells with HAT inhibitors prior to stimulation with an activator or latency reversing agent would decrease the "reactivatability" of these cells, evident by reduced EGFP expression. This would suggest HAT inhibitors are in part acting on histone architecture or by interactions with viral Tat. In disagreement with this hypothesis, treatment with HAT inhibitors did not reduce reactivation of latent HIV by HDAC inhibitors or TNFα (Ionomycin alone included as additional negative control). Unexpectedly, curcumin treatment boosted EGFP in all conditions and was able to reactivate HIV from latency 30% in the absence of stimulation (Figure 25). This data suggest that HAT inhibition does not significantly prevent transcription/reactivation of integrated proviruses (though we recognize the
2D10 model has several caveats when comparing it to our combination reporter virus system).

Figure 25: HAT inhibition does not prevent reactivation of HIV. HAT inhibitors garcinol, curcumin, and anacardic acid were tested for their ability to reactivate latent HIV in the jurkat 2D10 cell model. Following 18-hour treatment with HAT inhibitor, cells were stimulated with the HDAC inhibitor SAHA, activator TNF-alpha, or negative control ionomycin and incubated overnight prior to FACS analysis. Error bars represent SEM. asterisk represent p value < 0.005.

3.3.5 - Proteomic Analysis of Acetylation

We initially hypothesized that the antiviral effects of HAT inhibitors were due to epigenetic modifications of histones mediated by the inhibition of p300 and PCAF. Inhibition of these HATs reduces transcription of integrated viral
DNA. However, more recent experiments with an additional histone acetyltransferase inhibitor anacardic acid showed no change in HIV productive infection (Figure 26A). Anacardic acid also inhibits relevant HATs p300 and PCAF, questioning the role of p300 and PCAF in the antiviral effects of garcinol and curcumin. This data suggest that inhibition of lysine acetylation might reduce HIV productive infection independent of a direct histone acetyltransferase interaction. Indeed the promiscuity associated with many small molecule inhibitors would favor the notion of additional unknown targets. Consequently, we considered that other host proteins might be differentially acetylated in the presence of garcinol or curcumin. Identifying the scope of acetylated proteins would therefore help shed light on the molecular mechanisms behind these inhibitors.

To investigate the targets of garcinol and curcumin, we developed a mass spectrometry-based approach to enrich and quantify cellular acetylated proteins (Figure 26B). In brief, we subjected primary unstimulated cells to treatment with a mock control, garcinol, curcumin, or anacardic acid. Following 4-hour treatment, peptide libraries were generated using trypsin and lys-C digest. A fraction of total peptides was reserved for analysis and remaining peptide volumes were enriched with a pan acetyl-lysine antibody. Proteomic analysis will allow us to compare the abundances of total and acetylated proteins in each treatment condition.
An initial pilot experiment demonstrated that total protein levels were largely unchanged, suggesting that global transcription is likely unaffected. Proteomic analysis of acetylated proteins revealed that enrichment with acetyl-lysine antibody was successful. However, less than 70 acetyl proteins were identified in all conditions, suggesting low input of peptides into the spectrometer (data not shown). We also recognize that unstimulated primary cells may contain low abundances of acetylated proteins. Unfortunately, the low detection of this portion of the study prevented accurate comparison of each treatment condition. As such, the data is currently excluded from this analysis. We have since redesigned this experiment to include prolonged inhibitor treatments with the hopes of detecting maximal changes in response to each compound. We have also chosen to include HIV-infected conditions, as we are very interested in virally-induced alterations of cellular acetylation patterns. We believe this redesigned approach will allow us to more accurately determine the targets of HAT inhibitors in the presence and absence of HIV infection.
Figure 26: Identifying acetylated targets of HAT inhibitors. **A.** Evaluation of an additional p300/PCAF inhibitor anacardic acid revealed no significant effect on productive infection, suggesting non-HAT proteins are responsible for the effects observed with garcinol and curcumin. **B.** Workflow for identification of acetylated protein targets. CD4+ T cells are treated with indicated inhibitors and digested with trypsin and lys-C to generate peptide libraries. Peptides are enriched using pan acetyl-lysine antibodies and quantified by label free mass spectrometry. A fraction of unenriched total peptides is also reserved for proteomic analysis. Identification of differential protein expression and acetylation patterns between control, garcinol/curcumin, and negative control anacardic acid will determine the overall targets of these inhibitors.
Discussion

In this study, we sought to investigate the ability for histone acetyltransferase (HAT) inhibitors garcinol and curcumin to reduce susceptibility of primary CD4+ T cells to productive infection by HIV-1. This is a direct result of our previous studies demonstrating that inhibition of histone deacetylases significantly increases viral productive infection 200-300% [228]. In agreement with our hypothesis, both HAT inhibitors (garcinol and curcumin) were found to reduce HIV infection in a dose- and time-dependent fashion. Viral fusion was unaffected by HAT inhibitor treatment (supported by unchanged levels of CCR5 and CXCR4 expression on the T cell surface). Given that our previous studies with HDAC inhibitors suggested that microtubule acetylation was involved in promoting infection, we were interested in whether garcinol and curcumin will oppose this mechanism. However, HAT inhibition did not reduce microtubule acetylation despite the ability for these compounds to counteract HDAC inhibitor mediated enhancement of EGFP expression. Time-of-addition experiments assessing the kinetics of reverse transcription and integration also supported these results.

Pinpointing the exact mechanism of these compounds has proven challenging. Both garcinol and curcumin are reported to effectively inhibit histone acetyltransferases p300 and PCAF at the concentrations evaluated in this study. Importantly, p300 and PCAF have been shown to interact directly with
Tat and acetylate Tat at critical residues to regulate its activity[247]. For example, PCAF-mediated acetylation at lysine 28 leads to 3-fold enhancement of cyclin T1 interactions [248]. Cyclin T1 is a critical component of the p-TEFb complex recruited by Tat to promoter proximal paused RNAPII, enhancing viral transcription [249]. We sought to evaluate the ability of garcinol and curcumin to inhibit viral transcription in this manner by utilizing a latently infected jurkat T cell model carrying a fluorescent reporter EGFP. Neither garcinol nor curcumin prevented reactivation of integrated proviruses in this system when stimulated with TNF alpha or the HDAC inhibitor vorinostat. This data suggest that inhibition of PCAF and p300 and their interactions with Tat do not play a role in our observed effect. However, these experiments do not exclude the potential HAT inhibitor mediated condensation of chromatin resulting in dampened global transcription. Furthermore, the impact of p-TEFb in this latent 2D10 model should be assessed. Quantification of cellular gene expression and ChIP analysis of relevant nucleosomes in the HIV promoter would also shed light on these issues.

We believe our experiments with the HAT inhibitor anacardic acid support the hypothesis that p300 and PCAF are not responsible for this effect. Coupled with the well-documented promiscuity of many small molecule inhibitors, we hypothesized garcinol and curcumin may be targeting a currently unknown protein or cellular acetyltransferase. To test this possibility, we developed a mass spectrometry based approach to quantify the total and acetylated protein
abundances in response to treatment with HAT inhibitors garcinol, curcumin, and anacardic acid. Pilot experiments revealed lackluster results in regards to acetylated targets. Fewer than 70 acetylated proteins were identified in all conditions following enrichment with pan-acetyl lysine antibody. While this confirmed enrichment was successful, the low input of acetyl peptides in the spectrometer prevented accurate comparison between each treatment conditions. We are in the process of repeating this experiment in efforts to improve our detection.

Despite a lack of concrete mechanism, HAT inhibition reduces HIV productive infection in both single cycle and replication competent infections. Our experiments with curcumin showed a striking effect to prevent multiple rounds of viral replication. We believe this demonstrates a proof of concept principle that targeting cellular machinery to reduce HIV infection is possible. In cases of sexual HIV transmission, the local amplification of virus in mucosal CD4+ T cells has been shown to determine likelihood of dissemination [250, 251]. Reduction of productive infection by 50-80% (as seen with garcinol and curcumin) would significantly dampen the localized amplification of virus within mucosal T cells and could reduce or prevent dissemination. Overall, designing novel PrEP strategies that target cellular machinery would be an added defense against HIV transmission alongside direct acting anti-retroviral drugs.
CHAPTER FOUR:

A SMALL MOLECULE INHIBITOR SCREEN FOR HOST FACTORS
REGULATING CELLULAR SUSCEPTIBILITY TO HIV-1 INFECTION

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4.1 - Abstract

HIV-1 infection requires substantial use of host machinery to complete its replication cycle. Examples of this include cytoskeletal proteins for transport, importin proteins for nuclear import, transcription complexes for viral gene expression, and the ESCRT pathway for particle assembly and budding. In some instances, post-translational modifications of cellular proteins directly impact their function in these processes. We previously reported that inhibition of histone deacetylase inhibitors promotes HIV-1 replication by a mechanism in part involving acetylation of microtubules. Conversely, the reduction of cellular acetylation by inhibition of histone acetyltransferases yielded an opposing effect on viral infection efficiency. To uncover epigenetic or post-translational modifications associated with HIV-1 replication that fall outside the "axis" of acetylation, we conducted a flow-cytometric based screen to assess the effect of 80+ small molecule inhibitors on viral infection. Preliminary analysis exposed multiple mechanisms modulating HIV productive infection, including histone deacetylation, histone and DNA methylation, bromodomain family inhibition, and Rho GTPase family signaling. Importantly, several of these protein families are being clinically investigated as latency reversing agents and will merit further testing to exclude potential off-target effects. The identification of Rho and related Rho associated kinase (ROCK) signaling has sparked sizeable interest as this network was also identified in a related HIV-1 phosphoproteomics study.
Our outcomes from this screen are of two-fold importance. Firstly, it is apparent that epigenetic control of HIV infection extends beyond the currently known effects on viral transcription and latent reactivation; secondly, the use of low-cost pharmacological libraries coupled with medium-throughput analysis should not be overlooked as a method to identify novel mechanisms underlying host-pathogen interactions.
4.3 - Introduction

Despite our increased understanding into the pathogenesis of HIV-1/AIDS, an estimated 2 million new infections arise every year [12]. The advent of highly active anti-retroviral drugs has dramatically improved quality of life for those infected; however, current therapies are focused on targeting HIV-1 enzymes directly and introduce chance for development of escape mutations. Indeed this can be a significant problem in cases of ART monotherapy, scheduled treatment interruption, or general lack of adherence. Designing ART therapies that additionally target host proteins required for viral replication would be highly advantageous, as this would lessen selection pressure for the virus to acquire resistance. As such, identifying novel cellular factors that impact infection would be significant two-fold: Not only to increase our understanding of basic HIV-1 biology but to also open new therapeutic avenues for exploration.

The pursuit to expose both HIV-1 restriction and dependency factors is not a new one. There have been numerous high-throughput siRNA screens that have proven fruitful [252-258]. Most significant were the studies conducted by Brass et al, Zhou et al, and Konig et al. The first study utilized CD4-expressing HeLa cells in conjunction with a two-part siRNA screen. In short, siRNA-transfected cells were infected with lab adapted HIV and probed for intracellular p24 capsid expression as a surrogate for early post-entry event efficiency. To examine for late stage effects, infected cell supernatants were transferred to a
viral Tat-responsive cell line and analyzed for reporter gene expression. From their results, components of transcription machinery were identified, as well as previously unknown golgi transport and import proteins.

The second study by Zhou et al utilized a similar experimental approach with a large siRNA library. Despite identification of 232 genes that differentially regulated HIV replication, only 15 overlapped with that of the previous Brass study. However, the small overlap was noteworthy as once again similar transcription machinery was identified.

The final study by Konig chose a slightly altered approach, utilizing a single-cycle firefly luciferase expressing HIV construct coupled with two siRNA libraries. This study focused strictly on early events leading to viral protein synthesis and sought to characterize effects of siRNA knockdown on early reverse transcription, late reverse transcription, 2-LTR circle production, and integration. To strengthen their results, Konig and colleagues compared their results to pre-existing HIV screens, known host interaction networks, and gene array data sets. The significance of their comparative analysis should not be overlooked, as the authors suggest this reduced the likelihood of pursuing false-positive siRNA hits. This study identified ~60 genes in relation to the previous Brass report, as well as an additional 64 genes as being an interacting partner. Most significantly, the authors highlighted the roles of several cytoskeletal and nuclear import proteins during replication.
We chose to mention these milestone knockdown screens for several reasons. Despite the abundance of data acquired by each study, the slim degree of overlap amongst them was met with considerable confusion and inquiry. It was assumed that bonafide dependency or restriction factors would be identified across multiple studies, though it is possible that variables such as cell type and virus strain can perturb true comparisons. Along with the difficulties of data analysis, there are many technical challenges in performing these large genome-wide siRNA screens. The sizeable cost of libraries coupled with the task of experimental setup can be overwhelming. Furthermore, the difficulty of performing siRNA/RNA knockdown studies on primary cells forces the hand to a more convenient and less physiologically relevant cell line or infection model. At the end of the screen, finding scarce needles in the hefty haystack can require extensive validation to confirm a single observation.

To contrast these studies, several groups have taken to a different approach to identify potential HIV-cellular interactions [259, 260]. Budhiraja et al. chose a systems biology platform to analyze a large-scale immunoprecipitation mass spectrometry study that was performed in HeLa cell extracts [261]. Specifically, they focused on what they term the "nuclear complexome", with the goal of identifying cellular factors that regulate HIV mRNA export. By narrowing focus onto proteins that complex with the known Rev-interacting protein CRM1, the authors identified novel roles for the cellular protein RBM14 and long-noncoding RNA NEAT1 in HIV-1 replication. Most
significantly, this study demonstrated that a more focused exploration of previously generated data could uncover new HIV dependency factors and potential therapeutic targets.

A final study deserving of attention involved the roles of interferon-stimulated genes (ISGs) during viral replication [260]. In a very purposeful manner, Schoggins et al. generated a focused library of lentiviruses encoding single ISGs (389 in total). Following transduction, cells were infected in parallel with a panel of fluorescent reporter viruses including HIV-1, hepatitis C, yellow fever, venezuelan equine encephalitis virus, and chikungunya virus. Cell populations positive for ISG expression with an appreciable change in infection were selected as significant. Most notably, ISGs corresponding to innate immune sensors elicited broad-spectrum inhibition of viruses while several candidate ISGs such as ADAR, IDO1, and LY6E had surprising effects to amplify viral reporter expression. The original findings reported by Schoggins and colleagues points to yet another method of identifying host factors; one that does not require as many challenges as whole-genome knockdown/out studies.

Of all the studies compared above, a notable limitation is their reliance on measuring viral replication in response to a protein's absolute abundance. *These method lack the ability to examine the role of any post-translational or epigenetic modifications during HIV infection.* It is well established that post-translational and epigenetic machinery can have profound effects on cellular and pathogenic functions. For instance, it has been demonstrated that viruses induce numerous
cytoplasmic signaling events, many of which are mediated by phosphorylation of proteins [89, 262-264]. More recently, one group has examined this directly by analyzing the phosphorylated proteome of primary CD4 T cells following exposure to HIV-1 [265]. Their study identified more than 200 phosphorylation sites that were differentially expressed following exposure to HIV-1. Importantly, the majority of their findings would not be revealed by conventional genetic knockdown approaches.

Our findings with histone deacetylase and histone acetyltransferase inhibitors have uncovered a novel role for epigenetic/post-translational modification, particularly acetylation, in modulating the susceptibility of CD4+ T cells to HIV infection. Treatment with the HDAC inhibitor vorinostat revealed a significant increase in the susceptibility of cells to HIV productive infection. Further examination pointed to a cytoplasmic effect of this drug on histone deacetylase 6, a mechanism we believe related to its regulation of microtubule acetylation. Conversely, treatment with HAT inhibitors garcinol and curcumin had an opposing effect on HIV gene expression, which may have potential impacts on our understanding of viral transcription. Together these data highlight that cellular acetylation acts as a "seesaw" regulator of HIV-1 replication.

It has also been shown previously that other post-translational and epigenetic modifications play a role in HIV. For example, myristylation of matrix protein is critical for plasma membrane interaction and Gag recruitment [41]. HIV-1 integrase is subject to numerous post-translational modifications and has
ben more recently shown to be significantly controlled by SUMOylation [266]. Ubiquitination of HIV proteins can either signal their degradation or assist the virion budding process [135, 143, 267]. At the transcriptional level, epigenetic modifications greatly impact chromatin structure and recruitment of transcriptional machinery [201, 268]. CpG methylation of HIV promoters regulates maintenance of latency by increasing resistance to reactivation with latency reversing agents [157]. Additionally, the histone lysine methyltransferase EZH2, a component of polycomb repressive complex 2, is induced during latency [158].

Based on our findings with HDAC and HAT inhibitors, we sought to investigate additional post-translational/epigenetic modifying compounds for their ability to alter susceptibility of CD4+ T cells to HIV-1 fusion and LTR-driven EGFP expression. To achieve this, we selected a commercially available panel of small molecule inhibitors and tested them within our combination reporter virus system. Of all compounds tested, we identified the histone lysine methyltransferase chaetocin as a potential regulator of viral fusion. 39 compounds yielded >1.5 fold change in HIV productive infection (EGFP expression). Most notably, all histone deacetylase inhibitors tested increased cellular susceptibility (confirming our previous studies with vorinostat). Conversely, inhibitors of histone and DNA methylation predominated those compounds that reduced infection. Small molecule CCG-100602, which has been shown to inhibit Rho kinase signaling, was identified as a negative regulator of
HIV productive infection. Coupled with identification of the Rho signaling pathway in our recent HIV phosphoproteomics screen, this result stresses the importance of cytoplasmic signaling in mediating viral replication. Here we present our initial findings with the small molecule inhibitor library as well as validation of significant hits from our analyses.

4.4 - Methods and Materials

4.4.1 - Small Molecule Inhibitors

The 96-well "epigenetic screening library" was acquired from Cayman Chemical and was stored at -20°C as 10 mM stocks in DMSO (Item 11076, Batch 0449323). Small molecule inhibitors were serially diluted in RPMI 1640 with 10% FBS and 1% PenStrep for a final effective range of 10 nM to 10 µM. Dilutions were performed immediately prior to experimental setup to minimize freeze-thaw cycling and to prevent precipitation from solution.

4.4.2 - Infection Experiments

1 x 10^6 unstimulated primary CD4+ T cells were plated per well in a 96-well format and incubated for 4 hours at 37°C with media alone or media supplemented with 10 nM, 100 nM, 1 µM, or 10 µM of indicated small molecule inhibitor. Identical plates were prepared for viral fusion and productive infection analysis. Following incubation with small molecule inhibitors, cells were infected
with HIV-1 reporter virus strain NL4-3-deltaE-EGFP bearing CXCR4-tropic envelope JOTO.TA1.2247. Plates were spinoculated for two hours at 1200g and 25°C. Following centrifugation, cells were incubated at 37°C for 1 hour. The fusion plate was washed, loaded with CCF2-AM, and incubated overnight at room temperature as previously described. Productive infection plates were incubated at 37°C for a total of 72 hours prior to staining and analysis.

4.4.3 - Flow Cytometry

Cells were washed once with PBS containing 1% BSA and incubated with live/dead near-IR fixable viability dye (Invitrogen), CD3 Brilliant Violet 650 (BioLegend), and CD4 Allophycocyanin (eBioscience) at 4°C for 30 minutes. Cells were washed in PBS/BSA and fixed in PBS/BSA containing 1% paraformaldehyde prior to analysis. All samples were acquired using a BD Fortessa cell analyzer equipped with a High Throughput Sampler option. A minimum of 50,000 events was collected per sample at a flow rate of 2.5 µL/sec with 50 µL mixing and 3 x 200 µL washes between each collection. All experimental conditions were performed in triplicate. FlowJo version 9.7.6 (TreeStar, Inc) was used for analysis. For downstream validation of top hits, experiments above were mirrored in triplicate on a minimum of 3 separate healthy donors.

4.4.4 - βlam-mediated Cleavage of CCF2 in HEK293T Cells
Human embryonic kidney 293T/17 cells were seeded in 10 cm² dishes at a density of 3 x 10⁵ cells per dish. Cells were transfected with 7.5 mg β-lactamase vpr plasmid using previously establish calcium phosphate methods. Fresh media was added 6 hours post transfection. At 48 hours post transfection, cells were incubated with media alone or media containing the small molecule inhibitor chaetocin (10 µM) for 4 hours. Cells were subsequently loaded with CCF2-AM for 2 hours at room temperature and incubated overnight in CO2-independent media containing probenecid. The following day, cells were trypsinized, stained with viability dye, and analyzed for evidence of βeta lactamase mediated cleavage of fluorescent CCF2 substrate.

4.4.5 - Phosphoproteomic Analysis of HIV-exposed CD4+ T Cells

Memory CD4+ T cells were purified by negative selection from a leukapheresis pack using custom RosetteSep kits (Stemcell, Inc). Briefly, equal populations of 150 x 10⁶ cells were exposed to 20 ug/ml p24 equivalent AT2-inactivated HIV-1 or to protein equivalent concentrations of non-viral microvesicles (for control). After stimulation for 1 minute, 15 minute, and 60-minute periods, ice cold 1X PBS containing protease inhibitors was added. Cells were washed, lysed, and digested with trypsin. Phosphopeptides were enriched with titanium dioxide and purified by long gradient UPLC. Enriched samples were analyzed by LC-MS/MS.
4.5 - Results

4.5.1 - Epigenetic Screening Library

Numerous epigenetic modifying compounds have been studied in the context of HIV-1 reactivation from latency. Most significantly, the histone deacetylase family of enzymes has become a leading target for pharmacological inhibition in efforts to eliminate viral reservoirs in patients. However, our lab has previously reported that \textit{in vitro} treatment of primary CD4+ T lymphocytes with broad-spectrum HDAC inhibitors including vorinostat and panobinostat significantly increases cellular susceptibility to infection by HIV-1. This observation was independent of the effect on latent reactivation. Conversely, we also investigated the effects of small molecule inhibitors garcinol and curcumin, both of which have been published to inhibit the opposing histone acetyltransferase enzymes. To probe for additional mechanisms modulating HIV-1 replication and to extend studies beyond acetyl-lysine post-translational modification, we employed the use of a small molecule inhibitor library in consort with our combination reporter virus system (\textbf{Figure 27A}). Compounds within this library have reported effects on (but not limited to) epigenetic machinery, kinase signaling, and BET protein family members. All compounds were screened using equal infection conditions (\textbf{Figure 27B}) and were analyzed across a 3-log concentration to minimize variability of half-maximal inhibitory concentrations in the library.
<table>
<thead>
<tr>
<th>Identifier</th>
<th>Compound Name</th>
<th>Reported or predicted IC50s</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>3-Amino Benzamidine</td>
<td>1.8 μM</td>
</tr>
<tr>
<td>A3</td>
<td>Ellagic Acid</td>
<td>600-700 nM</td>
</tr>
<tr>
<td>A4</td>
<td>UNC0638</td>
<td>&lt;15 nM</td>
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<tr>
<td>A5</td>
<td>Decitabine</td>
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</tr>
<tr>
<td>A6</td>
<td>Lomeguatrib</td>
<td>3 nM</td>
</tr>
<tr>
<td>A7</td>
<td>Tenoacin-6</td>
<td>21 μM (SIRT1) / 10 μM (SIRT2) / 67 μM (SIRT3)</td>
</tr>
<tr>
<td>A8</td>
<td>M 344</td>
<td>46 nM</td>
</tr>
<tr>
<td>A9</td>
<td>2′,3′,5′-triacetyl-5-Azacytidine</td>
<td>200 nM</td>
</tr>
<tr>
<td>A10</td>
<td>trans-Resveratrol</td>
<td>15 μM (peroxidase) / 3.7 μM (COX-1)</td>
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<tr>
<td>A11</td>
<td>CAY10591</td>
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</tr>
<tr>
<td>B2</td>
<td>SB 939</td>
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<td>B3</td>
<td>Suberohydroxamic Acid</td>
<td>250-300 nM</td>
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<td>B4</td>
<td>Isoliquiritigenin</td>
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<td>B5</td>
<td>(+)-JQ1</td>
<td>18-77 nM</td>
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<td>B6</td>
<td>Daminozide</td>
<td>500 nM - 2.1 μM</td>
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<tr>
<td>B7</td>
<td>Sodium Butyrate</td>
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<tr>
<td>B8</td>
<td>Oxamflatin</td>
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<td>B9</td>
<td>S-Adenosylhomocysteine</td>
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<tr>
<td>B10</td>
<td>2,4-DPD</td>
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<tr>
<td>B11</td>
<td>EX-527</td>
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<tr>
<td>C2</td>
<td>PCI 34051</td>
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<td>C3</td>
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<td>C4</td>
<td>CCG-100602</td>
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<td>C5</td>
<td>(-)-JQ1</td>
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<td>D3</td>
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<tr>
<td>D5</td>
<td>BSI-201</td>
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<tr>
<td>D6</td>
<td>GSK-J2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>D8</td>
<td>Mirin</td>
<td>66 μM</td>
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<td>D9</td>
<td>Chidamide</td>
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<td>D10</td>
<td>Trichostatin A</td>
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<td>D11</td>
<td>2-PCPA (HCl)</td>
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<tr>
<td>E2</td>
<td>Sirtinol</td>
<td>38 (SIRT2) / 131 μM (SIRT1)</td>
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<tr>
<td>-----</td>
<td>---------------</td>
<td>-----------------------------</td>
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<td>E3</td>
<td>(-)-Neplanocin A</td>
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<td>Zebularine</td>
<td>IC20 = 5-20 μM</td>
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<td>E5</td>
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<tr>
<td>E6</td>
<td>GSK-J4 (HCl)</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>E7</td>
<td>CAY10603</td>
<td>.002 nM - 7 μM</td>
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<td>E8</td>
<td>Pimelic Diphenylamide 106</td>
<td>150 nM - 5 μM (HDAC 1) / 180 μM (HDAC II)</td>
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<td>E9</td>
<td>3-Deazaadenosine A</td>
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<td>E11</td>
<td>1-Naphthoic Acid</td>
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<td>F2</td>
<td>C646</td>
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<tr>
<td>F3</td>
<td>CI-Amidine</td>
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<tr>
<td>F4</td>
<td>Delphinidin Chloride</td>
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<tr>
<td>F5</td>
<td>IOX1</td>
<td>1.7-20.5 μM</td>
</tr>
<tr>
<td>F6</td>
<td>GSK-J5 (HCl)</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>F7</td>
<td>Chaetocin</td>
<td>0.8-3 μM</td>
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<tr>
<td>F8</td>
<td>(S)-HDAC-42</td>
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</tr>
<tr>
<td>F9</td>
<td>N-Oxalylglycine</td>
<td>2.1-5.6 μM (PHD1/2)</td>
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<tr>
<td>F10</td>
<td>2,4-Pyridinedicarboxylic Acid</td>
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<td>Nicotinamide</td>
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<td>Tubastatin A (trifluoroacetate Salt)</td>
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<td>G4</td>
<td>PFI-1</td>
<td>98 nM (BRD2) / 0.22 μM (BRD4)</td>
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<tr>
<td>G5</td>
<td>Mi-2 (HCl)</td>
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<td>G6</td>
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<td>G7</td>
<td>Splitomicin</td>
<td>60 μM (SIR2p)</td>
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<tr>
<td>G8</td>
<td>MS-275</td>
<td>300 nM (HDAC-1)</td>
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<tr>
<td>G9</td>
<td>AMI-1 (sodium salt)</td>
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</tr>
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<td>G10</td>
<td>CAY10433</td>
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<td>G11</td>
<td>Sinelfungin</td>
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<tr>
<td>H2</td>
<td>Garcinol</td>
<td>7 μM (p300) / 5 μM (PCAF)</td>
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<td>H3</td>
<td>JBG1741</td>
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<tr>
<td>H4</td>
<td>5-Azacytidine</td>
<td>.2μM</td>
</tr>
<tr>
<td>H5</td>
<td>Mi-negative control(HCl)</td>
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<td>H6</td>
<td>Tenovin-1</td>
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</tr>
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<td>H7</td>
<td>CBHA</td>
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<td>H8</td>
<td>RG-108</td>
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<tr>
<td>H9</td>
<td>UNCl1215</td>
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</tr>
<tr>
<td>H10</td>
<td>Piceatannol</td>
<td>25 μM</td>
</tr>
<tr>
<td>H11</td>
<td>Suramin (sodium salt)</td>
<td>4.9 μM</td>
</tr>
</tbody>
</table>

Quantification of viral replication stages

Viral Fusion

Viral Productive Infection
Figure 27: Epigenetic Screening Library. A. Small molecule inhibitors were screened within the combination reporter virus system [71]. Compounds were tested with an assigned identifier (left column) to remove analysis bias. Listed half-maximal inhibitory concentrations (IC50) were tabulated from published cell culture and in vitro binding studies. B. Primary, unstimulated CD4+ T cells were incubated with small molecule inhibitors for 4 hours and infected with a CXCR4-tropic reporter virus. Preliminary analysis of viral fusion and productive infection will provide significant targets for downstream validation and quantification of changes during viral replication.

4.5.2 - Viral Fusion Analysis and the Small Molecule Inhibitor Chaetocin

As was predicted, the majority of compounds yielded little to no effect on viral fusion (Figure 28). For our preliminary analysis, compounds exhibiting greater than 1.5 fold change in either fusion or productive infection were flagged as a significant hit.

Figure 28: Effect of Small Molecule Inhibitors on Viral Fusion. Analyses of βlam-mediated CCF2-AM cleavage reveal little to no effects on CXCR4-tropic...
viral fusion in response to small molecule inhibition. A fold change greater than 0.5 fold change was selected as the cutoff for significant hits in the study. Experimental conditions were performed in triplicate. Error bars represent SEM.

Unexpectedly, incubation of CD4+ T cells with the small molecular inhibitor chaetocin yielded a significant decrease in viral fusion. Chaetocin is a fungal metabolite (Figure 29A) that non-specifically inhibits histone lysine methyltransferases. Based on previous reports with this compound, we did not anticipate any effect on HIV entry.

![Figure 29: The fungal metabolite chaetocin decreases fusion of combination reporter viruses. A. Structure of the small molecule chaetocin.](image)
B. CD4+ T cells incubated with chaetocin for 4h and subsequently infected with CXCR4-tropic reporter viruses showed a dose-dependent decrease in % fusion. Error bars represent SEM.

To confirm this observation, we repeated experiments with chaetocin on primary CD4+ T cells from multiple donors and indeed observed a concentration-dependent effect on fusion (Figure 29B). However, given that chaetocin contains a similar nitrogen-carbon bond as the fluorescent CCF2-AM used in the viral fusion assay, we wanted to eliminate that chaetocin competed for beta-lactamase mediated cleavage of substrate. To test this, we transfected 293T cells with a plasmid encoding the fusion protein beta-lactamase vpr following chaetocin treatment (Figure 30). Incubation with 10 µM chaetocin resulted in a 40% reduction in βlam-vpr mediated cleavage of CCF2-AM, suggesting this effect is not related to viral fusion but instead a disruption of the enzymatic assay.

![Figure 30: Chaetocin competes for βlam-vpr mediated cleavage of CCF2-AM.](image)

HEK 293T/17 cells transfected with βlam-vpr expression plasmid were incubated in the presence or absence of chaetocin and assayed for cleavage of fluorescent substrate CCF2-AM.
4.5.3 - Productive Infection Analysis Reveals Multiple Enzymatic Targets

Our results with viral fusion established that small molecule inhibitor treatment did not affect HIV entry into CD4+ T cells. Contrary to this result, analyses of LTR-driven EGFP expression revealed numerous hits both increasing and decreasing cellular susceptibility to HIV-1 productive infection (Figure 31). In support of our previous studies, 17/39 (44%) of significant hits with greater than 1.5 fold change were inhibitors targeting the histone deacetylase class of enzymes. This profoundly validates our previously reported findings that HDAC inhibitors increase susceptibility to productive viral infection. Knowing this, we used the presence of HDAC inhibitors in this library as a positive internal control.

Figure 31: Effects of Small Molecular Inhibitors on Viral Productive Infection. Analyses of HIV LTR-driven EGFP expression reveal that a large
proportion of compounds present in library (49%) have greater than 1.5 fold change in viral productive infection. Further analysis revealed 17/39 (44%) of total compounds, or 17/23 (74%) of compounds that increased susceptibility, targeted the HDAC family of enzymes. Experimental conditions performed in triplicate. Error bars represent SEM.

A list of the 22/39 (56%) non-HDAC targeting compounds from our screen can be found in Figure 32. We observed that inhibition of multiple classes of enzymes (most prominently DNA methyltransferase, histone methyltransferase and histone acetyltransferase) resulted in a pronounced reduction in susceptibility to productive infection. In fact, the majority of total hits identified from this screen reduced productive infection, which is advantageous in the context of designing strategies to reduce HIV infection.

Conversely, inhibition of prolyl hydroxylase and chromatin associated bromodomain family members BRD2/BRD4 increased susceptibility to HIV productive infection. The BRD2/BRD4 findings are significant as BRD inhibitors are currently being evaluated in the context of "shock-and-kill" therapies to eradicate latent reservoirs.

The findings that multiple inhibitors of specific enzyme families influence HIV productive infection strengthens the indication that these proteins play an important role promoting or inhibiting the HIV replication cycle. It should be noted that several of the compounds found to decrease productive infection
susceptibility are validated nucleoside reverse transcriptase inhibitors and will be excluded from downstream validation.
### Figure 32: Significant hits identified with greater than 1.5 fold change

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
<th>Function</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decitabine</td>
<td>DNA MTase inhibitor</td>
<td>Inhibits L3MBTL3 chromatin repres</td>
<td>UNC1215</td>
</tr>
<tr>
<td>Lomeguatrib</td>
<td>O6-MeGu-DNA MTase inhibitor</td>
<td>BRD2, BRD4 inhibitor</td>
<td>PFI-1</td>
</tr>
<tr>
<td>UNC0638</td>
<td>H3K9 methylation inhibitor</td>
<td>JMJD2 inhibitor/Prolyl-hydroxylase</td>
<td>N-Oxalyl-Glycine</td>
</tr>
<tr>
<td>2,3,5 triacetyl-5-azacytidine</td>
<td>DNA MTase inhibitor</td>
<td>lysine demethyl/hydroxylase inhib</td>
<td>2,4-,Pyridinedicarboxylic Acid</td>
</tr>
<tr>
<td>trans-resveratrol</td>
<td>COX1-inhibitor</td>
<td>BRD4 inhibitor</td>
<td>(+)-JQ1</td>
</tr>
<tr>
<td>Tenovin-6</td>
<td>P53 activator</td>
<td>HIF-proyl hydroxylase inhibitor</td>
<td>DMOG</td>
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<td>CAY10591</td>
<td>Sirtuin 1 activator</td>
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<td>AG-014669</td>
<td>PARP1 inhibitor</td>
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<td>Histone ATase inhibitor</td>
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<td>Garcinol</td>
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<td>Chaetocin</td>
<td>Histone MTase inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCG-100602</td>
<td>Inhibitor of Rho signaling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>Guanylyl cyclase activator</td>
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<tr>
<td>MI-2</td>
<td>Merin binding compound</td>
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<tr>
<td>5-azacytidine</td>
<td>Ribonucleoside analog</td>
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</table>
4.5.4 - Validation of Significant Hits

To confirm effects of small molecule inhibitors found in our productive infection analyses, we validated the efficacy of each compound on a minimum of three healthy donors (Figure 33). In some cases, the range of concentrations tested was re-evaluated given conflicting reports of optimal IC$_{50}$ values (Changes will be noted when applicable). Small molecule inhibitors UNC1215, PFI-1, and (+)-JQ1 consistently increased productive infection across all donors tested. In fact, we observed that treatment with PFI-1 was highly potent with concentrations as low as 10 nM yielding at minimum 2-fold effect to increase productive infection.

Our validation experiments investigating prolyl hydroxylase inhibitors N-oxalylglycine, 2,4-PDCA, and DMOG were inconsistent at best yielding a range of results across concentrations. The significance of this finding has yet to be determined and will warrant careful examination of each compound independently. For the purposes of this study, these compounds will be excluded from downstream analysis.
Figure 33: Validation of small molecule inhibitors promoting viral productive infection. Significant hits identified from preliminary analysis were subjected to additional rounds of experiments using a minimum of (next page)
three healthy donors. All experimental conditions were performed in triplicate. Error bars represent SEM.

To validate small molecule inhibitors that successfully reduced viral productive infection, we first eliminated compounds with previously reported effects on HIV-1 replication (such as acetyltransferase inhibitors, or nucleoside analogs acting as reverse transcription inhibitors). Our final list included 9 compounds with effects on histone and DNA methylation, cellular cyclooxygenases, and Rho GTPase pathway signaling.

At highest concentrations tested, all 9 compounds exhibited significant reduction in EGFP expression with the exception of transresveratrol (Figure 34). Upon careful examination, the "parental" GFP-/CD4+ population as viewed by flow cytometric analysis was shifted in response to potent transresveratrol treatment (skewing the notable EGFP statistic, data not shown). Interestingly, transresveratrol retained a larger percentage of GFP+/CD4+, suggesting potential interference with HIV nef function or altered CD4 recycling (data not shown). Of all compounds evaluated in this portion of the study, we observed pronounced effects for the non-specific histone lysine methyltransferase chaetocin and JMJD3 histone 3 lysine 27 trimethylation inhibitor GSK-J4 at all concentrations.
**Figure 34: Validation of small molecule inhibitors reducing viral productive infection.** Significant hits identified from preliminary analysis were subjected to additional rounds of experiments using a minimum of three healthy donors. Nucleoside analogs and acetyltranferase inhibitors were excluded from this portion of the study. All experimental conditions were performed in triplicate. Error bars represent SEM.

**4.5.5 - Rho and Rho-associated kinase signaling regulates HIV infection**

A unique and unexpected observation from our analysis was that treatment with small molecule CCG-100602 reduced LTR-driven EGFP expression in a dose dependent fashion (*Figure 35A*). CCG-100602 is a specific inhibitor of Rho A/C mediated signaling, a pathway that regulates various cytoplasmic and nuclear processes [269]. Given our previous findings involving HIV infection and acetylated microtubules, we wanted to expand upon this finding by probing additional Rho family protein members. To do this, we acquired two additional inhibitors ML-141 and Y-27632, which target additional Rho family CDC42 and Rho associated protein kinase (ROCK), respectively. To our satisfaction, preliminary analysis with both inhibitors show pronounced reduction in EGFP+ cells, suggesting that Rho GTPases, associated ROCK kinase, and downstream signaling cascades may play a critical role during HIV-1 replication (*Figure 35B,C*).
During the time this study was conducted, our lab also undertook an unbiased proteomic approach to identify relevant cellular phosphoproteins that are dysregulated in response to HIV-1. To achieve this, purified memory CD4+ T cells were exposed in parallel to non-viral microvesicles, inactivated HIV-1 particles, or inactivated particles in the presence of CCR5 antagonist maraviroc. The ultimate goal of this study was to delineate the individual contributions of CD4 and CCR5 signaling cascades as a result of HIV exposure (ultimately virus to cell surface binding).

Importantly, prominent phosphoproteins in the RhoA, CDC42, and Rock signaling pathways were significantly altered in response to HIV-1 (Figure 36, purple). Interestingly, changes in several cascades occurred in both the
presence and absence of maraviroc suggesting that CD4 and CCR5 may both regulate this signaling (data not shown). Of notable interest from this analysis are the cytoskeletal proteins coflin, actin, and myosin. A link between virally-induced Rho/ROCK signaling and the regulation of cytoskeletal trafficking proteins is highly attractive to us and will be the focus of our future studies.

Figure 36: Proteomic analysis of phosphorylation cascades responsive to CCR5-tropic HIV. Phosphoproteomic analysis reveals that numerous Rho family GTPase and Rock signaling members are dysregulated (purple) in response to HIV exposure. Network analysis was performed using Ingenuity Pathway Analysis software.
4.6 - Discussion

As an obligate intracellular pathogen, HIV-1 is largely reliant on interactions with cellular proteins that will facilitate the completion of each step in its replication cycle. As such, understanding the cellular regulation of HIV-1 infection is critical to developing novel therapies to combat the virus. Pinpointing mechanisms that promote or restrict viral replication will not only impact latency reactivation studies but also shed light on attempts to prevent initial viral infection. Recent studies in our laboratory have led to identification of novel mechanisms that directly affect HIV-1 replication. To our surprise, these mechanisms were primarily epigenetic or post-translational as opposed to total protein abundance. For example, our investigation into small molecule inhibitors of histone deacetylases revealed that inhibition of HDACs enhanced initial infection nearly 300% [228]. This effect was independent of the previously known nuclear effects of HDACs and was instead attributed in part to cytoplasmic HDAC6, as specific inhibition with small molecule tubacin promoted the acetylation of microtubules and recapitulated the enhancement of initial infection. This post-translational modification of tubulin has been shown to increase microtubule stability and is thought to provide more secure trafficking networks during early stages of replication. This finding peaked our curiosity into how additional modifications outside of acetylation can impact the replication efficiency of HIV-1.
While the use of RNAi and gene knockout studies is an effective tool to identify novel host-pathogen interactions, the impact of protein modifications is lost in this process. Furthermore, studies into epigenetic/post-translational control of HIV infection have revolved almost exclusively around the establishment and maintenance of latency. We hypothesized that coupling this library with our combination reporter virus assay would allow us to assess the effects of these compounds outside HIV latency, instead probing both viral fusion and productive infection (LTR-driven EGFP expression, or our surrogate for active viral transcription and protein synthesis).

To investigate this, we employed the use of a commercially available small molecule inhibitor library that is tailored towards epigenetic and post-translational machinery. As with many of our previous studies, we performed these experiments utilizing primary, unstimulated CD4+ T cells in efforts to promote physiologic relevance. We used CXCR4-tropic reporter viruses in part due to higher density of co-receptor on T-cell subsets (and therefore higher achievable infection in our mixed subset population).

At the very least, we hypothesized that we would observe significant changes in productive infection in response to histone deacetylase and acetyltransferase inhibition. We were also aware that several DNA methylation inhibitors are known nucleoside analogs, which would restrict reverse transcription and subsequently decrease productive infection. Our findings not
only supported both notions but also revealed several unexpected findings. A few key examples will be highlighted below:

**Chaetocin** - Initial analysis of viral fusion sparked excitement into the histone lysine demethylase inhibitor chaetocin, as we observed a dose dependent effect in our assay. However, we began to expect that this compound might be acting as a competitive inhibitor given nitrogen-carbon bonding similarities between chaetocin and the CCF2 fluorescent substrate. Our preliminary experiments have suggested this is the case but will warrant repeated investigation for certainty. It is still important to note that chaetocin treatment abolished productive infection greater than 95% at the two highest concentrations, an effect independent of fusion. This finding is actually contradictory to a previous report that chaetocin induces latent proviral DNA [270] (also contradictory are our findings with lysine methyltransferase inhibitor UNC0638, not to be discussed). However, the viral construct used in the above mentioned study was highly modified and solely assessed LTR promoter-driven luciferase expression. An additional report evaluated chaetocin in the context of a larger inhibitor analysis and observed marginal effects to promote reactivation [158]. Nevertheless, our primary cell data presented here suggests chaetocin treatment largely antagonizes HIV-1 replication in primary CD4+ T cells. Furthermore, the use of reporter viruses that harbor clinical isolate envelopes, native viral LTRs, and encode all HIV-1 genes (with exception to altered env region), increase our confidence that this
effect is relevant to physiologic viral replication. It is important to note that this inhibitor has dose-dependent cytotoxicity, which cannot be ignored as we continue our interpretation of the data.

**Multiple methylation mechanisms** - We observed an ~2-fold increase in productive infection upon treatment with small molecule UNC1215. Developed as a "chemical probe" for lethal-3 malignant brain tumor-like protein (L3MBTL), UNC1215 displaces interacting peptides from the methyl lysine reader domain of L3MBTL [271]. The significance of this is not entirely understood. The functional characterization and interaction network of L3MBTL is weak at best with several studies proposing a role in transcriptional repression [272, 273]. Additional studies suggest that L3MBTL protein acts in consort with a polycomb complex and is recruited to mono and dimethylated histones. On the matter of polycomb, it is only recently that polycomb repressive complex members are being studied in the context of HIV-1. Specifically, disrupting polycomb repressive complex 2 proteins have been shown to reactivate proviruses [160]. Given the lack of data on the role of L3MBTL, this finding has important implications in studying the transcriptional regulation of HIV-1.

Conversely, treatment with small molecule GSK-J4 dramatically reduced productive infection. GSK-J4 is an inhibitor to the histone demethylase JMJD3, which has been demonstrated to promote transcription by reversing trimethylation at lysine 27 of histone 3 (H3K27me3) [274]. H3K27me3 is highly
associated with inactive gene promoters, and indeed inhibition or depletion of the methyltransferase EZH2 (member of polycomb complex 2) has been shown to promote transcription and reactivate latency in the context of HIV [158]. It is likely the case that inhibition of the opposing JMJD3 recapitulates this result and further strengthens the role for lysine residue 27 trimethylation.

**BRD inhibition** - Inhibition of bromodomain proteins BRD2 and BRD4 by compounds PFI-1 and (+)-JQ1 was found to increase productive infection in this study. This is relevant in the context of shock-and-kill eradication strategies, as JQ1 has been found to reverse latency and is currently being vetted as a clinical candidate [274]. This mechanism has been ascribed to interactions between BRD4 and the P-TEFb complex. Specifically, association of cyclin T1 and CDK9 with BRD4 negatively impact Tat-transactivation. Beyond latency reactivation, BRD4 inhibition is likely promoting efficiency of Tat-transactivation in our primary cell assays. While our findings are not entirely unexpected, the use of PFI-1 or JQ1 may prove useful when assessing the role of Tat transactivation in a particular mechanism.

**Rho/ROCK Signaling** - Perhaps the most interesting result from our small molecule inhibitor screen is the finding that inhibition of Rho family GTPases and Rho-associated kinase (ROCK) reduces productive infection of HIV-1. These family members were also significantly identified in a related HIV
phosphoproteomic study within the lab. Taken together, these results highlighted that 1) HIV mediated signaling through CD4 and co-receptor initiated a Rho/ROCK cascade, and 2) inhibition of this signaling pathway has a detrimental effect during viral replication. The mechanism behind this effect is highly intriguing to us. Rho/ROCK signaling has an extensive list of downstream effectors and regulates processes including endosomal trafficking, cytokinesis, cell cycle, and cell adhesion [269]. Most important to us however are the effects on cytoskeletal networks such as actin and microtubules. Signaling through ROCK has been shown to phosphorylate LIM kinases, which subsequently phosphorylate the actin regulator cofilin [275, 276]. Rho family members also interact with WASP and WAVE complexes, which have direct effects on actin polymerization [277]. In regards to microtubules, Rho/ROCK signaling may play an important role in polymerization and stability [278]. Firstly, CDC42 GTPase can regulate the translocation of the nucleus and the microtubule-organizing center (MTOC) [279]. Given the requirement for microtubules during HIV infection, it is tempting to propose that CDC42 signaling may reorient the MTOC near the nucleus, which could directly influence the transport of pre-integration complexes through MTOCs and downstream nuclear import.

Independently, the Ezrin-Radixin-Moesin family of proteins is regulated in part by ROCK phosphorylation [280]. This family has been implicated as negative regulators of microtubule stability and of HIV-1 replication [97]. In relation, several groups have determined that RhoA GTPase signals downstream
effector mDia and regulate stable microtubule networks from studies of cell motility [281-283]. Indeed mDia and related formin family members co-localize with stable microtubules, and interactions between Rho, mDia, and microtubule-associated proteins have been described [284].

To reiterate, inhibitions of RhoA, CDC42, and ROCK directly have detrimental effects on HIV productive infection. Phosphoproteomic analysis reveals that Rho/Rock signaling is dysregulated in response to HIV exposure. We believe our findings point to a tightly regulated signaling network controlled by RhoA, CDC42, and ROCK. We favor the hypothesis that HIV mediated signaling through these pathways primes a cell for viral replication in several ways: 1) Signaling actin reorganization, 2) promoting microtubule stability, and 3) through reorientation the MTOC and nucleus to facilitate PIC import. While these conclusions are broad and carry a high degree of conjecture, the union of our original data and numerous studies builds a strong case for these mechanisms to hold true.

To pursue these studies further, we will begin by asking several rudimentary questions: Does inhibition of Rho/ROCK signaling affect HIV trafficking efficiency or nuclear import? We can observe this by way of microscopic visualization, as well as measurement of nuclear 2-LTR circles. We are also very interested in identifying potential direct relationships between signaling and microtubule acetylation. Does Rho/ROCK signaling influence the degree of microtubule acetylation? We have initiated experiments in which we
temporally measure the induction of acetylated microtubules in response to infection. We believe the addition of the small molecule inhibitors in this system will very rapidly indicate if this is the case.

Overall, the results from this small molecule inhibitor screen have uncovered several distinct avenues to pursue. It is important to stress that epigenetic/post-translational control of HIV infection extends beyond our current knowledge and impacts processes outside latency reactivation. Furthermore, we also demonstrate here that the use of a low-cost pharmacological library coupled with semi-automated flow cytometry is a highly effective and rapid method to identify novel biology underlying viral infection. This point should not be overlooked given the inherent challenges with large scale RNAi or knockout studies.
CHAPTER FIVE:

FINAL CONCLUSIONS AND FUTURE DIRECTIONS
Despite three decades of research, our understanding of HIV-1 infection is incomplete. In fact, the scientific community is recognizing that the mechanisms underlying viral replication are more complex than previously appreciated as our scientific toolbox evolves to include the latest cutting-edge assays and techniques. For example, the achievement of advanced multi-color flow cytometry has allowed for simultaneous quantification of cell subsets, cell behavior, and protein expression. This has had dramatic impact in the HIV field by improving our understanding of immune system dysfunction, host-virus interactions, and viral latency at the individual cell level. In our hands, we have taken advantage of flow cytometry to develop our combination reporter virus system. Unlike previous reporters, this system allows for parallel quantification of viral fusion and LTR-driven protein expression (termed productive infection) using the same virus particle. The sensitive measurement of both of these critical steps in viral replication allows us to not only measure the susceptibility of cells to infection but also measure the efficiency of post-entry events in that same cell. We have highlighted the power of this system in a recent study characterizing the susceptibility of stem cell memory T cells to HIV infection [285]. We believe this finding has important clinical implications for viral latency given these cells are self-renewing and are thought to give rise to differentiated memory T cells. This data is supported by more recent integration site analyses of infected individuals, which reported that viral reservoirs were largely comprised of clonal viruses [286].
In addition to identifying novel cell subsets that are susceptible to viral infection, we sought to use the combination reporter virus to understand the efficiency of post-entry viral events in primary CD4+ T cells. With both CCR5 and CXCR4-tropic reporter viruses, we repeatedly observe that only 5-10% of cells that fuse with HIV will become productively infected [71]. This was striking to us in that it highlighted the inefficiency of viral infection. This is likely a direct consequence of the requirement for HIV to hijack cellular machinery to complete each step in its replication cycle (coupled with host anti-viral factors attempting to interrupt this process). There likely exist additional unknown proteins and mechanisms that impact viral replication. The combination reporter virus system is one sensitive method to probe for cellular mechanisms that modulate the post-entry efficiency of HIV.

5.1 - The Effects of HDAC inhibitors

During our characterization of the combination reporter viruses, we began to investigate the abundance of latently infected viruses in our system. This was achieved by addition of latency reversing agents such as T-cell activators or histone deacetylase inhibitors during infection. By quantifying the enhancement of EGFP production one can determine the relative contribution of latent HIV that is not actively transcribed in unstimulated cells. It was these experiments that sparked the first studies presented in this work. We initially observed that
addition of histone deacetylase inhibitors at 48 hours during infection led to a 30-50% boost in EGFP production. This time of addition is common as it coincides roughly with completion of viral integration. In stark contrast, we observed a 200-300% increase in EGFP when the HDAC inhibitor vorinostat was added prior to infection (Figure 11). This finding was unexpected and led us to further investigate this effect. More detailed analysis pointed to a cytoplasmic mechanism that was independent of latency reactivation (Figure 13). Specifically, the addition of vorinostat enhanced the kinetics of HIV reverse transcription and integration. These data were supported by measurement of nuclear 2-LTR circle products, a surrogate for the overall efficiency of nuclear import. Addition of vorinostat led to a 3-fold increase in 2-LTR circle production, suggesting an upstream effect that boosted nuclear import of viral DNA (Figure 14). Finally, experiments with the specific HDAC6 inhibitor tubacin led us to hypothesize that these inhibitors act by upregulating the acetylation of microtubule networks.

It has been known for more than a decade that HIV utilizes microtubule networks for trafficking. Only more recently, it was discovered the HIV can directly induce the formation of detyrosinated and acetylated microtubules during infection. These subsets of microtubules have been shown to be more stable, lending to the notion that HIV primes the cell to improve its own trafficking. We believe our findings with tubacin are the first to suggest that stable acetylated microtubule formation can dramatically improve post-entry viral efficiency. We
plan to expand upon these studies to characterize the kinetics of microtubule acetylation during HIV infection. It is likely that HIV (as well as other viruses) induce formation of stable microtubules through a combination of receptor-mediated signaling and direct viral-host protein interactions. Understanding these interactions could allow for development of therapeutics that prevent HIV productive infection by disrupting acetylated microtubule trafficking.

While induction of acetylated microtubules may be a negative effect in the context of promoting HIV infection, improving this phenomenon may be of benefit in the development of novel gene-therapy viral vectors. The use of viral vectors are currently limited in part by the poor transduction efficiency of the particular therapeutic. In the case of lentiviruses, the low 5-10% efficiency we observe in our HIV system would be unfavorable for gene therapy. Given our recent findings with tubacin, we hypothesized that we can improve this efficiency by designing a viral vector that boosts microtubule acetylation. Improving efficiency 200-300% would be a significant achievement and a proof of concept that one can engineer a more efficient gene therapy vector.

To demonstrate this as a proof of concept, we have designed lentiviral constructs that incorporate the enzyme alpha-tubulin acetyltransferase 1 (αTAT1). αTAT1 was identified more recently as the putative enzyme responsible for microtubule acetylation; specifically by addition of acetyl groups to lysine 40 residues of alpha tubulin [243]. We hypothesize that delivery of exogenous αTAT1 will increase acetylated microtubules only in cells that undergo
successful fusion with viruses. To ensure packaging of αTAT1 into the virus particle, we have designed constructs encoding αTAT1 linked to HIV Vpr (which has been shown to tolerate both N- and C-terminal additions). We recognize that this approach does not ensure proper folding of αTAT1, nor does it guarantee delivery of the enzyme to the correct intracellular compartment (it is suggested αTAT1 is localized to the microtubule lumen). Additionally, little is known regarding the post-translational modifications of this enzyme, and whether they are needed for activity. We can begin to address several of these questions if needed through our collaborations with the mass spectrometry and imaging/microscopy cores at Case Western Reserve University. Regardless, we believe that delivery of the enzyme responsible for microtubule acetylation will demonstrate a post-entry method to improve infection efficiency without causing significant off-target cell effects.

These studies have all stemmed from our initial investigations into investigate latency reactivation. While our findings with HDAC inhibitors do raise concern for their clinical use as latency reversing agents, we unexpectedly uncovered fascinating cell biology that underlies HIV infection. In turn, we have positively applied these findings in attempts to improve development of gene-therapy relevant viral vectors. We hope to continue these studies as a means to expand into the field of gene therapy while continuing to understand basic HIV biology.
5.2 - HAT Inhibitors Garcinol and Curcumin

Our findings with HDAC inhibitors led us to investigate the effects of histone acetyltransferase (HAT) inhibition on HIV infection (Chapter 3). We hypothesized that HAT inhibition will have opposing effects of HDAC inhibitors, in that they will reduce HIV productive infection. For these studies, we chose to investigate HAT inhibitors garcinol and curcumin, both naturally derived from plant products. Our studies demonstrated that garcinol and curcumin both significantly reduce HIV productive infection, albeit curcumin to a greater extent. Mechanistic evaluation of both inhibitors has proven challenging. We have systemically shown these compounds do not alter viral fusion, microtubule acetylation, or the kinetics of reverse transcription and integration. Our experiments with the latent jurkat cell line 2D10 suggested these compounds are not acting to simply reduce transcription of integrated DNA. However, we are unable to say with certainty that histone/chromatin architecture play a significant role in this model of latent infection.

The finding that the HAT inhibitor anacardic acid does not reduce productive infection added an additional degree of complexity to our study. This compound also targets the relevant HATs p300 and PCAF at similar half maximal inhibitory concentration, suggesting that these acetyltransferases are not responsible for effect observed here. In efforts to identify additional targets of these inhibitors, we designed a proteomics-based experiment to quantify total
and acetylated proteins in response to HAT inhibition. These experiments are ongoing and as such are not included in this work. Interestingly, our initial pilot study identified a surprisingly low number of acetylated proteins when compared to other studies though this may be a consequence of using primary unstimulated CD4+ T cells (many of which are in a resting state). We have since redesigned this experiment to probe for targets following a longer incubation with HAT inhibitors.

Despite the challenge of pinpointing exact mechanism, our findings with HAT inhibitors demonstrate that cellular machinery can be targeted to reduce productive HIV infection. Experiments with replication-competent HIV demonstrated that curcumin treatment dramatically reduced multiple rounds of infection. In the context of pre-exposure prophylaxis (PreP), reducing spreading infection would be highly advantageous during the time of initial transmission. For example, vaginal acquisition of HIV has been shown to occur in approximately 1 in 200-2000 virus exposures. It has also been shown that the dissemination of virus from the cervico-vaginal tissue is highly dependent on the resident mucosal CD4+ T cell population. Reduction of productive infection by 50-80% (as seen with garcinol and curcumin) may significantly dampen the localized amplification of virus within mucosal T cells and prevent dissemination. We are currently in progress of optimizing conditions to infect primary CD4+ T cells isolated from ectocervical biopsies in order to test this hypothesis in a tissue-relevant cell type. Designing strategies to significantly prevent productive
HIV infection of first infected cells or to drive these cells immediately into latency could be used in conjunction with current direct anti-retroviral therapy to prevent transmission.

5.3 - Probing the Epigenetic Screening Library

Based on our studies with HDAC and HAT inhibitors, we sought to expand our investigation beyond acetylation-based control of HIV infection. Specifically we were interested in identifying additional post-translational/epigenetic modifying compounds that modulate the susceptibility of CD4+ T cells to HIV infection. By probing a commercially available small molecule library, we identified numerous hits that both positively and negatively modulate productive infection. Several of these hits were expected given their previously reported roles as reverse transcription inhibitors. However, we identified several compounds related to cellular methylation that reduce productive infection and are in disagreement with prior reports.

Most notable from this screen is the finding that Rho family GTPase and Rho-associated kinase (ROCK) inhibition reduces productive infection of HIV-1. In consort with a recent phosphoproteomic study, these data highlight the importance of Rho/ROCK during HIV infection. To pursue these studies further, we will begin by asking several fundamentalase.edu questions: Does inhibition of Rho/Rock signaling affect HIV trafficking efficiency or nuclear import? We can
measure this by way of microscopic visualization and quantification of nuclear 2-LTR circle DNA. Given the well-established relationships between Rho/Rock signaling and cytoskeletal organization, we are planning to investigate the connection between Rho/ROCK and microtubule dynamics. Does Rho/Rock signaling influence the degree of microtubule acetylation? We have initiated experiments in which we temporally measure the induction of acetylated microtubules in response to infection. We believe the addition of the small molecule inhibitors in this system will very rapidly indicate if Rho/ROCK signaling acts upstream of microtubule formation.

5.4 - Assembling the Experimental Tacklebox

Each of the studies summarized above plays to the strengths of the combination reporter virus system. As a tool, it is extremely useful to assess the direct effects of small molecules on viral fusion and productive infection. However, this is just a starting point. The journey through each of these projects has allowed us to amass an "experimental tacklebox" to sequentially narrow down the search for a particular mechanism. For example, we have used the combination reporter virus system in conjunction with direct acting anti-retroviral drugs to probe the kinetics of various stages of the HIV life cycle. We have also quantified the degree of nuclear import and integration to determine whether an effect occurs early or late during infection. Finally, with the
combination of microscopy, mass spectrometry, immunoprecipitation, and western blotting we can accurately determine protein abundance and localization within a cell. We believe that this experimental tacklebox should be used as a comprehensive system to dissect novel mechanisms underlying HIV-1 infection.
Title: The Histone Deacetylase Inhibitor Vorinostat (SAHA) Increases the Susceptibility of Uninfected CD4 T Cells to HIV by Increasing the Kinetics and Efficiency of Postentry Viral Events

Author: Mark B. Lucera, Carisa A. Tilton, Hongxia Mao et al.

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