Small molecules effective for disruption of HIV-1 latency

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Small molecules effective for disruption of HIV-1 latency

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ABSTRACT

Although antiretroviral therapies have improved the outlook of the HIV epidemic, they do not provide a cure. The major barrier to development of a cure lies in the virus’s ability to become transcriptionally silent as chromosomally integrated provirus. The presence of latently infected cells that harbor transcriptionally repressed viral genomes, gives rise to cellular reservoirs that are impenetrable by current therapies. Therefore, devising ways to selectively target these latent reservoirs is imperative for the long-term management of the disease.

This thesis focuses on the shock phase of a proposed cure strategy known as “shock and kill,” which aims to induce latent HIV-1 reservoirs that could then be purged via a boosted immune response, specific targeting of infected cells, or by viral-induced apoptosis. Accordingly, research over the past decade has resulted in identification of small molecules capable of inducing HIV-1 latent reservoirs, by reactivation of viral transcription. Molecules with this capability, known as latency-reversing agents (LRAs). Thus far, none of the LRAs examined in clinical trials have reduced the size of persistent HIV-1 infection. Therefore, new classes of LRAs must be identified. To this end, I identified five novel LRAs, that are capable of reversing HIV latency without affecting the general T cell activation state. These compounds exhibit synergy for reactivation of latent provirus with other LRAs, in particular, ingenol-3-angelate. One compound, designated PH02, was efficient at reactivating viral transcription in several in vitro cell lines bearing HIV-1 reporters at different integration sites. Furthermore, this compound was capable of reversing latency in resting CD4+ T lymphocytes from patients on antiretroviral therapy. The combination of PH02 and ingenol-3-angelate produces a strong synergistic effect of
reactivation, as demonstrated by a quantitative viral outgrowth assay on CD4+ T lymphocytes from HIV-1-infected individuals.

A comparison of similar efforts from other groups is provided, with the goal of illustrating the diversity of molecular scaffolds that can produce HIV-1 latency reversing activity. I expect these results will contribute to a deeper understanding of mechanisms regulating HIV-1 latency but also will provide insight towards design of optimized structures for development of highly effective LRAs capable of forcing a purge of the persistent HIV-1 infection.
LAY SUMMARY

Despite intensive scientific research in the HIV/AIDS field, there remains no cure. The major challenge in developing a cure is associated with the population of cells carrying silenced HIV genomes. This population is unrecognizable by the patient’s immune system and unaffected by current antiretroviral therapies. Upon cessation of therapy, replication becomes induced from these cellular reservoirs and virus particles can repopulate the blood. The overall goal of this thesis was to develop a novel means to reawaken these silent viruses to expose the latently infected cells to a boosted immune defense capable of eliminating these cells. Towards this goal, I found five new small molecules that are capable of reactivating HIV from latency. Additionally, I discovered that treatment with one of my compounds in combination with a previously characterized chemical compound provides a particularly effective means of disrupting viral latency. These results will ultimately lead to more effective therapeutic interventions for purging latent virus from HIV infected patients.
PREFACE

This thesis is presented in four chapters.

Chapter 1 presents a literature review of the research studies in the HIV field.


Chapter 3 is based on a first author published paper. Hashemi, P.; Barreto, K.; Bernhard, W.; Lomness, A.; Honson, N.; Pfeifer, T.A.; Harrigan, P.R.; Sadowski, I. Compounds producing an effective combinatorial regimen for disruption of HIV-1 latency. EMBO Mol. Med. 2017. PH conceived and designed the in vitro and ex vivo experiments, performed the experiments, analyzed the data, and wrote and revised the manuscript. KB, WB, and NH designed and performed the high-throughput screening. TAP supervised the HTS and edited the manuscript. PRH coordinated the HIV-infected patient samples and edited the manuscript. IS supervised the experiments, wrote and revised the manuscript.

HIV-infected patient samples used in the study presented in chapter 3, were provided through the BC Centre for Excellence in HIV/AIDS at St. Paul’s Hospital, Vancouver. All subjects provided written, informed consent prior to their inclusion in the study (Ethics approval issued by the UBC Clinical Research Ethics Board, the project title: modulation of HIV latency, and the certificate number: H13-00357).
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LIST OF ABBREVIATIONS

°C  Degree Celsius
×  g  Times gravity
5HN  5-hydroxynaphthalene-1,4-dione
AIDS  Acquired immunodeficiency syndrome
ALDH  Aldehyde dehydrogenase
AP1  Activator protein 1
AP4  Activator protein 4
APC  Antigen presenting cell
APOBEC3G  Apolipoprotein B mRNA-editing enzyme–catalytic polypeptide-like-3G
AV6  Antiviral 6
BAF  BRG1-associated factor
Bcl-2  B- cell lymphoma 2
BD  Bromodomain
BET  Bromodomain and extra-terminal
BLT  Bone marrow, liver, thymus
Bp  Base pair
Brd4  Bromodomain protein 4
BSO  Buthionine sulfoximine
CA  Capsid
Calm  Calmodulin
CaN  Calcineurin
CAPE  Caffeic acid phenethyl ester
CBP  Cyclic AMP response element binding protein (CREB)-binding protein
CCBN  Canadian Chemical Biology Network
CCK-A  Cholecystokinin-A
CCR5  C-C chemokine receptor type 5
CD  Cluster of differentiation
Cdk9  Cyclin-dependent kinase 9
cDNA  complementary deoxyribonucleic acid
cdNIPP1  central domain of nuclear inhibitor of PP1
CETSA  Cellular thermal shift assay
ChiP  Chromatin immunoprecipitation
cpz  chimpanzee
CTD  C-terminal domain
CTIP2  COUP-TF interacting protein 2
CTL  Cytotoxic T lymphocyte
CU  Connection unit
CXCR4  C-X-C chemokine receptor type 4
DAG               Diacylglycerol

dCA               didehydrocortistatin A

DDTC             Diethyldithiocarbamic acid

DH                Dopamine beta-hydroxylase

DHFR             Dihydrofolate reductase

DIS               Dimerization initiation site

DSIF             DRB sensitivity-inducing factor

DTT               Dithiothreitol

EC50             Effective concentration 50

EDTA             Ethylenediaminetetraacetic acid

eGFP             Enhanced green fluorescent protein

Env               Envelope

ER               Endoplasmic reticulum

ERK              extracellular signal-regulated kinase

ESCRT            Endosomal sorting complexes required for transport

ET               Extra-terminal

EZH2             Enhancer of zeste homolog 2

FACS             Fluorescence-activated cell sorting

FBS               Fetal bovine serum

FLT3             FMS-like tyrosine kinase-3

GABP             GA-binding protein

Gag               Group-specific antigen

GFP               Green fluorescent protein

gp               glycoprotein

GSH               Glutathione

GTF               General transcription factor

GVHD             Graft versus host disease

HAART            Highly active antiretroviral therapy

HAT               Histone acetyltransferase

HDAC             Histone deacetylase

HDACI            HDAC inhibitor

HEXIM 1          Hexamethylene bisacetamide-induced protein 1

HI-FBS           Heat-inactivated- fetal bovine serum

HIV               Human immunodeficiency virus

HLA-DR           Human leukocyte antigen- DR isotype

HMBA             Hexamethylene bisacetamide

HMT              Histone methyltransferase

HMTi             Histone methyltransferase inhibitor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>HODHBt</td>
<td>3-hydroxy-1,2,3-benzotriazin-4(3H)-one</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Hydrophobic spacer</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screen</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor-Kappa B</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor kappa B kinase</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International union of pure and applied chemistry</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus family kinases</td>
</tr>
<tr>
<td>JHDL</td>
<td>Johns Hopkins drug library</td>
</tr>
<tr>
<td>Jkt</td>
<td>Jurkat</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LCGC</td>
<td>LIMR Chemical Genomics Center</td>
</tr>
<tr>
<td>LEDGF</td>
<td>Lens epithelium-derived growth factor</td>
</tr>
<tr>
<td>LRA</td>
<td>Latency reversing agent</td>
</tr>
<tr>
<td>LSF</td>
<td>Late Simian Factor</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBD2</td>
<td>Methyl-CpG-binding domain containing protein-2</td>
</tr>
<tr>
<td>mdHIV</td>
<td>mini-dual fluorescent HIV-1 LTR reporter</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ER kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMQO</td>
<td>8-methoxy-6-methylquinolin-4-ol</td>
</tr>
<tr>
<td>MPC</td>
<td>Multi-catalytic proteinase complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MW (g/mol)</td>
<td>Molecular weight (gram/mole)</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative effector</td>
</tr>
<tr>
<td>NELF</td>
<td>Negative elongation factor</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NI</td>
<td>Not investigated</td>
</tr>
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<td>NIK</td>
<td>NF-kappa B inducing kinase</td>
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<td>Nanometer</td>
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<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside/nucleotide reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>Nuc</td>
<td>Nucleosome</td>
</tr>
<tr>
<td>PASTAA</td>
<td>Predicting Associated Transcription factors from Annotated Affinities</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Primer binding site</td>
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<td>PCAF</td>
<td>P300/CBP-associated factor</td>
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<td>RAS guanyl nucleotide-releasing protein</td>
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<td>Ras-responsive binding element</td>
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<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>sm</td>
<td>Sooty Mangabey</td>
</tr>
<tr>
<td>SMAPP1</td>
<td>Small molecule activator of PP1</td>
</tr>
<tr>
<td>SMN2</td>
<td>SMN2 survival of motor neuron 2</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
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<td>Super T-cell media</td>
</tr>
<tr>
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<td>Surface glycoprotein 120</td>
</tr>
<tr>
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<td>SWItch/Sucrose Non-Fermentable</td>
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<td>Tumor necrosis factor-alpha</td>
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<tr>
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<tr>
<td>U5</td>
<td>Unique sequence at 5’ end of the genome</td>
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</tr>
<tr>
<td>USF2</td>
<td>Upstream-stimulatory factor 2</td>
</tr>
<tr>
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<td>Vascular endothelial growth factor-receptor 2</td>
</tr>
<tr>
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<td>Viral infectivity factor</td>
</tr>
<tr>
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<td>Viral protein R</td>
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<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus-G protein</td>
</tr>
<tr>
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<td>Weight per volume</td>
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<td>World Health Organization</td>
</tr>
<tr>
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<td>Yin Yang 1</td>
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<tr>
<td>ZAP-70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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<tr>
<td>ZNF 198-FGFR1</td>
<td>Zinc finger 198-fibroblast growth factor receptor 1</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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DEDICATION

To my beloved family
CHAPTER 1 INTRODUCTION

1.1 HIV-1

Human immunodeficiency virus-1 (HIV-1), the etiologic agent of the disease known as “acquired immunodeficiency syndrome (AIDS)”, is a major global health issue. Despite advances in understanding the biology of HIV-1, as well as significant efforts by the global health community, the World Health Organization (WHO) estimated that 37.9 million people worldwide were living with HIV-1 at the end of 2018 (http://www.who.int/gho/hiv/en/). In that same year, approximately 1.7 million individuals became newly infected with HIV, and 770,000 million people died from AIDS-related illnesses. Sub-Saharan Africa remains the most severely affected region, representing 66% of all new infections. The complexity of the disease poses formidable challenges toward development of a cure, making HIV/AIDS one of the biggest health concerns for humans to date.

1.2 EPIDEMIOLOGY OF HIV/AIDS IN CANADA

According to the Public Health Agency of Canada (PHAC), there were an estimated 63,110 individuals living with HIV at the end of 2016, and, in the same year, an estimated 2,165 new HIV infections in Canada. The HIV prevalence rate was an estimated 173 per 100,000 Canadians, and it continues to rise as new infections occur simultaneously with a decrease in the number of AIDS-related deaths which has resulted from the effectiveness of current anti-retroviral therapies in controlling virus replication. The HIV epidemic is concentrated in certain populations. As one might expect, HIV infections are most prevalent in the gay, bisexual, and other men who have sex with men.
(gbMSM) community, which represents 51.9% of the total HIV-infected population in Canada1.

1.3 VIRUS ORIGIN

Several pathogens have emerged from cross-species transmission of the simian immunodeficiency viruses (SIVs) to greater apes and humans2,3. For instance, SIV_{sm} (where {sm} indicates Sooty Mangabey, the primate species of origin) has crossed over to humans and evolved into human immunodeficiency virus-2 (HIV-2), and SIV_{cpz} from chimpanzees in south-eastern Cameroon has crossed over and evolved into HIV-12. These viruses are members of the lentivirus genus within the retroviridae family. HIV-1 is represented by a series genetically diverse subtype variants classified into several major phylogenetic groups, including M (main), O (outlier), and N (non-M/ non-O)3,4. In particular, HIV-1 group M, the pandemic form of HIV-1, causes most infections around the world, and is the focus of this thesis.

1.4 HIV-1 GENOME AND STRUCTURE

1.4.1 HIV-1 genome

As shown in Figure 1.1, the HIV-1 genome, a plus polarity-single stranded RNA (ribonucleic acid), has nine open reading frames and ultimately produces 15 distinct viral proteins5. Most proteins are formed by the cleavage of three main polyproteins, Gag (group-specific antigen), Pol (polymerase), and Env (envelope), and can be classified into two major groups of viral components with either structural or enzymatic functions6. Gag and Env polyproteins become proteolytically cleaved to produce the envelope and capsid
proteins involved in forming the outer membrane and virus core. Proteins cleaved from the Pol precursor, including a protease, the reverse transcriptase and integrase, facilitate enzymatic reactions associated with different stages during the viral replication cycle. In addition to these proteins which are typically produced by retroviruses, the HIV-1 genome encodes six accessory proteins, namely, Tat (trans-activator of transcription), Rev (regulator of virion gene expression), Nef (negative effector), Vif (viral infectivity), Vpr (viral protein r), and Vpu (viral protein u); these play major roles in the virus replication cycle and pathogenesis.

Figure 1.1 Schematic representation of the HIV-1 genome.
The HIV genome encodes three major polyproteins, indicated as Gag, Pol and Env. The Gag precursor is cleaved into the Matrix (MA p17), capsid (CA p24), and nucleocapsid (NC p7) proteins. Pol is cleaved into protease (PR p10), reverse transcriptase (RT p51/p66), and integrase (IN p32), while the Env precursor is cleaved into surface glycoprotein 120 (SU gp120) and transmembrane glycoprotein 41 (TM gp41). Accessory factors encoded by alternative reading frames and sub-genomic RNA splicing include virion infectivity factor (Vif), viral protein U (Vpu), viral protein R (Vpr), negative regulatory factor (Nef), trans-activator of transcription (Tat), and regulator of expression of virion proteins (Rev). The 5’ and 3’ Long Terminal Repeat (LTR) transcriptional and regulatory regions are indicated. Adapted from.
1.4.2 Virus Structure

The spherical HIV-1 particle has a diameter of 120–200 nm, and is enclosed in a lipoprotein membrane. Figure 1.2 indicates that each viral particle contains glycoprotein (gp) spikes comprised of gp120, the docking protein on the outside of the viral membrane, and gp41, the transmembrane protein anchored into the viral membrane. Upon virus budding from the infected cell, some of the host proteins located within the host cellular membrane are also incorporated into the viral lipoprotein layer, including HLA-DR (human leukocyte antigen- DR isotype) molecules and CD54/ICAM-1 (cluster of differentiation 54/ intercellular adhesion molecule 1) adhesion proteins. Matrix p17 proteins are located inside the viral membrane. The cone-shaped viral capsid core, comprising protein p24 monomers encloses two single-stranded viral RNAs. The viral genomes are tightly bound to the nucleocapsid proteins p7 that protect them from nucleases. Additional viral proteins required for replication are also present inside the viral particle, including reverse transcriptase, integrase, and protease.
Figure 1.2 HIV-1 structure.
Shown is a schematic representation of an HIV-1 particle. Viral molecules include: surface envelope glycoprotein 120 (SU gp120), transmembrane glycoprotein 41 (TM gp41), matrix protein 17 (MA p17), nucleocapsid protein 7 (NC p7), protease protein 10 (PR p10), positive sense RNA (+sRNA), capsid protein 24 (CA p24), integrase protein 32 (IN p32), reverse transcriptase p51/p66 (RT p51/p66). Adapted from (https://web.stanford.edu/group/virus/retro/2005gongishmail/HIV.html).

1.5 VIRUS REPLICATION CYCLE
1.5.1 Viral attachment and fusion

Virus entry into the host cell is the first step upon which the HIV-1 infection starts (Figure 1.3). The virus, through its gp120 subunit of the env protein, binds to its primary receptor CD4, present on the cell surface of T lymphocytes, which are the main target cells for HIV-1 infection11-13. The initial binding causes conformational changes in gp120 which enable further interactions with the seven-transmembrane domain chemokine receptor, referred to as the co-receptor14. The interaction between gp120 and the co-receptors CCR5 (C-C chemokine receptor type 5) or CXCR4 (C-X-C chemokine receptor type 4), depending on tropism of the virus strain, leads to a dramatic rearrangement of gp41 which catalyses formation of a fusion pore and entry of the viral contents into the host cell11,15.
Figure 1.3 HIV-1 replication cycle.

Stages of the virus replication cycle from viral entry to host cell release of new virus are illustrated. The HIV-1 virion binds the target T-cell through the engagement of the envelope proteins (Env shown in yellow) with the CD4 receptor (shown in red) on the cell surface, in conjunction with a seven-transmembrane co-receptor, either CCR5 or CXCR4 (shown in blue). The virus fuses with the cell membrane and its contents are released into the cytoplasm. The viral RNA genome is transcribed into cDNA in the cytoplasm and the double stranded DNA genome is transported into the nucleus via the TNPO3, RanBP2/Nup358 nuclear pore complex. Inside the nucleus, the viral integrase protein, in association with the transcription co-factor LEDGF/p75 (shown in light green) promotes integration into actively transcribed regions of the genome. In activated T cells, the viral genome is expressed, and mRNAs translated to produce viral proteins, components of which translocate to the cell membrane, where viral particles are assembled and then released. Upon release, the viral polyproteins are further cleaved to form structural components of the mature virus.
CCR5, C-C chemokine receptor type 5; CXCR4, C-X-C chemokine receptor type 4; PIC, pre-integration complex; TNPO3, transportin 3; RanBP2, ran binding protein 2; LEDGF, lens epithelium-derived growth factor. Adapted from 13.

1.5.2 Uncoating and reverse transcription of viral RNA

Once introduced into the cytoplasm, the viral genome becomes organised into a complex with the reverse transcriptase and microtubules, thereby facilitating delivery to the nuclear membrane (Figure 1.3)\textsuperscript{16,17}, where the viral genome is reversed transcribed into cDNA (complementary deoxyribonucleic acid); this process presumably occurs within an intact capsid core\textsuperscript{18}. Although early studies suggested that uncoating occurs immediately after viral entry, recent investigations have highlighted the importance of the capsid for the process of transporting the viral genome to the nucleus while synthesis of the cDNA occurs\textsuperscript{18-20}. For instance, it has been shown that efficient viral reverse transcription occurs within the capsid core, which counteracts the tendency of the reverse transcriptase enzyme to dissociate from the RNA template\textsuperscript{20,21}. Although most studies favour a proposed model in which the complete disassembly of capsid most likely occurs upon completion of reverse transcription close to the nuclear pore, the precise timing of capsid uncoating still needs to be determined.

Figure 1.4 shows a schematic representation of the reverse transcription process to generate a double stranded cDNA copy of the genome. This process is initiated by the synthesis of the (-) strand DNA, which initiates from an annealed tRNA (transfer RNA) at the primer binding site located between U5 (unique sequence at 5’ end of genome) and the gag region at the 5’ end, and is extended by reverse transcriptase to the end of the R (repeat sequence) region\textsuperscript{22}. Strand transfer then occurs where the newly synthesized (-)
strand DNA anneals to the R region at the 3' end of either the same RNA strand or a second copy of the RNA genome. Synthesis of the (-) strand DNA is then continued while the RNA template is degraded by RNase H. The synthesis of positive strand DNA is initiated from a free 3' end produced by degradation of the genome template. Synthesis of the cDNA is completed by production of full 5' and 3' LTRs (long terminal repeats) at either end. After the synthesis of the double stranded genomic DNA, the pre-integration complex (PIC) is formed, which mediates entry of the viral cDNA into the nucleus.
Figure 1.4 Synthesis of ds HIV-1 genomic DNA by reverse transcription.

Events in synthesis of the HIV-1 double stranded DNA genome are indicated. The blue line indicates viral genomic RNA, and indicated are the repeat, R; unique 5’ sequence, U5; primer binding site, PBS; polypurine tract, PPT; and unique 3’ sequence, U3. The green line indicates minus (-) strand cDNA, and the red line plus (+) strand cDNA. 1) The Lys3 tRNA primer anneals with the primer binding site 2) Minus strand DNA synthesis is initiated by viral reverse transcriptase from the 3’ end of the tRNA primer and proceeds to the 5’ end of R. 3) Newly synthesized - strand DNA anneals to the 3’ R region of the same RNA strand or the second copy of the RNA genome carried by the virus. 4) Minus strand DNA synthesis continues, while the 3’ end of the genomic template RNA from the PPT is degraded by RNase H. 5) Plus strand DNA synthesis is initiated from the 3’ end of the remaining genomic RNA. 6) The remaining genomic RNA template is degraded. 7) Plus stand U3-R-U5-PBS DNA anneals to the PBS of the minus strand template. 8) Synthesis of both DNA strands is completed. Adapted from 13.

1.5.3 Nuclear import and integration of viral genome

The capsid detaches upon the PIC reaching the nuclear pore (Figure 1.3)23. Unlike other retroviruses for which genome entry into the nucleus is dependent on cell replication, HIV-1, like all lentiviruses, can infect non-dividing cells and the PIC is delivered across the nuclear membrane24. The PIC is translocated into the nucleus through the nuclear importer TNPO3/Ranbp2 (Transportin 3/ Ran binding protein 2) where, in association with another host cellular protein LEDGF (lens epithelium-derived growth factor)/p75, it is targeted to predominately actively transcribed regions within the host genome where viral integration occurs25-27. The viral integrase binds to the host DNA and cuts both strands, thus initiating a strand transfer reaction in which the viral cDNA is ligated to the host genome through gap repair28.
1.5.4 Viral expression, assembly and budding

Once the HIV-1 genome is integrated into the host chromosome, it relies on the host’s transcriptional machinery to synthesize its genomic RNA (Figure 1.3). Viral transcription produces three classes of mRNA (messenger RNA) molecules resulting from complex alternative splicing\(^{29,30}\). The HIV-1 genome contains four different splice donor sites (5’ splice sites) and eight splice acceptor sites (3’ splice sites), enabling the production of more than 40 mRNA species\(^{29,30}\). Un-spliced mRNAs represent the genomic and gag/pol-encoding mRNAs (about 9 kb), while transcripts that have undergone one and two or more splicing events produce singly spliced mRNAs (about 4 kb; Vif, Vpr, Vpu and Env encoding mRNAs) and multiply spliced mRNAs (about 2 kb; Tat, Rev and Nef mRNAs), respectively, which encode the additional viral structural and accessory proteins. Multiply spliced transcripts encoding Tat, Rev and Nef are the first mRNAs produced. Un-spliced and singly spliced transcripts encoding the other viral components are produced later during infection following accumulation of Rev protein which exports un-spliced and incompletely spliced HIV-1 RNA to the cytoplasm. As it is of particular importance in this thesis, the process of HIV-1 transcription is discussed in great detail later in this chapter.

With the exception of Env-encoding mRNA, HIV-1 transcripts are translated on host cytoplasmic ribosomes\(^{31}\). Viral Env mRNAs are translated in the rough endoplasmic reticulum (ER), which leads to synthesis of the Env polyprotein precursor, gp160\(^{32}\). Simultaneous with translation, gp160 is glycosylated with N-linked oligosaccharide side chains on the amino group of asparagine residues, in addition to O-linked oligosaccharide side chains on hydroxyl groups of serine/threonine residues\(^{33,34}\). The gp160 monomers
oligomerize in the ER into trimers which facilitate movement to the Golgi complex. Processing of gp160 through the secretory pathway includes modification by high-mannose oligosaccharide side chains in the trans-Golgi network. In the Golgi complex, gp160 is proteolytically cleaved at a highly conserved motif (K/R-X-K/R-R,49) by the host cell protease furin to yield the mature surface (SU) glycoprotein gp120 and transmembrane (TM) glycoprotein gp41. After cleavage, gp120 and gp41 remain associated via noncovalent interactions; three molecules each of gp120 and gp41 form a heterotrimeric HIV-1 glycoprotein spike. Once located at the host plasma membrane, Env is rapidly recycled via endocytosis, which is driven by interactions with the clathrin adaptor complexes. The internalization of Env and the shedding of gp120 from the cell surface due to the relatively weak and non-covalent nature of the gp120-gp41 interaction contribute to relatively low levels of Env incorporation into virus particles (~10 spikes/virion), a feature that likely helps the virus evade the host immune response and decreases virus-induced cytotoxicity.

Assembly of the virus occurs at regions near the host plasma membrane called lipid rafts, and is initiated by dimerization of two + strand RNAs through complementary sequences near the 5' end of each RNA, known as the dimerization initiation site (DIS) (Figure 1.3). Simultaneously, Gag and Gag-Pol polyproteins, bound to the packaging signal site in the viral RNA genome, interact with proteins at the plasma membrane to initiate creation of spherical virion particles. During budding from the plasma membrane, the viral protein Vif plays an important role in excluding incorporation of the host cell enzyme “APOBEC3G (apolipoprotein B mRNA-editing enzyme–catalytic polypeptide-like-3G)” present within the membrane, into the virion. The packaging of this host protein
into the newly made virions negatively affects reverse transcription in the next virus replication cycle. APOBEC3G causes deamination of deoxycytidine to deoxyuridine in the minus strand cDNA during reverse transcription, which causes degradation of viral cDNA through the activity of uracil DNA glycosylase, or can result in hypermutations or defective cDNA synthesis\textsuperscript{40,41}. Another viral infectivity factor, Vpu inhibits retention of the virus at the membrane by interfering with tetherin\textsuperscript{42,43}. Viral budding is mediated through the host ESCRT (endosomal sorting complexes required for transport) pathway\textsuperscript{44}. The ESCRT-associated proteins required for budding interact with the C-terminal domain of Gag to complete separation of the virus from the cell membrane. ESCRT-I and ESCRT-II proteins induce bud formation and stabilize the bud, while ESCRT-III is responsible for membrane scission\textsuperscript{44}.

1.6 HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

The introduction of highly active antiretroviral therapy (HAART) in the mid-1990s truly revolutionized the treatment of HIV-1 infections\textsuperscript{45,46}. With this therapy, patients are administrated a drug regimen consisting of two or three therapeutic agents that interfere with different stages of the virus replication cycle discussed above\textsuperscript{45,46}. Based on their structure and mechanism of action, these agents are commonly divided into the following classes, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors, fusion inhibitors, and co-receptor antagonists. Table 1.1 lists some of the most commonly prescribed drugs in each class. The combination of these drugs not only suppresses viral
load to a clinically undetectable level (below 50 RNA copies/mL) in the plasma of HIV-1 infected patients, but also lowers the pace at which resistant viral strains emerge.\textsuperscript{47-49}

Table 1.1 Commonly prescribed antiretroviral drugs for HIV-1 infected patients

<table>
<thead>
<tr>
<th>Class\textsuperscript{a}</th>
<th>Generic Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRTIs</td>
<td>Abacavir; Tenofovir; Emtricitabine</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>Delavirdine; Efavirenz</td>
</tr>
<tr>
<td>PIs</td>
<td>Ritonavir; Atazanavir</td>
</tr>
<tr>
<td>Integrase inhibitors</td>
<td>Dolutegravir; Raltegravir</td>
</tr>
<tr>
<td>Co-receptor antagonists</td>
<td>Maraviroc, Aplaviroc</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Class of antiretroviral agent(s) based on their structure and mechanism of action. Abbreviations: NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors.

1.7 T CELL SIGNALLING PATHWAYS

During HIV infection, the viral genome integrates into the host chromosome and therefore viral gene expression becomes dependent upon the host cell transcription machinery and gene regulatory mechanisms.\textsuperscript{50} As primary targets for HIV-1 infection, the activation status of T lymphocytes plays a critical role in determining the fate of the virus.\textsuperscript{51-53} As shown in Figure 1.5, T cell stimulation is initiated when the CD4 T cell receptor (TCR), in association with the CD3 receptor and zeta chains recognizes an antigen presented by a major histocompatibility complex (MHC) class II molecule anchored in the cellular membrane of the antigen presenting cell (APC).\textsuperscript{52,54,55} Recognition of the presented antigen causes recruitment of the CD4 receptor to the site, which initiates a cascade of phosphorylation events through which the T cell activation signal is transduced to the nucleus. The CD28 receptor can enhance T cell response by engaging CD80 on the APC. Upon antigen presentation, CD4 becomes associated with p56\textsuperscript{lo}, a member of the Src family of tyrosine kinases, which phosphorylates zeta chains of the CD4 TCR/CD3 complex, leading to recruitment of ZAP-70 (Zeta-chain-associated protein
kinase 70) kinase. Phosphorylation of ZAP-70 causes activation of phospholipase C (PLC), which hydrolyses phosphatidylinositol (4,5) bisphosphate (PIP2) into the secondary messengers diacylglycerol (DAG) and inositol triphosphate (IP3). These secondary messengers stimulate three downstream signalling pathways with different transcriptional targets52,54,55. In T cells, DAG activates protein kinase C (PKC) and the Ras/Raf/MAPK/ERK pathways, while IP3 triggers release of intracellular calcium and consequently activates the calcineurin pathway. PKC activation results in activation of the inhibitor-κB kinase (IKK) and subsequent phosphorylation of the inhibitor of-κB (IκB) (Figure 1.5)52. Dissociation and degradation of IκB causes release of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) p65 subunit which forms heterodimers with the NFκB p50 subunit in the nucleus to form active NFκB. Activation of the Ras/Raf/MAPK/ERK pathways leads to AP1 (activator protein 1) (cFos-cJun) phosphorylation which enhances its transactivation capacity. With the other parallel pathway, Ca2+ influx into cytoplasm leads to activation of calmodulin (Calm) and the calmodulin-dependent phosphatase calcineurin (CaN)56. Calcineurin dephosphorylates cytoplasmic NFAT (nuclear factor of activated T-cells) which allows its translocation to the nucleus56. Activated forms of NFκB, AP1, and NFAT bind to cis-elements within the HIV-1 5’ LTR enhancer region and cooperatively stimulate expression of the HIV-1 mRNAs. It should be stated that there are other host transcription factors not directly regulated by these signalling pathways that also bind to the HIV-1 LTR in both unstimulated and stimulated T cells.
Figure 1.5 Signalling pathways activated by engagement of the TCR/CD3 receptor complex with the antigen presenting complex in dendritic cells.

Engagement of the T cell receptor (CD3/CD4) causes activation of associated p56\textsuperscript{lck} and ZAP-70 protein kinases and consequently production of the secondary messengers DAG and IP\textsubscript{3} through PIP\textsubscript{2} hydrolysis by PLC\textsubscript{γ}. IP\textsubscript{3} causes calcium release and activation of calcineurin (CaN), which subsequently dephosphorylates NFAT to allow its accumulation in the nucleus. DAG activates protein kinase C θ (PKC θ) and the Ras/Raf/MEK/ERK pathways through RasGRP. The transcription factor AP1 (cFos-cJun) is phosphorylated and activated by ERK, while NFκB is activated upon destruction of the IκB through a phosphorylation mediated by the I-κB kinase (IKK).

APC, antigen presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor; ZAP-70, zeta-chain-associated protein kinase 70; PIP\textsubscript{2}, phosphatidylinositol (4,5) bisphosphate; PLC\textsubscript{γ}, Phospholipase C gamma; PI3K, phosphatidylinositol 3-kinase; DAG, diacylglycerol; IP\textsubscript{3}, inositol triphosphate; ER, endoplasmic reticulum; IP\textsubscript{3}R, inositol
triphosphate receptor; Calm, calmodulin; NFAT, nuclear factor of activated T-cells; RasGRP, RAS guanyl nucleotide-releasing protein; MEK1/2, MAPK/ERK kinase 1/2; ERK, extracellular signal-regulated kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP1, Activator protein 1.


1.8 REGULATION OF HIV-1 TRANSCRIPTION

The 5' LTR of the HIV-1 genome functions as the regulatory region for viral transcription\(^{57}\). A number of transcription factors that are regulated by T cell signaling pathways, as discussed above, bind to their cis-element(s) within the U3 (unique sequence at the 3' end of genome) LTR region and promote transcription (Figure 1.6)\(^{58}\).

Many of these factors which function as transcriptional activators have been shown to be involved in recruiting or interacting with histone acetyltransferases (HATs) which consequently facilitate transcription by creating an accessible chromatin conformation\(^ {59-61}\). Furthermore, the interaction of such activators with the coactivator complex mediator directs assembly of the transcription pre-initiation complex (tPIC) which is comprised of the general transcription factors (GTFs) associated with RNA polymerase II (RNA pol II), on the viral core promoter\(^ {62,63}\). These transcriptional activator-mediator interactions as well as the direct contact of the mediator complex with the RNA pol II, are the key steps for both recruiting and further stabilizing RNA pol II at the promoter region\(^ {63}\). Accordingly, the formation of a stable tPIC is initiated by binding of TBP (TATA-binding protein), a subunit of the general transcription factor TFIID, to the TATA box motif within the HIV-1 core promoter, which allows viral transcription to initiate at a basal level\(^ {62}\).
**Figure 1.6 Schematic representation of regulatory elements within the 5' HIV-1 LTR.**

The enhancer region of the 5' HIV-1 LTR binds to the multiple transcriptional activators, including NFAT, NFκB, AP1, GABP/Ets, SP1, and RBF2 (TFII-I, USF1, USF2). Upon binding of these transcriptional factors to their corresponding cis-regulatory element, general transcription factors and co-activators are recruited to the HIV-1 core promoter region to stimulate transcription. The transcriptional start site is denoted by a green arrow and +1.

Nuc0, nucleosome; ac, acetylation tag; LTR, long terminal repeat; U3, unique sequence at 3’ end of the genome; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP1, activator protein 1; GABP, GA-binding protein; SP1, specificity protein 1; RBF2, Ras-responsive element binding factor 2; TFII-1, transcription factor II-I; USF1/2, upstream-stimulatory factor 1/2. Adapted from 58.

Multiply spliced-mRNAs, encoding Tat, Rev, and Nef, are produced early, likely by sporadic basal transcription that escapes pausing mechanisms imposed on RNA Pol II as described below. These RNAs are processed by the host cell’s regular mRNA export pathway to translocate into the cytoplasm where they are translated into Tat, Rev, and Nef proteins. Tat and Rev viral proteins play vital roles in regulating transcription and RNA processing to enable full expression of the HIV-1 genome. In the absence of Tat, the RNA pol II complex becomes paused downstream of the transcription start site (TSS) through the interaction of the transcriptional inhibitory factors, NELF (negative elongation factor).
and DSIF (DRB sensitivity-inducing factor)\textsuperscript{64}. HIV-1 overcomes this inhibition through the function of the Tat transactivator protein\textsuperscript{65}. Unlike conventional activators that bind to specific DNA sequences, Tat interacts with the RNA element TAR (trans-activation response) that is located at the 5’ end of the nascent viral RNA transcript. Tat–TAR interaction promotes recruitment of the positive transcription elongation factor b (pTEFb), consisting of Cyclin T1 and Cdk9 (cyclin-dependent kinase 9), to the site where the RNA pol II is paused. Cdk9 phosphorylates the transcriptional pausing factors (NELF and DSIF) and the C-terminal domain (CTD) of RNA pol II. Phosphorylation of NELF and DSIF prevents their inhibitory effects on transcription elongation which triggers RNA pol II processivity. In addition, phosphorylation of serine 2 (Ser2) residues within the CTD heptad repeats of RNA pol II enables promoter escape\textsuperscript{29}. Tat also recruits chromatin remodelers, including SWI/SNF (SWItch/sucrose non-fermentable) complexes, and histone acetyltransferases (HATs), such as p300/CBP (cyclic AMP response element-binding protein) and P/CAF (p300/CBP-associated factor), to further enhance viral transcription through post-translational modification of histones flanking the promoter\textsuperscript{66,67}. The Rev protein allows synthesis of additional viral proteins by binding to the Rev response element (RRE), which is only present in unspliced (genomic and gag/pol) and singly-spliced (vif, vpr, vpu, and env) mRNAs, and guides translocation of these unspliced transcripts from the nucleus to the cytoplasm, where the remaining viral proteins are synthesized\textsuperscript{68}. Rev is then recycled to the nucleus with the help of importin $\beta$. 
1.9 HIV-1 LATENCY ESTABLISHMENT AND MAINTENANCE

Active viral transcription, as discussed above, reflects a productive viral infection, which occurs in activated T cells. However, upon clearance of the antigen to which the host T helper cell is programmed, these cells revert to a resting G₀ state. This leads to generation of long-lived resting memory CD4⁺ T cells, harboring stably integrated but transcriptionally repressed or latent proviruses⁶⁹. The major molecular mechanisms that enforce viral latency are discussed below.

1.9.1 Chromatin

The human genome is packaged in a highly organized complex within the cell nucleus called chromatin⁷⁰,⁷¹. The fundamental unit of chromatin is the nucleosome, consisting of two superhelical turns of DNA wrapped around the histone core composed of a histone protein H3/H4 tetramer, and histone protein H2A/H2B dimers. This protein-DNA structure creates a dynamic regulatory mechanism for gene expression⁷¹. Post-translational modifications of the histone tails, particularly acetylation and methylation⁷² as well as chromatin remodeling BAF (BRG1-associated factor) complexes (SWI/SNF), modify nucleosome structure in ways that enable regulation of accessibility of DNA cis-regulatory elements with their corresponding cellular factors⁷³. Strikingly, two nucleosomes, regardless of the viral integration site, are precisely positioned at regions within the HIV-1 5' LTR⁷⁴,⁷⁵. They are Nuc0, which is located at the distal regulatory region spanning nucleotides -415 to -255, with respect to the TSS, and Nuc1, which is located at the proximal core promoter spanning nucleotides -3 to +141 (Figure 1.7)⁵⁸. In unstimulated cells, viral transcription is inhibited by the presence of Nuc1, which blocks
formation of the tPIC, and activation of transcription from the LTR requires displacement of Nuc1. Rafati et al. have shown that the BAF chromatin remodeler is a necessary for positioning of Nuc1 near the TSS, and its absence causes Nuc1 displacement from the core promoter\textsuperscript{76,77}.

**Figure 1.7 Factors regulating repression of the HIV-1 LTR.**

In unstimulated cells, activator proteins are replaced by transcriptional repressors such as NF\(\kappa\)B p50, that recruit histone deacetylase and histone methyltransferase complexes. LTR-bound factors, including SP1, and RBF2 (TFII-I, USF1, and USF2) are converted from activators to repressors that recruit HDAC enzymes (HDAC1/2/3). Transcription factor YY1 is associated with the latent provirus 5‘ LTR which also recruits HDACs. CTIP2 recruits histone methyltransferase Suv39H1 that enforces transcriptional silencing. The 5‘ LTR is also associated with two strongly positioned nucleosomes, designated nuc0 and nuc1.

Nuc0/1, nucleosome 0/1; me, methylation tag; HDAC1/2/3, histone deacetylase 1/2/3; RBF2, Ras-responsive element binding factor 2; TFII-1, transcription factor II-I; USF1/2, upstream-stimulatory factor 1/2; YY1, yin yang 1; CTIP2, COUP-TF interacting protein 2; Sp1, specificity protein 1; LSF, late Simian Factor. Adapted from\textsuperscript{58}. 
Studies demonstrating that histone deacetylase (HDAC) inhibitors are capable of causing elevation of viral transcription by promoting Nuc-1 remodeling, have suggested that maintenance of Nuc-1 is highly dependent on histone acetylation status; in unstimulated cells, HDAC proteins are recruited to the HIV-1 core promoter to repress viral transcription by maintaining Nuc-1 positioning\(^{78,79}\). The recruitment of HDACs by multiple host cellular factors bound to specific sequences in the viral 5' LTR has been demonstrated in latently infected cells. In particular, HDAC1 is recruited to the viral core promoter by transcription factors, including NFκB p50, YY1 (yin yang 1), LSF (late simian factor), AP-4 (activator protein 4), SP1 (specificity protein 1), and CTIP2 (COUP-TF interacting protein 2), while HDAC3 is recruited by the transcription factor Ras-responsive element binding factor 2 (RBF2) which is a protein complex comprised of TFII-I (transcription factor II-I), USF1 (upstream stimulatory factor 1), and USF2 (upstream stimulatory factor 2) (Figure 1.7)\(^{58,80-84}\).

Upon removal of acetyl groups, the lysine residues can undergo further modification, such as by methylation, which is catalyzed by histone methyltransferases (HMTs)\(^{85}\). Lysine methylation can have both activating or repressive effects on transcription, depending on which lysine residue is methylated. For instance, H3K9-me3 and H3K27-me3 are two well-characterized histone modifications associated with gene repression, while 3K4-me3 is associated with gene activation\(^{86}\). With regard to HIV-1 transcription, it has been shown that the Suv39H1 methyltransferase, recruited by SP1/CITP2 to the HIV-1 5' LTR, causes histone H3K9-me3 and the subsequent recruitment of heterochromatin protein 1 (HP1), which further maintains and propagates H3K9-me3\(^{87}\). The G9a methyltransferase is also involved in repressing HIV-1
transcription by dimethylation of the H3K9. Trimethylation of the H3K27 by another histone methyltransferase, enhancer of zeste homolog 2 (EZH2), has also been shown to cause repression of HIV-1 transcription.

1.9.2 Integration site

The existence of distinct chromatin environments (euchromatin and heterochromatin) within the human genome has important consequences for expression of viruses that integrate within the host cell’s genome. Transcribed genes are usually found within euchromatin, a relaxed conformation of chromosomal DNA, while non-expressed and/or poorly transcribed genes reside in the condensed chromosomal DNA, heterochromatin. Studies of HIV-1 integration sites in cell lines, as well as resting CD4+ T cells from patients, have revealed that HIV-1 predominately integrates into transcriptionally active genes. This is likely because the transcriptional co-activator LEDGF/p75 binds to integrase associated with the viral PIC and targets integration to intronic regions within highly expressed genes. However, latent proviruses can result from integrations in heterochromatin regions, such as centromeric alphoid repeats and centromeric regions, as shown with an in vitro J-Lat cell line model of HIV-1 latency. Despite predominately integrating with actively transcribed chromosomal regions, HIV-1 invariably produces latent infections, either immediately through mechanisms that have yet to be elucidated, or gradually through epigenetic silencing; this implies the site of integration does not predominately dictate mechanisms contributing to viral silencing but rather the capability to produce latent infection is a feature of the virus itself. Nevertheless, several studies have shown that transcriptional phenotypes are influenced by the
integration site, as variable HIV expression phenotypes are observed for both basal and stimulated levels for individual integrated clones, presumably due to different chromatin landscapes flanking the site of integration98-100.

1.9.3 DNA methylation

DNA methylation of promoters in metazoans is generally associated with permanently repressed genes, and HIV-1 latency could be reinforced by DNA methylation101. It has been shown that two CpG islands flanking the viral TSS are methylated in the J-Lat and primary CD4+ T cells model of HIV-1 latency102,103. Methyl-CpG-binding domain containing protein-2 (MBD2) and HDAC2 have been detected at one of these sites, which could be associated with HIV-1 repression103. However, its exact role during in vivo viral infection still needs further elucidation.

1.9.4 MicroRNAs

As another evolutionarily conserved cellular mechanism to control gene expression, mature microRNAs anneal to complementary sequences located at the 3’ end of their target mRNAs, resulting in mRNA degradation and/or inefficient translation104,105. With respect to establishment of HIV-1 latency, it has been observed that several miRNAs have repressive effects on viral transcription by restricting expression of host cellular factors and/or viral proteins required for transcription106,107. For example, the microRNAs miR-198 and miR-17/92 cause a reduction in the cellular levels of cyclin T1 and p300/CBP-associated factor (PCAF), respectively108,109. Furthermore, a cluster of
microRNAs enriched in resting HIV-1 infected CD4+ T cells directly target the 3’ end of the viral transcripts, reducing expression of both Tat and Rev\textsuperscript{110}.

1.9.5 Role of host cell transcriptional activators and viral Tat in the establishment of viral latency

As discussed above, the initiation of HIV-1 transcription is dependent on host transcription factors, some of which are activated by extracellular stimuli through the TCR and stimulation of downstream T cell signaling pathways. Importantly, a loss of signaling from the TCR causes sequestration of the p65 NFκB subunit by IκB in the cytoplasm of resting cells, and thereby limits the availability of the active form of NFκB (p65/p50) within the nucleus\textsuperscript{65}. Furthermore, in un-activated cells, p50-p50 homodimers, representing a repressive form of NFκB, occupy the same elements on the viral 5’ LTR as the p65-p50 heterodimer in activated cells, which encourages the repression of viral transcription through the recruitment of HDACs (Figure 1.7)\textsuperscript{65}. As posttranslational modifications of histone tails play a vital role in regulating HIV-1 transcription, the acetylation status of non-histone substrates, in particular lysines 221 and 310 of the NFκB p65, also regulates viral expression\textsuperscript{111-113}. It has been shown that the deacetylases HDAC3 and SIRT1 (sirtuin 1) deacetylate p65 at these residues, which promotes association with IκB to inhibit transcriptional activation by NFκB\textsuperscript{111,114}. Notably, viral Tat protein inhibits SIRT1-mediated deacetylation of p65 K310, thereby enhancing the NFκB transcriptional activation function in stimulated cells\textsuperscript{115}.

Similarly, NFAT and AP1 activity are down-regulated in unstimulated T cells, and consequently this decreases transcription from the virus LTR promoter, which eventually
prevents the production of Tat protein. Loss of Tat from the cell is an important event in the production of HIV-1 latency because this prevents recruitment of pTEFb and transcriptional elongation of the viral RNAs. pTEFb is also inhibited in unstimulated cells by association with the inhibitory HEXIM1 (hexamethylene bisacetamide-induced protein/7SK snRNP (small nuclear ribonucleoprotein) complex) in the cytoplasm, which further contributes to viral latency. Additionally, phosphorylation of T186 (threonine 186) at the active site of Cdk9 of pTEFb, which is required for kinase activity, is inhibited in unstimulated cells, which results in inability to phosphorylate Ser2 RNA Pol II CTD, which inhibits elongation of polymerase, thus contributing to viral latency.

1.10 NON-T CELL HIV-1 LATENT RESERVOIRS

In addition to CD4+ T lymphocytes a variety of additional cell types are susceptible to the HIV-1 infection, including monocytes, macrophages, dendrocytes, microglia and human hematopoietic stem cells. Notably, monocyte-derived macrophages and microglial cells play an important role in long-term HIV-1 infection. Due to their capacity to infiltrate a variety of tissues that can act as sanctuaries inaccessible to antiretroviral drugs, these cells can contribute to the persistence of HIV-1 infections. Specifically, latently infected microglial cells, with 3- to 10-year life spans, are resistant to current antiretroviral therapies, and thus representing isolated reservoirs for virus maintenance that would eventually cause neurological disorders in HIV-1 infected individuals. Other cell types, such as epithelial cells and astrocytes lacking CD4 receptors, can also be infected with HIV-1 through syncytial fusion with infected CD4+ T cells. However, their role in the long-term persistence of the virus has not been elucidated.
1.11 REVERSING HIV-1 LATENCY

Although current antiretroviral therapies have rendered HIV-1 infection a manageable disease, ongoing efforts to develop a cure could profoundly affect the outlook of the HIV/AIDS epidemic\textsuperscript{121}. Phenotypically indistinguishable from uninfected cells, latently infected cells harbouring a stable genome-integrated virus represent a reservoir that is insensitive to the immune system and current antiretroviral treatments. Reappearance of virus from this population can lead to disease progression upon cessation of therapy, thus making patients dependent on treatment for the remainder of their lives\textsuperscript{122}. Due to the accumulation of side effects caused by prolonged consumption of daily medications and, ultimately, the inevitable emergence of viral strains resistant to the antiretroviral drugs, effective curative strategies must be developed to eradicate virus from infected patients\textsuperscript{122,123}.

One proposed therapeutic strategy to purge the latent proviruses is generally referred to as "shock and kill." The rationale for this strategy is that stimulation of transcription from the repressed provirus, and subsequent production of viral proteins in the latently infected cellular reservoirs (shock phase) would theoretically make these cells vulnerable to clearance, either through virus-induced cytopathic effects or immune-mediated mechanisms (kill phase; Figure 1.8)\textsuperscript{124}. Initial clinical investigations on stimulating latent viral transcription involved the use of reagents that produced T cell activation\textsuperscript{125}. For example, OKT3, a murine monoclonal antibody that recognizes CD3 of the T cell receptor complex, causes an activation response that partially mimics engagement of the receptor and correspondingly activates downstream signalling.
However, treatment with OKT3 was observed to cause toxicity due to off-target effects resulting from general T cell activation\textsuperscript{126}.

![Diagram of HIV infection and treatment](https://example.com/diagram.png)

**Figure 1.8 Elimination of latently infected cells by the proposed shock and kill strategy.**

Stimulation of the transcriptionally-latent HIV-1 reservoirs, predominately integrated in genome of the CD4$^+$ T cells, results in the production of viral particles (shock phase) which would subsequently mark those latently infected-cells for elimination through immune defence mechanisms (kill phase). Simultaneous presence of antiretroviral drugs would also protect other cells from infection by newly released virions. Adapted from\textsuperscript{124}.

Following these unsuccessful initial trials, most efforts focused on identifying small molecular compounds with the capacity to reactivate transcription from the 5' LTR to a level that would enable production of sufficient levels of the viral transactivator Tat, which produces strong positive feedback, thereby resulting in expression of significant levels of viral proteins\textsuperscript{127}. Small molecules with this capacity are known as latency-reversing agents (LRAs). These pharmacological agents mainly function by targeting host-dependent molecular mechanisms involved in gene regulation. Two predominant mechanisms through which well-studied LRAs stimulate viral transcription are discussed below.
1.11.1 PKC-NFκB signalling

PKC-NFκB signalling represents an important regulatory pathway for regulation of HIV-1 transcription, and consequently LRA compounds known as PKC agonists have been studied extensively as a potential means to reverse HIV latency. PKC agonists can act as DAG mimetics capable of binding to and stimulating activity of one or more PKC isoforms, causing release of NFκB p65 from IκB, allowing its translocation to the nucleus where it induces gene expression as a heterodimer with p50 (Figure 1.5) \(^{128}\). Compounds with this activity are among the most potent LRAs identified to date, and notable examples include PMA, prostratin, bryostatin-1, and ingenol-3-angelate. For example, prostratin induced viral transcription in a variety of in vitro cell lines with latent HIV-1 provirus, as well as in primary CD4+ T cells isolated from HIV-infected patients on anti-retroviral therapy\(^ {129,130}\). However, due to side effects resulting from general T cell activation, this compound has not been examined in clinical trials for HIV-1 therapy. The safety and efficacy of bryostatin-1 was examined in one clinical investigation where a single administration of 10 µg/m\(^2\) or 20 µg/m\(^2\) was given to 12 infected patients on ART. In this study, no adverse effects were observed, but also, neither dose caused an increase in production of cell-associated viral RNAs\(^ {131}\). Ingenole-3-angelate, which has FDA-approval for treatment of actinic keratosis, has shown significant HIV latency reversing activity in various in vitro cellular lines with integrated provirus\(^ {132-134}\). It has been previously demonstrated that the latency-reversal activity of PEP005, which is of particular interest in this thesis, is mediated through the PKC–NFκB signaling pathway, where the phosphorylation of PKCδ/θ and IκBα/IκBε is induced by PEP005 as early as 0.5–2 h post-treatment\(^ {135}\). This observation suggests that this compound is involved in
mechanism(s) inducing transcription at the level of initiation. However, a recent study indicates that PEP005 promotes accumulation of HIV-1 RNAs by promoting transcriptional elongation, rather than initiation\textsuperscript{136}. This study employed reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) assays which indicated that PEP005 causes accumulation of polyadenylated and spliced transcripts, relative to RNAs from the 5' leader region, which suggests that activation of transcriptional elongation rather than initiation\textsuperscript{136}.

1.11.2 Epigenetic modifications

Since post-translational modifications, particularly at the core histone tails, play a critical role in maintaining gene expression and the establishment of viral latency, the effect of agents that affect epigenetic modification, such as various HDAC inhibitors (HDACIs) and histone methyltransferase inhibitors (HMTIs), on inducing viral transcription has been investigated in several pre-clinical and clinical studies. For instance, the well-studied non-class HDACIs (pan-HDACIs) valproic acid, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) have been shown to reactivate latent HIV-1 in various reporter cell lines as well as primary cells isolated from HIV-infected patient samples\textsuperscript{137-140}. Particularly, SAHA, which is an FDA-approved HDACi for the treatment of cutaneous T cell lymphoma, has shown efficacy to reactivate viral transcription in \textit{in vitro} cellular models of HIV-1 latency, including J89, ACH-2, U1, and J-Lat, \textit{in vitro}-infected primary CD4+ T cells, and primary CD4+ T cells isolated from patient samples\textsuperscript{139,141,142}. Furthermore, SAHA was the first HDACi showing potency to stimulate viral production from latently infected cells in patients treated in clinical trials \textsuperscript{143,144}. 
However, HDACIs are generally considered weak LRAs, as they mainly enhance basal levels of viral transcription. In addition to HDACIs, HMTIs such as the Suv39H1 inhibitor chaetocin\textsuperscript{145-147}, the G9a inhibitor BIX01294\textsuperscript{88}, and the EZH2 inhibitor DZNep\textsuperscript{89}, have also been effective in activating viral transcription in \emph{in vitro} cell lines with HIV provirus. HDACIs and HMTIs have been reported to induce expression of latent HIV-1 synergistically in combination, although such findings have been largely obtained from \emph{in vitro} studies.

1.12 RESEARCH OBJECTIVES

Long-lived populations of latently infected resting CD4\textsuperscript{+} T cells are a major barrier to developing an HIV-1 cure. To overcome this challenge, many studies have aimed to identify a novel means to purge these latent viral reservoirs. To this end, this thesis focuses on the shock phase of the HIV the proposed curative strategy known as "shock and kill".

In this study, the identification of novel LRAs with pharmacological features required for disruption of HIV-1 latency was investigated. Towards this goal, a high-throughput screen of small molecule libraries representing a broad range of chemical structures was conducted, through which five new LRAs (PH01–PH05) were identified. The \emph{in vitro} and \emph{ex vivo} analyses presented in this study demonstrated that each of these agents could serve as potential starting points for the development of therapeutic candidates for future clinical investigations of potential to eliminate latently infected cells from patients. Furthermore, one particular combination of LRA, was PH02, identified in this study, and a previously known LRA (PEP005), found to produce significant synergy
for inducing viral transcription. I also review efforts from the research community as a whole to discover novel LRAs via low and high throughput small molecule screens. Characteristics and biochemical properties of chemical structures with latency reversing activity from these efforts are summarized, and this analysis may provide insight for further research into optimized designs for new classes of more potent LRAs. Furthermore, characterizing the mechanisms through which novel LRAs induce viral transcription will provide a better understanding of mechanisms regulating HIV-1 latency and pathogenesis.
CHAPTER 2 HIV-1 LATENCY REVERSING AGENTS WITH DIVERSE CHEMICAL STRUCTURES TARGET VARIOUS MOLECULAR PATHWAYS

2.1 INTRODUCTION

Central to the success of the shock and kill strategy as a proposed HIV curative strategy, is the development of an effective means for reactivating HIV provirus expression in the latently infected population. Discovery of novel LRAs, and redirecting previously characterized bioactive molecules for this purpose have been the focus of many studies over the past decade\textsuperscript{148}.

As discussed in Chapter 1, many studies directed at reversing viral latency, have employed PKC agonists that activate the PKC-NFκB cell signalling and chromatin-modifying agents, mostly histone deacetylase inhibitors\textsuperscript{149}. Results from these studies have revealed that successful implementation of this general approach will likely depend on several crucial factors relating to influence of the chromosomal integration site on viral transcriptional responses, and enhanced killing of cells where viral expression has become reactivated. Trials using HDACIs as LRAs have indicated that although viral replication can be induced in patients’ T cells, this treatment alone is not capable of reducing the latently infected population\textsuperscript{150-153}. This limitation can likely be attributed to inadequate anti-HIV-1 cellular immune response in these patients, but also because of limitations of the HDACIs, which are only capable of inducing a subset of provirus integrations. Several studies have confirmed this limitation using cell lines with HIV-1 reporter virus where it was shown that the site of chromosomal integration influences responsiveness to signaling agonists and chromatin-modifying agents\textsuperscript{98,100,154}. 

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Importantly therefore, a critical property of LRAs is the capacity to induce a broad range of provirus integrated at diverse chromosomal locations. Consequently, identification of effective novel LRAs must involve screens that employ multiple different *in vitro* latency cell lines, in addition to infected normal CD4+ T cells, and latently infected cells from patients on antiretroviral therapy. Additional important considerations include a requirement for minimal toxicity, and negligible effects on global T cell activation. Furthermore, there is increasing recognition that no single LRA is likely capable of inducing the full spectrum of latent provirus required to purge a sufficient fraction of infected cells, and therefore most recent efforts have focused on developing and characterizing combinations of compounds that produce synergistic effects on reactivation of individual provirus, and exert a broader effect on the latently infected population as a whole\textsuperscript{155-159}.

Thus far, none of the LRAs examined in clinical trials have reduced the size of persistent HIV-1 infection\textsuperscript{160-164}. Therefore, new classes and combinations of molecules with optimized pharmacological features must be developed to improve the shock parameter of the potential shock and kill strategy for therapeutic purposes. To this end, we review results of efforts to date towards discovering novel effective LRAs using low- and high-throughput screening of small molecule libraries. We compare structural properties and pharmacological features of the most effective compounds from these screens, and their capacity to produce synergistic responses with previously characterised LRAs. Comparison of novel molecules identified in these screens may reveal important chemical scaffolds, functional groups and specific moieties that provide important features required for anti-latency activity of particular classes of compounds.
Therefore, we expect this analysis, will aid in the design of optimized chemical structures for future research towards development of more potent LRAs for use in potential strategies to purge HIV-1 infections.

2.2 LRAS IDENTIFIED FROM SMALL MOLECULE SCREENS EXHIBIT DIVERSE CHEMICAL STRUCTURES

We examined results from the 18 separate studies published to date (Table 2.1) involving screens of small molecule compound libraries for the capability to induce transcription of latent HIV-1 reporter provirus. Most of these studies employed primary screens involving previously characterized cell line models for latency, most commonly the Jurkat T cell leukemia-derived J-LAT lines, where GFP expression is dependent on expression from the HIV-1 5' LTR. Several screens were performed with partially immortalized Bcl-2-transduced resting CD4+ T cells infected with a GFP-expressing HIV-1 reporter virus. Most screens have involved smaller libraries of compounds with known biological activities, natural compounds, or compounds with defined targets, such as epigenetic modifiers, and protein kinases or phosphatases. Five screens have involved libraries of synthetic chemical compounds with diverse structures (Table 2.1). As detailed above, compounds capable of inducing HIV-1 provirus expression in initial screens, and which produce a dose dependent response in re-analysis, are typically re-examined for this activity using additional reporter virus cell lines to determine whether the compound(s) are capable of reactivating a broad spectrum of HIV-1 provirus integrants. Importantly, most studies also examine effectiveness for induction of replication-competent virus from CD4+ T cells isolated from aviremic patients on antiretroviral
therapy, using a quantitative viral outgrowth assay (qVOA), or induction of viral RNA by quantitative RT-PCR (reverse transcription-polymerase chain reaction) (Tables 2.1, 2.2). Finally, the capacity of novel LRAs to produce synergistic induction of provirus expression in combination with previously characterized LRAs, typically signaling agonists or HDAC inhibitors, are often assessed. The efficacy of these novel compounds for induction of HIV-1 transcription in reporter cell lines, reactivation of virus replication in primary cells from aviremic infected patients on ART, and their capacity to produce synergistic responses are summarized in Table 2.2.

To summarize effects of LRAs identified and characterized in these screens, we have classified the compounds into four groups, that include epigenetic modifiers, chromatin modulators, signaling effectors or modulators, and transcriptional elongation modulators (Table 2.3). Precise identification of cellular targets affected by previously uncharacterized compounds and, consequently, the molecular pathway(s) involved, is challenging and sometimes can remain elusive for decades. Furthermore, identification of molecular targets also requires detailed analysis of off-target and secondary effects that may influence HIV-1 transcription. Therefore, the groupings indicated here are intended to represent major pathways in which the compounds are most likely involved (Figure 2.1), but in many cases details of molecular effects have not been completely elucidated.
Table 2.1 Overview of studies to identify novel Latency Reversing Agents (LRA)

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<th>Ref.</th>
<th>Library; Initial # compounds</th>
<th>LRA(s)</th>
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<td>Institutional library of HDACIs; 32</td>
<td>MS-275</td>
<td>ACH-2, U1, J-Lat A1</td>
<td>NI</td>
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<td>166</td>
<td>MicroSource Spectrum, JHDL; ~5,000</td>
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<td>Bcl-2 transduced-rCD4+ T cells, J-Lat</td>
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<td>J89GFP, A5, EF7, HIV-1 NL4-3 infected CD8+ T cell-depleted PBMCs</td>
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<td>CEM T cells, Jkt-pHR'P-luc cells, THP1-pHR'P-luc cells, HIV pHR'P-PNL-H13LTat-δNef-GFP-infected CD4+ T/PBMCs</td>
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<td>Epigenetic modulating compounds; 94</td>
<td>Chidamide; MS-275</td>
<td>gGn-p* infected rCD4+ T cells, 24STNLEG</td>
<td>N</td>
</tr>
<tr>
<td>176</td>
<td>Panel of Isoform-targeted HDACIs; 15</td>
<td>Largazole</td>
<td>J-Lat 10.6, Greene cell model</td>
<td>N</td>
</tr>
<tr>
<td>177</td>
<td>Natural Compounds; ~100</td>
<td>EK-16A</td>
<td>C11 Jkt, J-Lat clones (10.6; 6.3)</td>
<td>N</td>
</tr>
<tr>
<td>178</td>
<td>Chemical Kinase inhibitors; 378</td>
<td>PH01-PH05</td>
<td>J-Lat A1, Jkt T-Luc, Jkt T-LTR-DSRed clones (11; 131)</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>a</sup>Study reference; <sup>b</sup>Novel LRA(s) identified; <sup>c</sup>in vitro HIV-1 latency cellular models utilized; <sup>d</sup>Compound(s) induce T cell activation. Abbreviations: LRA, latency reversing agent; TCA, T cell activation; HDACI, histone deacetylase inhibitor; N, no; NI, not investigated; JHDL, Johns Hopkins Drug Library; rCD4+ T cells, resting CD4+ T cells; Jkt, Jurkat.
<table>
<thead>
<tr>
<th>LRA(s)</th>
<th>Cell linea</th>
<th>Time (h)b</th>
<th>EC (µM)c</th>
<th>Synergyd</th>
<th>Patiente</th>
<th>C (µM) [Time]f</th>
<th>Techniqueg</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-275165</td>
<td>ACH2</td>
<td>72</td>
<td>0.10</td>
<td></td>
<td>BSO</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>5HN166</td>
<td>Bcl-2 transduced-rCD4+ T cells</td>
<td>40-48</td>
<td>0.5</td>
<td>NI</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AV6167</td>
<td>24STNLSG</td>
<td>48</td>
<td>12</td>
<td>VA</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disulfiram168</td>
<td>Bcl-2 transduced-rCD4+ T cells</td>
<td>24/48</td>
<td>0.3-0.5, 5-10</td>
<td>NI</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMQO170</td>
<td>J-Lat</td>
<td>24</td>
<td>80</td>
<td>TNF-α, PMA, TSA</td>
<td>PBMCs</td>
<td>80 [36h]</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>Aclacinomycin155</td>
<td>CA5 cells</td>
<td>24</td>
<td>0.5</td>
<td>TNF-α</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57704171</td>
<td>U1</td>
<td>24</td>
<td>5-9</td>
<td></td>
<td>Prostratin, SAHA, DSF, BIX-01294</td>
<td>CD8-depleted MNCs</td>
<td>1/5 [7 days]</td>
</tr>
<tr>
<td>SMAPP1172</td>
<td>CEM T cells</td>
<td>24</td>
<td>10</td>
<td></td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine; CAPE196</td>
<td>J-Lat 11.1</td>
<td>24</td>
<td>2</td>
<td></td>
<td>CD4+ T cells</td>
<td>20/1 [12h]</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>PKC412173</td>
<td>ACH2</td>
<td>48</td>
<td>0.5</td>
<td>VOR</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HODHB174</td>
<td>Primary TCM model</td>
<td>24</td>
<td>100</td>
<td>NI</td>
<td>rCD4+ T cells</td>
<td>100+IL-2 [24h]</td>
<td>VOA, ELISA</td>
</tr>
<tr>
<td>Chidamide; MS-275175</td>
<td>gGn-p* infected rCD4+ T cells</td>
<td>48</td>
<td>2, 20</td>
<td></td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Largazole158</td>
<td>J-Lat 10.6</td>
<td>24</td>
<td>0.15</td>
<td></td>
<td>Bryostatin, SUW133, SUW124</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>EK-16A176</td>
<td>J-Lat 10.6</td>
<td>28</td>
<td>4.06</td>
<td></td>
<td>5-Aza, JQ1, I-Bet151, Romidepsin, SAHA</td>
<td>rCD4+ T cells</td>
<td>0.05 [18h]</td>
</tr>
<tr>
<td>PH01-PH05159</td>
<td>Jurkat/LTR-Luciferase cells</td>
<td>24</td>
<td>0.1-5.9</td>
<td>SAHA, Ionomycin, PEP005</td>
<td>CD4+ T cells</td>
<td>1 [24h]</td>
<td>VOA, RT-qPCR</td>
</tr>
<tr>
<td>BI-2536; BI-6727177</td>
<td>ACH2/U1</td>
<td>16</td>
<td>1</td>
<td>SAHA, Prostratin</td>
<td>PBMCs</td>
<td>1 [16]</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>Psammaplin A; Aplysiatoxin; Debropanamapsiatoxin178</td>
<td>J-Lat 9.2</td>
<td>24</td>
<td>1.9</td>
<td>TNF-α, Prostratin; TNF-α, Panobinostat; TNF-α, Panobinostat</td>
<td>PBMCs</td>
<td>3.8 [24h]; 1.1 [24h]; 1.3 [24h]</td>
<td>ELISA</td>
</tr>
</tbody>
</table>

aHIV-1 reporter cell line utilized for dose-response study; bTreatment time (hours); cEffective concentration for induction of HIV expression; dTreatment(s) found to produce synergistic HIV expression response in combination with the LRA; eEffect of LRA for reactivation of HIV from cells purified from aviremic patients on ART; fConcentration of LRA examined on primary cells for the indicated time period [ ]; gTechnique for measuring viral production (viral mRNA/ p24) post treatment. Abbreviations: LRA, latency reversing agent; EC, effective concentration; C, concentration; BSO, buthionine sulfoximine; NI, not investigated; VA, valproic acid; TNF-α, tumor necrosis factor alpha; PMA, phorbol 12 myristate 13-acetate; TSA, trichostatin A; PBMCs, peripheral blood mononuclear cell; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; DSF, disulfiram; VOR, vorinostat; VOA, viral outgrowth assay; ELISA, enzyme-linked immunosorbent assay; 5-Aza, 5-azacytidine.
Table 2.3 Summary of Small Molecule LRAs identified in Screens

<table>
<thead>
<tr>
<th>Group 1: Epigenetic modifiers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LRA</strong></td>
</tr>
<tr>
<td>MS-275</td>
</tr>
<tr>
<td>Chidamide</td>
</tr>
<tr>
<td>Largazole</td>
</tr>
<tr>
<td>Psammaplin A</td>
</tr>
<tr>
<td>MMQQ</td>
</tr>
<tr>
<td>BI-2536</td>
</tr>
<tr>
<td>BI-6727</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2: Chromatin modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pyrimethamine</strong></td>
</tr>
<tr>
<td><strong>CAPE</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3: Signaling effectors/ modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5HN</strong></td>
</tr>
</tbody>
</table>

*Latency Reversing Agent, common designation; International union of pure and applied chemistry designation; Molecular weight (grams/mole); Known or suspected biological target(s); References for screen and other compound’s related studies. Abbreviations: HDAC, histone deacetylase; Brd4, bromodomain-containing protein 4; PLK, polo-like kinase; DHFR, dihydrofolate reductase; BAF, BRG1/BRM associated factor; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPlase, peptidylprolyl isomerase; Pin 1, peptidylprolyl Cis/Trans isomerase, NIMA-interacting 1; HIV-RT, Human Immunodeficiency Virus- reverse transcriptase.
### Table 2.3 Summary of Small Molecule LRAs identified in Screens (continued)

<table>
<thead>
<tr>
<th>LRAa</th>
<th>IUPAC Designationb</th>
<th>M.W. c</th>
<th>Target(s)d</th>
<th>Ref(s)e</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV6</td>
<td>4-3,4′-dichloroanilino-6-methoxyquinoline</td>
<td>333.05</td>
<td>NFAT-signaling pathway</td>
<td>167</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>diethylcarbamothioylsulfanyl N,N-diethylcarbamodithioate</td>
<td>296.54</td>
<td>ALDH, ΔβH, Akt-signaling (PTEN)</td>
<td>204-207</td>
</tr>
<tr>
<td>57704</td>
<td>1,2,9,10-tetramethoxy-7H dibenzo[de,g]quinolin-7-one</td>
<td>351.35</td>
<td>Akt-signaling pathway (PI3K p110)</td>
<td>171</td>
</tr>
<tr>
<td>PKC412</td>
<td>N-(9S,10R,11R,13R)-10-methoxy-9-methyl-1-oxo-2,3,10,11,12,13-hexahydro-9,13-epoxy-1H,9H-diindolo(1,2,3-GH:3′,2′,1′-lm)pyrrolo(3,4-j)(1,7)benzodiazinon-11-yl)-n-methylbenzamide</td>
<td>570.6</td>
<td>PKC, FLT3, ZNF198-FGFR1, PDGFR-, c-Kit receptor, VEGF-R2, JNK pathway, NFkB signaling pathway</td>
<td>173,208-211</td>
</tr>
<tr>
<td>HODHBt</td>
<td>3-hydroxy-1,2,3-benzotriazin-4(3H)-one</td>
<td>163.13</td>
<td>STAT5 SUMOylation</td>
<td>174</td>
</tr>
<tr>
<td>EK-16A</td>
<td>[(1S,4S,5S,6R,9S,10R,12R,14R)-5,6-dihydroxy-7-(hydroxymethyl)-3,11,11,14-tetramethyl-15-oxo-4-tetracyclo[7.5.1.01,5.010,12]pentadeca-2,7-dienyl] (Z)-2-methylbut-2-enoate</td>
<td>658.89</td>
<td>PKC (PKC-NFkB signaling pathway, pTEFb</td>
<td>176</td>
</tr>
</tbody>
</table>

#### Group 4: Transcriptional elongation modulators

| SMAPP1       | 1,2,3,4-Tetrahydro-2-[(4-methylphenyl)sulfonyl]-N-[(2-pyrimidylamino)sulfonyl]phenyl]-3-isoquinolinecarboxamide | 467.12 | PP1 | 172     |

*aLatency Reversing Agent, common designation; bInternational Union of Pure and Applied Chemistry designation; cMolecular weight (grams/mole); dKnown or suspected biological target(s); eReferences for screen and other compound’s related studies. Abbreviations: NFAT, nuclear factor of activated T-cells; ALDH, aldehyde dehydrogenase; ΔβH, dopamine beta-hydroxylase; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; FLT3, FMS-like tyrosine kinase-3; ZNF198-FGFR1, zinc finger 198-FGF receptor 1 fusion tyrosine kinase; PDGFA-, platelet-derived growth factor-alpha-; VEGF-R2, vascular endothelial growth factor-receptor 2; JNK, c-Jun N-terminal kinases; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; pTEFb, positive transcription elongation factor b; STAT5, signal transducer and activator of transcription 5; MPC, multicatalytic proteinase complex; PP1, protein phosphatase 1.
Figure 2.1 Latency reversing agents induce HIV-1 provirus replication by diverse mechanisms.

Small molecule compounds identified as latency reversing agents (LRAs) cause reactivation of HIV-1 transcription/replication through at least four distinct mechanisms, organized into Groups (Table 3), including: epigenetic modifiers (boxed in blue); chromatin modulators (purple); signaling effectors/modulators (orange); and RNA Pol II transcriptional elongation modulators (green). Epigenetic modifiers (blue), include HDAC and BET inhibitors that enhance viral transcription by modulating post-translational modifications of core histone N-terminal tails. Chromatin modulators (purple), include BAF...
inhibitors that regulate nucleosome positioning on the HIV-1 5' LTR (Nuc 0/1) and specifically disrupt Nuc 1 to allow activated transcription by RNA Polymerase II. Signaling effectors/modulators (orange) affect PI3K-Akt, PKC-NFκB, and Jak-STAT5 pathways. Agonists activating PKC stimulate the transcription factor NFκB through activation of IKK and degradation of the inhibitor of NFκB (IκB). Signalling modulator 5HN also induces HIV-1 expression through ROS-stimulated IKK, and consequently NFκB activation. PI3K agonists directly activate PI3K, while disulfiram induces activation through this pathway by inhibiting the negative regulator PTEN, which also regulates IKK through the function of Akt. The JAK-STAT pathway stimulates HIV expression through STAT5; benzotriazoles indirectly activate this effect by impairing STAT5 SUMOylation causing its retention in the nucleus. Signaling effector AV6 stimulates viral transcription by enhancing NFAT activity. Transcriptional elongation modulators (green) include SMAPP1, which enhances viral gene expression by activating Cdk9 of pTEFb, while aclacinomycin causes the dissociation of pTEFb from the inhibitory 7S snRNP complex.

Abbreviations: TCR, T cell receptor; TLR, Toll-like receptor; PKC, protein kinase C; 5HN, 5-hydroxynaphthalene-1,4-dion; ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinases; Jak, janus kinase; STAT5, signal transducer and activator of transcription 5; IKK, IκB kinase; IκB, inhibitor of kappa B; PIP2, Phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; HADC, histone deacetylase; HDACi, histone deacetylase inhibitors; BETi, Bromodomain and extra-terminal (BET) protein inhibitors; AV6, antiviral 6; NFAT, nuclear factor of activated T-cells; TFs, transcription factors; Nuc-0/1, nucleosome-0/1; Brd4, bromodomain containing 4; CTD, C-terminal domain; RNA Pol II, ribonucleic acid polymerase II; snRNP, small nuclear ribonucleoprotein; CycT1, cyclinT1; HEXIM1, hexamethylene bisacetamide inducible protein 1; LARP7, La ribonucleoprotein domain family member 7; MePCE, methylphosphate capping enzyme; PP1, protein phosphatase 1; SMAPP1, small molecule activator of protein phosphatase 1; BAFi, BAF complex inhibitor; LTR, long terminal repeat.
2.2.1 Group 1: Epigenetic modifiers

**MS-275/ Entinostat; Chidamide/ Epidaza; Largazole; and Psammaplin**

HDACIs have attracted most initial focus as LRAs in clinical trials, because several compounds with this activity had previously been employed as mood stabilizers and anti-epileptics; the compounds have also been used in clinical trials for treatment of severe depression\textsuperscript{216,217}, as well as various cancers\textsuperscript{218-220}. Similarly, several groups observed that HDACIs could elevate the expression of HIV-1 provirus in cell line models of latency, and these observations led to clinical trials with patients on antiretroviral therapy, with the goal of purging latently infected cells via "shock and kill"\textsuperscript{220,221}. Structurally, HDACIs are classified into four main groups: hydroxamates, cyclic peptides, aliphatic acids and benzamides\textsuperscript{220,221}. Although their full mechanism of action needs elucidation, these compounds cause accumulation of acetylated histones and non-histone proteins involved in the regulation of gene expression, cell proliferation, differentiation and cell death\textsuperscript{220,221}.

Eighteen human HDACs have been identified to date, which are organized into four classes based on structural identity with their yeast orthologues\textsuperscript{220}. The class I HDACs, primarily localized to the nucleus include HDAC 1, 2, 3, and 8, and are ubiquitously expressed in mammalian cell lines and tissues. HDACs in classes I, II, and IV share common features, including dependence on zinc\textsuperscript{2+} for enzymatic activity, while class III HDAC proteins have catalytic activity that requires a nicotinamide adenine dinucleotide (NAD+) cofactor\textsuperscript{220}. Class I HDACs play a large role for establishment of HIV-1 latency in resting CD4\textsuperscript{+} T cells\textsuperscript{149}. These proteins are recruited to the HIV-1 promoter by multiple transcription factors acting as transcriptional repressors in unstimulated cells, including YY1 (yin yang 1), RBF2 (Ras-responsive element binding factor 2) which is a protein
complex made up of proteins TFII-I (transcription factor II-I), and USF1/2 (upstream-stimulatory factor 1/2)), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) p50, and Sp1 (specificity protein 1), which cause repression of transcription\textsuperscript{149}. Therefore, the identification and/or development of potent inhibitors which specifically target these enzymes would be beneficial for disruption of HIV-1 latency (Figure 2.1, HDACI)\textsuperscript{222}. Towards this goal, the compound MS-275 (Figure 2.2, Table 2.3) was found to be a potent LRA, identified by screening a panel of HDACIs consisting of both non-class and class-specific HDACIs using in vitro latency cell models (Table 2.1-2.2)\textsuperscript{165}. This compound is a benzamide derivative, has affinity for class I HDACs and is particularly selective for HDAC 1 relative to HDAC 3 or HDAC 8 (Table 2.3). MS-275 was previously characterized as an anti-tumour agent in several cancer-related clinical studies as well as for the treatment of psychiatric disorders, and was shown to selectively cause elevated histone H3-acetylation in a mouse brain model\textsuperscript{218,223}. Structure-activity relationship (SAR) studies examining potency of HDACIs for reversing HIV-1 latency revealed essential features for this class of LRAs which include a cap group, polar connection unit (CU), and a hydrophobic spacer (HS) with a hydroxamate or benzamide Zn\textsuperscript{2+} binding group coupled to the HS\textsuperscript{165}. Similar requirements for benzamide-containing HDACIs, including MS-275 and chidamide (Figure 2.2, Table 2.3) were identified for latency reversing activity among epigenetic modifiers examined using a primary cell model of HIV-1 latency (Table 2.1-2.2)\textsuperscript{175}. Latency reversing activity of MS-275 was examined in combination with other molecules including buthionine sulfoximine (BSO), a glutathione-synthesis inhibitor; prostratin, a non-tumour-promoting phorbol ester; and 5-aza-2’deoxycytidine, a DNA demethylating agent\textsuperscript{165,224,225}, and these studies reported various levels of potency, which
is likely the result of differences in HIV-1 reporter virus at different chromosomal regions. The related compound chidamide (Figure 2.2), showed better cell tolerance than the commonly used broad-spectrum HDAC inhibitor SAHA, and was also able to disrupt viral silencing in primary CD4+ T cells isolated from patients.184,185

A number of HDACIs with latency reversing activity have emerged from screens involving compounds from natural sources, including largazole and psammaplin A (Figure 2.2, Table 2.3), which were identified in screens of class-specific HDACIs, and chemical libraries from marine invertebrates and microorganisms (Table 2.1-2.2).158,178 Largazole (Figure 2.2), originally isolated from marine cyanobacteria, is a well-known anti-proliferation agent with specificity towards class I HDAC enzymes, and showed significant synergy for HIV-1 provirus reactivation with analogs of bryostatin-1, and produced minimal toxicity, when examined on J-Lat reporter cell lines and resting CD4+ T cells.158 Psammaplin (Figure 2.2), which is also a class I HDAC inhibitor with anti-tumour activity was identified in a screen for LRAs from a library of natural compounds derived from marine invertebrates and microorganisms (Table 2.1-2.2).178 These recently identified LRAs, largazole and psammaplin A, are structurally divergent from previously characterized HDACIs in that they possess a thioester linkage and intramolecular disulfide bond, respectively.186,188,226 (Figure 2.2). Largazole is rapidly hydrolyzed to a thiol in the presence of plasma and active cellular proteins.226 In contrast, HDACIs with an intramolecular disulfide bond, like psammaplin A, are susceptible to reduction after cell uptake, leading to the production of monomers with thiol groups.41 The presence of a thiol group, in the hydrolyzed form of largazole and in the disulfide-reduced form of
psammaplin A, is essential for their inhibitory activity against HDAC molecules, as they chelate zinc ions present at the active site, which inhibits enzymatic activity\textsuperscript{39,41,227}.

**Figure 2.2 Chemical structures of Group 1 LRAs, epigenetic modifiers.**

Shown are the chemical structures small molecule compounds identified as LRAs causing alteration of histone modifications. MS-275, Chidamide, Larazole, and Psammaplin A were determined to inhibit class I HDACs. MMQO (8-methoxy-6-methylquinolin-4-ol), BI-2536, and BI-6727 were identified as BET inhibitors.

**MMQO, BI-2536, and BI-6727/ Volasertib**

Nuclear bromodomain and extra-terminal (BET) family proteins (bromodomain protein 2 (BRD2, BRD3, BRD4 and mBRDT), which recognize the acetylated N-terminal tail of histones through their bromodomains (BDs), act as readers of acetylated lysine and are involved in regulating gene expression\textsuperscript{228}. They serve as recruitment platforms for transcriptional regulatory factors as well as chromatin re-modellers. Generally, these
proteins contain two tandem bromodomains, BD1 and BD2, in addition to a C-terminal extra-terminal (ET) domain. BET proteins have attracted recent attention as therapeutic targets in various disorders, such as cancer, inflammation, neurological diseases and HIV-1 persistent infection. Computational analysis of compounds with structural similarity to known BET-inhibitor LRAs, and analysis using the J-Lat A2 cell line, identified 8-methoxy-6-methylquinolin-4-ol (MMQO) as a novel LRA (Table 2.1-2.2), which was demonstrated to disrupt HIV-1 latency via inhibition of the BET protein BRD4 (Figure 2.1, BETi, Figure 2.2, Table 2.3). MMQO directly binds the BRD4 BD1 domain, where the aromatic ring with two methyl groups (ring A) intercalates into the BD1 acetyl-lysine binding pocket, while the quinoline pyridine ring of MMQO is exposed outside of the BD1 binding pocket.

Several additional BRD4/ BET inhibitors have been identified as LRAs, namely BI-2536 and BI-6727 (Figure 2.1, BETi, Figure 2.2, Table 2.3). Both of these compounds have pteridine groups (Figure 2), and were identified in screens of protein kinase inhibitors using latently infected monocytic THP-1 cells (Table 2.1-2.2). BI-2536 was initially identified as an inhibitor of polo-like kinase 1 (PLK1), which has a key regulatory function for mitotic progression. More recently, BI-2536 was also shown to bind bromodomains, and structural analyses revealed specific interactions of BI-2536 with the BRD4 hydrophobic cavity. The latency reversing activity of BI-2536 and BI-6727 are likely associated with inhibition of BRD4, rather than an inhibitory effect on protein kinases; accordingly, other PLK inhibitors were unable to cause significant activation of HIV-1 transcription in the same HIV-1 reporter cell lines utilized for the initial screen. Furthermore, ChIP analyses indicates that treatment with either BI compound causes
reduction in BRD4 recruitment to the HIV-1 promoter\textsuperscript{177}, similar to the effect of JQ1, a previously identified LRA with known BRD4 inhibitory function\textsuperscript{230,231}.

2.2.2 Group 2: Chromatin modulators

\textit{Pyrimethamine/ Daraprim; and Caffeic acid phenethyl ester (CAPE)}

The HIV-1 LTR is known to have two strongly positioned nucleosomes, Nuc-0 and Nuc-1, flanking conserved elements within the viral 5’ LTR enhancer region\textsuperscript{58,232}. Nuc-1 is positioned immediately downstream of the transcriptional start site, and is restrictive to HIV-1 transcription\textsuperscript{74,79} (Figure 2.1, Nuc 0/1). Upon T cell activation, Nuc-1 is disrupted through the function of nucleosome remodelers, such as the SWI/SNF (SWItch/sucrose non-fermentable) family member, PBAF (polybromo-associated BRG1- or hBRM-associated factors (BAF)), which enables activation of gene expression\textsuperscript{79}. Importantly, the ATP-dependent BAF chromatin-remodeling complex plays a vital role in establishing and maintaining viral latency by actively positioning Nuc-1 near the transcription start site\textsuperscript{77,233}, which implicates the BAF chromatin remodeler as a putative target for disruption of silencing. Accordingly, several BAF inhibitors were shown to reactivate viral transcription (Figure 2.1, BAFi)\textsuperscript{196,234}, including pyrimethamine and caffeic acid phenethyl ester (CAPE), in the J-Lat reporter cell lines and in CD4\textsuperscript{+} T cells from HIV-1-infected patients (Figure 2.3, Tables 2.1-2.3). The effect of these compounds for reactivation of latency is mediated through inhibition of BAF250a which causes displacement of the complex from the viral promoter\textsuperscript{196}. Among these, pyrimethamine has anti/protozoan activity, and is FDA-approved for treatment of HIV-1-infected patients prone to opportunistic infections\textsuperscript{235}. An unexpected finding was that the BAF inhibitor CAPE\textsuperscript{196}, a bioactive compound isolated from honey bee propolis, also inhibits activation of NFkB at relatively
high concentrations\textsuperscript{198-200}, and at lower concentrations causes reactivation of latent provirus, but not NF\kappa B inhibition\textsuperscript{196}. Furthermore, both of these BAF inhibitors were also shown to be capable of preventing establishment of latent proviruses; Jurkat cells pre-treated with these compounds produce decreased numbers of latently infected cells\textsuperscript{196}.

![Chemical structures of Group 2 LRAs, chromatin modulators.](image)

**Figure 2.3 Chemical structures of Group 2 LRAs, chromatin modulators.**
Shown are the structures of Pyrimethamine and caffeic acid phenethyl ester (CAPE), that cause reactivation of HIV transcription through inhibition of the chromatin remodeling complex BAF.

2.2.3 Group 3: Signaling effectors/ modulators

*5HN/ Juglone*

Reactive oxygen species (ROS), such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), superoxide anions (O\textsubscript{2} -) and hydroxyl radicals (OH) cause oxidative stress and induce different biological responses depending on the level of ROS\textsuperscript{236}. High ROS levels cause damage to cellular macromolecules, which can lead to apoptosis or necrosis, while low and intermediate ROS levels can act as second messengers which stimulate anti-oxidative and inflammatory responses\textsuperscript{236,237}. Specifically, it is known that NF\kappa B becomes activated by intermediate levels of ROS that trigger inflammation\textsuperscript{237-239}. Accordingly, reporter genes...
under control of the HIV-1 LTR promoter, and HIV-1 provirus in latently infected Jurkat T cells, are induced in response to treatment with H$_2$O$_2$, and this effect is dependent upon NFκB binding sites within the HIV-1 5' LTR enhancer$^{240}$. Furthermore, intermediate levels of ROS were shown to activate IKK (inhibitor of kappa B (IkB) kinase) and cause degradation of IkB (Figure 2.1, ROS), in a cell type-dependent manner, which in T cells may involve the SH2 Domain-Containing Inositol 5'-Phosphatase 1 (SHIP-1)$^{237}$. Acetylation of p65 at specific residues enhances NFκB transcriptional activation function$^{112}$, and, accordingly, it was shown that histone acetyltransferases (HATs), such as CBP (cyclic AMP response element-binding protein(CREB)-binding protein) /p300, may also be involved in ROS-induced HIV-1 LTR activation through acetylation of NFκB p65$^{241}$. Consistent with these observations, the compound 5HN (5-hydroxynaphthalene-1,4-dione), was identified in a high-throughput LRA screen of natural small molecules, and shown to induce HIV-1 transcription through ROS generation (Figure 2.4, Tables 2.1-2.3)$^{166}$. This compound, found in leaves, roots and bark of the black walnut tree, is a quinone which can be reduced to a semiquinone radical by NADPH (nicotinamide adenine dinucleotide phosphate) oxidoreductase. Under aerobic environmental conditions, semiquinone radicals generate ROS and induce oxidative stress$^{242}$. Consistent with the observations described above, it was shown that 5HN induces HIV-1 transcription through ROS-stimulated NFκB activity (Figure 2.1, ROS), through a mechanism that is not dependent upon activation of NFAT or PKCθ (protein kinase C θ)$^{166}$. 
Figure 2.4 Chemical structures of Group 3 LRAs, signaling effectors or modulators. Structures of compounds affecting signaling pathways controlling transcription factors regulating HIV-1 transcription are illustrated. 5HN (5-hydroxynaphthalene-1,4-dione) causes generation of reactive oxygen species (ROS) and activation of NFκB. AV6 (antiviral 6) enhances activity of the nuclear factor of activated T-cells (NFAT). Disulfiram and 57704 activate Akt signaling. The kinase inhibitor PKC412 promotes phosphorylation of NFκB p65. The benzotriazole derivative 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HODHBt) inhibits STAT5 SUMOylation, promoting nuclear retention. EK-16A, aplysia toxin and debromoaplysia toxin act as PKC agonists.

**AV6**

One of the largest high-throughput screens of small molecules for LRAs to date, involved 200,000 structurally diverse compounds, using primary CD4+ T cells infected with a lentivirus expressing secretable alkaline phosphatase (*seap*) and *egfp* reporter genes under control of the HIV-1 LTR (Table 2.1). One compound identified in this
screen, AV6 (antiviral 6) was found to cause enhanced binding of NFAT (nuclear factor of activated T-cells) to the viral promoter in J-Lat cells (clone 9.2) (Figure 2.4, Table 2.3). Notably, this new LRA was shown to cause synergistic induction of HIV-1 provirus expression in combination with the HDAC inhibitor valproic acid (Table 2.2). A subsequent study described development of structural analogs with a linear alky linker and HDAC inhibitor functional group attached to the quinoline ring C-6 position of the parental AV6 structure244. Of these, structures carrying a CONHOH HDAC inhibitor functional group, connected by oxygen to the quinoline ring produced the greatest effect for reversing viral latency. Furthermore, these AV6 analogs were shown to enhance viral transcription mediated through both inhibition of HDAC activity, and stimulation of NFAT DNA binding, but also cause dissociation of pTEFb (positive transcription elongation factor b) from the inhibitory HEXIM (hexamethylene bisacetamide-induced protein) 7SK snRNP (small nuclear ribonucleoprotein) complex244.

**Disulfiram/ Antabuse; 57704/ Oxaglaucine**

The latency reversing activity of several hybrid polar compounds, including the HDACIs SAHA and HMBA (hexamethylene bisacetamide) was initially shown to be dependent upon the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway141,245. Subsequent screens for latency reversing activities identified compounds that activate the PI3K-Akt signaling pathway (Figure 2.1, PI3K, Akt)171,207, including disulfiram, a thiuram disulfide-containing compound, which was identified as a LRA in a screen of compounds with previously characterized biological activity (Figure 2.4, Tables 2.1-2.2). Disulfiram is an FDA-approved drug prescribed to patients afflicted with alcoholism because it inhibits aldehyde dehydrogenase, leading to increased levels of acetaldehyde, causing an
aversive effect that discourages alcohol consumption\textsuperscript{246}. Subsequent to identification as a LRA, disulfiram was also shown to inhibit PTEN (phosphatase and tensin homology), a negative regulator of the Akt signaling pathway (Figure 2.1, PTEN)\textsuperscript{207}, which can account for its effect on reactivation of HIV-1 transcription\textsuperscript{168,207,247}. Disulfiram is rapidly converted to diethylthiocarbamic acid (DDTC) \textit{in vivo}\textsuperscript{248}, and this metabolite was shown to act as a LRA. Because disulfiram had already been in clinical use, it attracted attention for clinical studies aimed at eliminating latent HIV-1 reservoirs. Although disulfiram administration was shown to induce a transient increase in viremia on its own, no change in the size of latent reservoirs was observed\textsuperscript{249}.

A quinoline-containing compound, designated 57704, was identified as a LRA from a screen of natural products, using HIV-1 reporter cell lines and CD8\textsuperscript{+}-depleted MNCs (mononuclear cells) isolated from HIV-1-infected patient samples (Figure 2.4, Tables 2.1-2.3)\textsuperscript{171}. Interestingly, the ability of 57704 to activate viral transcription was decreased in cells treated with the PI3K inhibitor wortmannin or the Akt inhibitor IV, and also this compound caused increased phosphorylation of Akt. These observations indicate that it may act as a PI3K-Akt agonist (Figure 2.1, Akt), and may specifically target the PI3K p110 isoform $\alpha$\textsuperscript{171}.

\textbf{PKC412}

PKC412 is a derivative of the alkaloid staurosporine, and was identified as a LRA in screens of synthetic and naturally occurring compounds (Figure 2.4, Tables 2.1-2.3)\textsuperscript{173}. This compound is a broad-spectrum kinase inhibitor, including for PKC and various protein-tyrosine kinases\textsuperscript{173}, and has anti-tumour activity against human myeloma cells,
non-small cell-lung cancer cells, and towards a murine model of myeloproliferative disease. PKC412 also induces apoptosis in human multiple myeloma cells, by an effect mediated through Jun N-terminal kinase (JNK) activation and up-regulation of the transcriptional activator AP1 (activator protein 1). Efforts investigating the anti-HIV-1 latency reversing activity of this compound suggest PKC412 stimulates HIV-1 transcription by a mechanism involving phosphorylation of NFκB p65, which suggests that one or more PKC isoforms, or related enzymes, may have inhibitory effects on this pathway (Figure 2.1).

**HODHBt**

The HIV-1 LTR has three cis-elements for the signal transducer and activator of transcription 5 (STAT5, Figure 2.1), which cause activation of transcription in response to γc-related cytokines (IL-2, IL-4, IL-7 and IL-15). Binding of these cytokines to their corresponding receptors results in tyrosine phosphorylation and subsequent activation of the associated Janus family kinases (JAKs). IL-2 predominately activates the JAK1 and JAK3 kinases, which phosphorylate cytoplasmic STAT5, causing translocation to the nucleus where it activates genes mainly involved in proliferation, differentiation and inflammation. Benzotriazole derivatives, particularly 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HODHBt), were shown to reactivate latent HIV-1 by a mechanism dependent on IL-2 treatment, which is also required for survival of TCM (central memory) cells in *in vitro* cultures (Figure 2.4, Tables 2.1-2.3). Using the PASTAA (Predicting Associated Transcription factors from Annotated Affinities) software program to analyze the differentially expressed genes in HODHBt-treated cells, the STAT transcription factors were predicted to be affected by this class of compounds for induction of viral
transcription\textsuperscript{174}. Accordingly, ChIP analyses from HODHBt-treated cells indicated recruitment of STAT5A to the HIV-1 LTR promoter (Figure 2.1, STAT5), which confirmed direct involvement of STATs in stimulating viral transcription\textsuperscript{174}. However, the benzotriazole derivatives did not induce STAT5 phosphorylation at tyrosine 694 on their own, which is required for activation, but rather cause accumulation of IL-2-induced phosphorylated STAT5 by impairing SUMOylation and causing increased levels of phosphorylated STAT5 in the nucleus (Figure 2.1)\textsuperscript{174}. Based on these observations, it might be predicted that benzotriazole compounds may also enhance produce latency reversing activity of the additional γC-cytokines (IL-7, IL-21 and IL-15)\textsuperscript{256-258} through activation of the STATs.

**EK-16A, Aplysiatoxin, and Debromoaplysiaotoxin**

HIV-1 transcription is tightly linked to signals generated from the T cell receptor (TCR) in CD4\textsuperscript{+} T cells\textsuperscript{51,52}, which is activated by engagement with major histocompatibility complexes (MHC) of antigen-presenting cells (APC)\textsuperscript{52,54}. Antigen presentation causes association of p56\textsubscript{lck}, and phoshorylation of the CD4 TCR/CD3 complex, leading to recruitment of ZAP-70 kinase (zeta-chain-associated protein kinase 70). ZAP-70 in turn activates phospholipase C\textgreek{y} (PLC\textgreek{y}), which hydrolyses phosphatidylinositol (4,5) bisphosphate (PIP\textsubscript{2}) into the secondary messengers diacylglycerol (DAG) and inositol triphosphate (IP\textsubscript{3}), that stimulate three downstream signaling pathways with different transcriptional activator targets\textsuperscript{52,54,55,58}. In T cells, DAG activates PKC\textgreek{θ} and the Ras/ Raf/ MAPK/ ERK - AP1 pathways\textsuperscript{52}. Because it regulates at least two divergent pathways downstream of the T cell receptor that consequently affect virus expression, PKC represents an important target for modulation by small molecules (Figure 2.1, PKC)\textsuperscript{259}. 
Accordingly, PKC agonists act as DAG mimetics, and comprise three structural categories, including phorbol esters, cyclic lactones and diterpenes, which include ingenol compounds. Notably, PKC enzymes have an N-terminal regulatory domain in which a conserved cysteine-rich motif, referred to as the C1 domain serves as a docking site for DAG as well as phorbol esters.

EK-16A, an ingenol derivative purified from the root of *Euphorbia kansai*, was identified as a LRA in a screen of compounds from traditional Chinese medicinal herbs, was shown to have more potent activity for reactivation of latent HIV-1 than prostratin (Figure 2.4, Tables 2.1-2.3), and found to predominately activate PKCγ. Two additional PKC activators aplysiatoxin and debromoaplysiatoxin were identified in a screen of natural compounds purified from marine invertebrates and microorganisms (Figure 2.4, Tables 2.1-2.3). These compounds are produced by blue-green algae, and were initially identified as agents with proinflammatory and tumour-promoting activities. Similar to phorbol esters, these compounds bind the C1 regulatory domain of PKC which facilitates interaction with the cell membrane phospholipid bilayer causing activation of the enzyme.

### 2.2.4 Group 4: Transcriptional elongation modulators

**Aclacinomycin/ Aclarubicin**

As indicated above, because any single LRA is likely incapable of inducing a sufficiently broad spectrum of latent provirus, a recent focus for identification of novel activities involves identifying combinations of LRAs that target parallel pathways regulating expression from the provirus genome, with the expectation that this would produce a more robust means of eradicating persistent viral infection. For example,
aclacinomycin was identified as a compound that increases the latency-reversing activity of TNF-α (tumor necrosis factor receptor-alpha) at low concentrations (Figure 2.5, Tables 2.1-2.3)\textsuperscript{155}. Aclacinomycin, is an FDA-approved compound which causes cell-differentiation, and has anticancer activity as a DNA intercalation agent and transcription inhibitor\textsuperscript{266,267}. However, these properties do not seem to contribute to latency reversing activity for HIV-1, but rather this compound was shown to cause dissociation of the elongation factor pTEFb from its inhibitory subunit HEXIM1, and this effect can account for its effect on enhancing viral transcription (Figure 2.1, pTEFb)\textsuperscript{155}.

![Figure 2.5 Chemical structures of Group 4 LRAs, transcriptional elongation modulators.](image)

Illustrated are the chemical structures LRAs identified in screens shown to promote transcriptional elongation from the HIV-1 LTR. Aclacinomycin causes dissociation of pTEFb from the inhibitory HEXIM1 7SK snRNP complex. SMAPP1 (small molecule activator of PP1) enhances activity of Cdk9 of the pTEFb transcriptional elongation complex.

**SMAPP1**

Protein phosphatase-1 (PP1) was shown to modulate HIV-1 transcription by interaction with the viral transactivator TAT, which promotes translocation of PP1 to the
nucleus where it indirectly enhances viral transcription by increasing catalytic activity of the pTEFb CDK9 subunit (Figure 2.1, pTEFb). Accordingly, expression of a short PP1-binding peptide derived from the nuclear inhibitor of PP1 (cdNIPP1) disrupts the TAT-PP1 interaction, causing alteration of CDK9 (cyclin-dependent kinase 9) phosphorylation which inhibits viral transcriptional elongation. Based on these observations a panel of small molecules targeting a PP1 non-catalytic subunit was evaluated for effects on both the inhibition and activation of viral transcription, and in these efforts compounds with a sulfonamide linker were found to be particularly effective for inducing viral transcription. This observation led to development of a compound library of sulfonamide-containing small molecules, from which a novel compound, designated small molecule activator of PP1 (Figure 2.1, SMAPP1), was identified as a potent stimulator of HIV-1 transcription in latently infected primary T cells (Figure 2.5, Tables 2.1-2.3).

Accordingly, SMAPP1 was shown to enhance phosphorylation of CDK9 at the regulatory Thr186 (Threonine 186) phosphorylation site (Figure 2.1). Subsequent structural analysis demonstrated direct interaction of SMAPP1 with the PP1 C-terminal groove. This compound was also subsequently combined with a nanoparticle delivery system to increase in vivo bioavailability and enhance activity towards latent provirus.

2.3 CHEMICAL BIOLOGY OF HIV PROVIRUS LATENCY REVERSING AGENTS

Identification of novel compounds with HIV latency reversing activity is a relatively young endeavor; we describe results from 18 small molecule screens, but only 2 of which have involved extensive unbiased libraries of 100K compounds or more (Table 2.1). Considering that 100's of high throughput screens involving very large libraries have been
focused toward identification of novel cancer drugs over the past 20 years\textsuperscript{273,274}, efforts towards identification of novel HIV LRAs by comparable strategies appear to be in their infancy. Furthermore, most drugs targeting cancer biology, initially identified in HTP small molecule screens, have typically undergone multiple rounds of medicinal chemistry optimization, subsequent to SAR analysis\textsuperscript{273,274}. However, to date only a few LRA compounds have been subjected to SAR studies to produce optimized activities\textsuperscript{165,244,275}. Therefore, we suggest that SAR of novel LRAs, and implementation of additional medicinal chemistry tools, such as scaffold hopping\textsuperscript{276}, fragment-based drug design\textsuperscript{277}, Lipinski’s rule of five\textsuperscript{278}, and analog-based drug design\textsuperscript{279}, will also contribute to identification of novel effective agents for reactivation of latent HIV provirus.

The HIV-1 5’ long terminal repeat is, arguably, among the most thoroughly characterized promoters in human cells, and in this respect, it is interesting that novel LRAs identified in unbiased HTP small molecule screens were found to affect well-characterized mechanisms for regulation of HIV-1 transcription, including PKC-NFκB signaling, histone deacetylases, and regulation of transcriptional elongation, but also previously less well-characterized mechanisms that have received less prior attention. For example, novel LRAs have illustrated the role of JAK-STAT signaling, and non-canonical NFκB signaling initiated by ROS and PTEN-Akt for regulation of HIV transcription\textsuperscript{174,280-282}. Furthermore, the effect of novel LRAs may illuminate previously unrecognized regulatory mechanisms, for example understanding the effect of PKC412 for reactivation of LTR expression\textsuperscript{173}, will likely reveal novel mechanisms for regulation of NFκB function. Considering the complexity and combination of mechanisms that control
HIV expression, we expect that many additional novel mechanisms will be revealed by results from further large scale HTP small molecule library screens.

2.4 LRAS WITH A COMMON PRIVILEGED STRUCTURE AFFECT VARIOUS TARGETS

The latency reversing agents MMQO, AV6, 57704, and PH03 were identified in four separate screens, but carry a common central quinoline structure\textsuperscript{159,167,170,171}, which we propose may represent a privileged structural motif. In chemical biology and medicinal chemistry, privileged structures are represented by a semi-rigid molecular scaffold that when modified by multiple hydrophobic residues or functional groups produce versatile binding properties that confer the capacity to interact with diverse biochemical targets\textsuperscript{283,284}. The term “privileged structure” was initially coined by Evans in 1988, to describe affinity of asperlicin derivatives toward a diverse collection of receptors (Figure 2.6A)\textsuperscript{285}. This term has subsequently been used frequently, but there are no strict rules defining particular structures as "privileged"\textsuperscript{286,287}. Typically, they carry either two or three rings connected through a ring-fusion or single bonds. Figure 2.6B illustrates examples of common privileged structures found in drugs and natural products. For discovery of novel LRAs, derivatives of the related privileged scaffold quinolin-8-ol, were screened for more potent latency reversing activity, which revealed that compounds carrying either a morpholine or piperdine ring at position 7 and a chloro group at position 5 of the quinoline ring, induced viral transcription at relatively lower concentrations than the parental compound (Figure 2.6C)\textsuperscript{169}. 
Figure 2.6 Privileged chemical structures relating to HIV-1 latency reversing activities.

Therapeutic agents recognizing receptors that bind the natural product asperlicin (A) include compounds related to cholecystokinin (CCK), 3-(acylamino)-5-phenyl-2H-1,4-benzodiazepines. Derivatives of this core structure exhibit affinity toward diverse biochemical targets. Screens for latency reversing agents have identified three examples of chemical scaffolds (B) that may represent privileged structures for novel drug discovery. Chemical structures of two HIV-1 latency reversing agents derived the privileged quinolin-8-ol structure are illustrated (C).

The four quinoline-containing LRAs identified in small molecule screens have various biological targets. MMQO activates HIV-1 transcription through inhibition of the BET protein BRD4. BET inhibitors, typified by the compound JQ1, had previously been used as anti-inflammatory and anti-cancer agents, but later several groups demonstrated HIV-1 latency reversing activity for JQ1 and compounds with related structures through inhibition of BRD4 (Figure 2.1, BETI). MMQO has significant structural
differences to previously characterized BET inhibitors\textsuperscript{190}, and unlike JQ1, HIV-1 latency reversing activity of MMQO is TAT-independent (Figure 2.1, Tat), which suggests that BRD4 is likely involved in additional mechanisms repressing viral transcription than competing for binding of TAT with pTEFb. Two additional structurally divergent pteridine-containing compounds identified as LRAs, BI-2536 and BI-6727\textsuperscript{177}, also have bromodomain inhibitory function, but the precise mechanism through which they stimulate viral transcription has not been elucidated.

The additional quinolone-containing compounds identified as LRAs appear to have additional distinct targets. Compound 57704 (Figure 2.4) acts as a LRA through activation of PI3K and PI3K-Akt signaling (Figure 2.1, Akt)\textsuperscript{171}, but through a different mechanism than disulfiram, which has a completely different structure (Figure 2.1). AV6 causes activation of LTR transcription through NFAT, but again, by a mechanism that has not been determined. The fourth quinoline-containing LRA, designated PH03\textsuperscript{159}, has previously been examined as a potential probe for \textit{in vivo} imaging of tau pathology in Alzheimer’s disease\textsuperscript{291-293}. This compound, also designated BF-170, seems to affect diverse molecular functions as it produced activity in a variety of additional screens, including for agonists of steroid receptors, enhancers of survival of motor neuron 2 (SMN2) expression, inhibitors of Marburg virus entry, inhibitors of human muscle pyruvate kinase, and inhibitors of the histone lysine methyltransferase G9a\textsuperscript{294-307}. However, whether any of these effects account for its latency reversing activity for HIV-1 provirus has not yet been elucidated.
2.5 PROSPECTIVE FOR FUTURE LRA IDENTIFICATION AND DEVELOPMENT

Critical features for effective latency reversing agents include a capacity to broadly reactivate viral transcription, independent of the chromosomal integration site, and without causing adverse effects on cellular homeostasis, T cell activation or toxicity. With this consideration, some currently well studied LRAs, including PKC agonists and non-class HDACIs may not be ideal for this purpose. PKC agonists typically cause broad-scale cytokine production\textsuperscript{308,309}, and non-class HDACIs can produce global alterations in transcription\textsuperscript{310,311}, that may cause significant toxic effects. Consequently, the development of LRAs that target unique, and currently less-characterized, mechanisms that modulate HIV-1 provirus transcription may be a priority for future investigations. As outlined, currently characterized LRAs function through diverse mechanisms of action (Figure 2.1), all of which also affect cellular transcriptional regulatory mechanisms. An ideal LRA for HIV "shock and kill" therapy would specifically reactivate HIV-1 transcription without causing alterations of cellular genes; a transcriptional activator that specifically regulates HIV-1 transcription would represent an ideal target for this purpose. HIV-1 does encode its own transactivator TAT (Figure 2.1, Tat), but which functions by binding the nascent TAR RNA to activate elongation of paused RNA Polymerase II complexes\textsuperscript{312,313}. Several LRAs function to indirectly enhance TAT activity by up-regulating function of Cdk9/cyclinT of the pTEFb elongation complex\textsuperscript{155,172,314,315} (Figure 2.1, pTEFb); however, these factors also regulate numerous cellular genes, and consequently compounds affecting this mechanism do not specifically target HIV-1 transcription. Consequently, development of compounds or agents capable of enhancing TAT function, or inhibiting factors that negatively regulate TAT, would provide a highly specific means of enhancing
provirus expression. Latently infected cells harboring transcriptionally silent provirus theoretically would not express TAT protein\textsuperscript{312}. However, the fact that BET inhibitors, and Cdk9 agonists (Figure 2.1, BETi, Cdk9), induce HIV-1 provirus expression supports the contention that latent HIV-1 must produce sporadic transcripts to maintain low levels of virus gene products in these cells\textsuperscript{155,314,315}. In this context, perhaps the most effective combination of latency reversing agents, to produce an effective "shock", would include agents that elevate basal HIV-1 provirus transcription, plus simultaneously stimulating activity of HIV-1 TAT protein.

An alternative strategy toward specifically reactivating HIV expression, relative to global transcription, would involve stimulating combinations of factors that wouldn’t affect most cellular genes but which would cause induction of expression from the HIV-1 LTR. This may represent an "Achilles heel" of the HIV-1 provirus, in that the 5' regulatory region contains binding sites for more than 30 different sequence-specific transcriptional regulatory factors\textsuperscript{58,316,317}. Effects on combinations of factors that bind the LTR typically produce synergistic effects on provirus reactivation\textsuperscript{58,316,317}. Consequently, it may be productive to implement small molecule screens to specifically identify compounds that are effective for provirus reactivation in combination with previously FDA approved LRAs. Additionally, we note that most of the small molecule screens for novel LRAs (Table 2.1), except for two small scale studies\textsuperscript{165,177}, were performed using T-cell lines or primary cells, and there has not yet been significant effort towards identifying LRAs that may be specifically effective in monocyte-macrophage lineages. Because these cell types represent important reservoirs for virus in patients on ART\textsuperscript{318-320}, it may be necessary to target unique aspects of provirus regulation in these cells.
2.6 CONCLUDING REMARKS

While current antiretroviral therapies effectively control HIV-1 replication and productive viral infection, they do not affect latently infected cells bearing transcriptionally repressed provirus, and development of a therapeutic means to eliminate these latent reservoirs is necessary for long-term management of the HIV-1 epidemic. Therapies devised to specifically target the latently infected population will require a means of distinguishing these cells from their uninfected counterparts. Considerable research effort has been directed at identifying biomarkers of cells latently infected with HIV-1 but to date no suitable macromolecules have been identified. Currently, the only known means of exposing the latently infected population is to force reactivation of HIV-1 expression to produce viral proteins that mark these cells as infected. This reality has driven efforts to identify novel LRAs with more potent and globally encompassing effects. Results from pre-clinical and clinical analysis of FDA approved LRAs has revealed a need for optimization of compounds with this activity to produce sufficiently effective responses to enable elimination of the latently infected population. Importantly however, all of the clinical trials performed to date were performed using drugs that had been repurposed from treatment of other conditions; and consequently, the full capability of LRAs with respect to the "shock and kill" strategy in general may not be realized until trials with compounds specifically designed and optimized for this purpose. Furthermore, application of recent developments in drug delivery involving nanocarriers, such as polymeric nanoparticles, liposomes, lipid nanoparticles, and dendrimers, to encapsulate LRAs, could increase their circulation or tissue retention time, solubility and
bioavailability, enhance drug potency, and reduce cellular toxicity\textsuperscript{330} and thereby improve overall success of this approach.

In addition to improving effectiveness of LRAs, success of the proposed shock and kill strategy will require combined intervention to improve killing of cells where virus replication has been reactivated\textsuperscript{152,153,331,332,333}. The failure of LRAs to reduce the latently infected population in initial clinical trials is likely a consequence of multiple factors, including that resting memory CD4\textsuperscript{+} T cells express elevated levels of anti-apoptotic molecules such as Bcl-2\textsuperscript{334}, which may interfere with virus-induced cell death. Additionally, some LRAs used in these trials have negative effects on CD8\textsuperscript{+} T cell function and cause upregulation of T-cell exhaustion markers on latently infected cells which reduces response to cell mediated immunity\textsuperscript{335-337}. Additionally, the majority of latent provirus in patients on anti-retroviral therapy were shown to express \textit{gag} escape mutations that cause insensitivity to the cytotoxic T lymphocyte (CTL) response\textsuperscript{338}. Additional interventions to overcome these barriers may include passive immunotherapy, therapeutic vaccination, or manipulation of apoptosis regulatory pathways\textsuperscript{339,340}.

Finally, a variety of additional strategies to purge HIV-1 infection have been proposed and are currently under development, including the use of broadly neutralizing monoclonal antibodies, T-cell immunotherapy, chimeric antigen receptor T cell therapy, gene editing, immune cell depletion and transplantation, as well as strategies intended to prevent re-activation of HIV-1 expression, referred to as "lock and block"\textsuperscript{341,342}. Because of the extent that HIV-1 infection is thought to penetrate multiple cell types and tissue compartments\textsuperscript{120,343-346}, it seems likely that no single strategy will provide a "cure all"
therapy, and combinations of various treatments may be necessary for routine curative intervention.
CHAPTER 3 SMALL MOLECULE COMPOUNDS DEMONSTRATING EFFECTIVE HIV-1 LATENCY REVERSING ACTIVITY

3.1 INTRODUCTION

The unique latency phenomenon of HIV-1 creates a major obstacle to eliminate persistent viral infection because the transcriptionally repressed provirus that resides within the genome of the memory CD4+ T cells is shielded from both host immune mechanisms and current antiretroviral therapy. Forced reactivation of viral transcription to cause production of viral proteins could mark these cells and render them susceptible to boosted immune defences for elimination. In this regard, a growing number of LRAs with capacity to reactivate silenced HIV provirus have been identified over the past decade. However, the inconsistency between in vitro and in vivo evaluations about such compounds’ efficacy in purging latent HIV-1 reservoirs, and/or compounds' toxic effects observed to date, limit their clinical interventions. Therefore, the identification of new small molecules with pharmacological features selectively and efficiently capable of stimulating viral transcription is still an unmet need for viral eradication.

The work in this chapter describes the identification of new LRAs that demonstrate promising ability to disrupt HIV-1 latency. Using high-throughput screening of synthetic small molecule libraries, five new compounds representing diverse chemical scaffolds were discovered. These compounds show efficacy for the disruption of viral latency in cell-based in vitro reporter models and, importantly, in resting CD4+ T lymphocytes isolated from pooled HIV-1 infected patient samples collected from aviremic individuals on antiretroviral therapy. Additionally, significant synergistic effects of these compounds
with the LRAs, ingenol-3-angelate/ PEP005, were observed. Overall, this study introduces the identification of new LRAs as well as an effective new combination treatment with high potential to disrupt HIV-1 latency.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 High-throughput screening of compounds

In total, 180,000 small molecules represented by the KD-2 (known drug library-2), CCBN (Canadian Chemical Biology Network), DIVERSet (Chembridge, San Diego, CA, USA), and LCGC (LIMR Chemical Genomics Center, Wynnewood, PA, USA) libraries were screened in 384-well plate format at the CDRD (Center for Drug Research and Development, Vancouver, BC, Canada). For the screen, 12,500 reporter cells (J-Lat Tat-GFP (A1) cells, carrying an integrated HIV-1 reporter LTR-Tat-IRES-GFP) were resuspended in phenol red-free Roswell Park Memorial Institute (RPMI)1640 media (Sigma-Aldrich, Cat # R8755) supplemented with 10% heat-inactivated (HI)-fetal bovine serum (FBS; Sigma-Aldrich, Cat # F1051) and dispensed into wells. Each compound was pinned in the wells using a PlateMate robot employing FP3 pins for a final concentration between 1 and 7 µM, depending on library, and the plates were then incubated overnight at 37°C, 5% CO₂. 10 µl of 1 µg/ml Hoechst and 500 ng/ml propidium iodide (PI) (Hoechst 33342 Trihydrochloride, Invitrogen, Cat # H3570 and PI, Invitrogen, Cat # P1384MP) were added to wells and incubated at 37°C, 5% CO₂ for 30 min. The plates were then scanned using a Cellomics Arrayscan (Thermo Scientific) with three channels to measure Hoechst, GFP, and PI. The results are presented as a percent activation of the GFP reporter normalized to the positive control, 50 nM PMA (Sigma-Aldrich, Cat # P1585),
such that \( \% \text{ activation} = \left[ \frac{(X - C^{-})}{(C^{+} - C^{-})} \right] \times 100 \); \( X \) = individual well reading where \( C^{-} \) = the average negative control reading, and \( C^{+} \) = the average positive control reading.

Compounds, including structural analogs of the compound PH02, were purchased from Chembridge (San Diego, CA, USA), Maybridge (Cambridge, UK), LCGC (Wynnewood, PA, USA), and Sigma-Aldrich. The compound ID numbers corresponding to each PH compound and PH02 analog is summarized in Table 3.1, and 3.2, respectively. The concentrations EC50/4, EC50, and EC50*4 stated throughout this study have been calculated based on the effective concentration 50 (EC50) value obtained from dose–response analysis for each PH compound using the Jurkat\textsuperscript{Tat} LTR-Luciferase cell line. GraphPad Prism software was used for calculating the EC50 value, which is defined as the concentration of compound provoking a response halfway between the baseline and maximum response in a dose-response analysis. Unless otherwise indicated, suberoylanilide hydroxamic acid (SAHA; Toronto Research Chemical, Cat # C755500) was used at 300 nM, chaetocin (Sigma-Aldrich, Cat # C9492) at 100 nM, ionomycin (Sigma-Aldrich, Cat # 10634) at 1 µM, and ingenol-3-angelate/PEP005 (Cayman Chemical, Cat # 16207) at 1–10 nM.

### 3.2.2 Recombinant DNA molecules, cell lines, and cell culture

Clonal cell lines bearing the pTY-LAI-luciferase and mini-dual HIV-1 reporter virus were described previously\textsuperscript{145,348}. J-Lat Tat-GFP (A1) cells, carrying an integrated HIV-1 reporter LTR-Tat-IRES-GFP, were obtained from the NIH-AIDS reagent resource. Cell lines and healthy peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 (Sigma-Aldrich, Cat # R8758) supplemented with 10% FBS (Sigma-Aldrich, Cat #
F1051), 100 U/ml penicillin (Thermo Fisher Scientific, Cat # 15070063), 100 µg/ml streptomycin (Thermo Fisher Scientific, Cat # 15070063), and 0.8 mg/ml Genetecin (G418 sulfate; VWR, Cat # CA45000-630) and incubated in a humidified 37°C and 5% CO₂ atmosphere. Primary CD4+ T lymphocytes were cultured in Super T-cell media (STCM) consisting of RPMI 1640 (Sigma-Aldrich, Cat # R8758) supplemented with 10% HI- FBS (Sigma-Aldrich, Cat # F1051), 100 U/ml IL-2 (Sigma-Aldrich, Cat # SRP6170), 100 U/ml penicillin (Thermo Fisher Scientific, Cat # 15070063), 100 µg/ml streptomycin (Thermo Fisher Scientific, Cat # 15070063), and 2% conditioned culture supernatant (T-cell growth factor, TCGF) from healthy PBMCs treated with 2 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich, Cat # 11249738001) and 5 ng/ml PMA (Sigma-Aldrich, Cat # P1585) for 4 h.

3.2.3 Reporter assays, ELISA, and flow cytometry

Luciferase assays were performed in 96-well plate format, where 100 µl of cell culture containing 1 × 10⁵ HIV-l LTR luciferase reporter cells was plated per well. After the indicated time of treatment, 100 µl of luciferase assay reagent (Superlight™ luciferase reporter Gene Assay Kit; BioAssay Systems, Cat # SLDL-100) was added per well, and incubated for 2-5 min at RT, when luciferase activity was measured on a Victor™ X3 Multilabel Plate Reader (PerkinElmer). Results were presented relative to the positive control samples treated with 50 nM PMA (Sigma-Aldrich, Cat # P1585). T-cell activation was measured using 1 × 10⁵ wild-type JurkatTat cells or PBMCs purified from healthy donors, treated as indicated. After 24 h, cells were incubated with 2.4G2 antibody (Abcam, Cat # ab210219) to block nonspecific binding and further stained with 5 µl of
anti-human CD69 (BioLegend, Cat # 310911) or an IgG1 isotype control antibody (BioLegend, Cat # 400125) conjugated with PE-Cy7m7. After 30-min incubation on ice, the cells were washed twice with phosphate-buffered saline (pH 7.4) and analyzed using a Becton Dickinson LSRII flow cytometer (BD Biosciences) to determine mean fluorescence intensity (MFI), using FlowJo software. The mean fluorescence intensity (MFI) obtained from cells stained with IgG isotype antibody control was subtracted from cells stained with CD69 to compensate for background (MFI_{CD69}-MFI_{IgG}). IL-2 production was measured in cell culture supernatants using a human IL-2 ELISA System (eBioscience). JurkatT_{Tat} clones bearing integrations of the mini-dual HIV reporter virus, clone #11 and #131, were plated at 1 × 10^5 per well in a 96-well plate format, treated for the indicated time, washed with 1 ml phosphate-buffered saline (pH 7.4), and then transferred into flow cytometry tubes, where they were fixed with freshly made 1% formaldehyde for 10 min at RT prior to analysis by flow cytometry. Results are presented as ΔMFI, where MFI obtained from parental JurkatT_{Tat} cells undergoing the same treatment was subtracted from the MFI corresponding to each cell line; ΔMFI = (MFI compound-treated cell line 131/11 - MFI compound-treated WT JurkatT_{Tat} cells). We applied the Bliss independence model to assess the latency reversing activity of drug combinations, which was calculated by the equation $f_{axy,P} = f_{ax} + f_{ay} - (f_{ax}) (f_{ay})$, where $f_{ax} = \text{fraction affected, drug x}$ and $f_{ay} = \text{fraction affected, drug y}$. The experimentally observed fraction affected ($f_{axy,O}$) was compared with the calculated predicted value ($f_{axy,P}$ and $\Delta f_{axy} = f_{axy,O} - f_{axy,P}$) if the compounds produced an additive effect. Based on this model, an $\Delta f_{axy}$ greater than 0 ($\Delta f_{axy} > 0$) indicates a synergistic effect when administrated together.
3.2.4 Chromatin immunoprecipitation (ChIP) assays

Jurkat\textsuperscript{Tat} LTR-DsRed cells, clone #11, were treated with 3 µM PH02 and/or 10 nM PEP005, for 24 h, and the cells cross-linked with 1% formaldehyde for 10 min at RT. The cells were resuspended in cold NP-40 lysis buffer (0.5% NP-40, 10 mM Tris–HCl pH 7.8, 3 mM MgCl\textsubscript{2}) containing freshly added protease inhibitor cocktail (Sigma-Aldrich, Cat # 04693159001) and spun to collect nuclei. The nuclei were sonicated in buffer containing 10 mM Tris–HCl (pH 7.8), 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS) to obtain DNA fragments of 200–2,000 bp using a Bioruptor sonicator (Diagenode). The chromatin fractions were precleared with protein A/G agarose beads (Millipore, Cat # 16-266) and immune-precipitated with anti-H3K9-me3 (Abcam, cat # ab8898), anti-H3K9-ac (Abcam, Cat # ab4441), or anti- H3 (Abcam, Cat # ab18521) antibodies according to the manufacturer's instructions. Cross-links were reversed by incubating the samples at 68°C for 2 h, and DNA was purified using QIAquick PCR columns (QIAquick PCR Purification Kit, Cat # 28104) and assayed by qPCR using primers specific to the conserved region designated RBEI (Ras-responsive binding element-1) which is located within the HIV-1 core promoter at the LTR U3 sub-region: AGTGGCGAGCCCTCAGAT, and AGAGCTCCAGGCTCAAAATC. Results are presented as normalized relative to that for histone H3 immunoprecipitates.

3.2.5 Immunoblot analyses

5 × 10\textsuperscript{6} luciferase reporter cells/Jurkat\textsuperscript{Tat} LTR DsRed, clone 11, were treated with the indicated concentration of the compounds. After treatment, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl,
1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with 1× protease inhibitor cocktail (Sigma-Aldrich, Cat # 04693159001) and 1 mM Dithiothreitol (DTT), to collect the whole-cell protein extract, which was further quantified using Bradford assay. A 30µg equivalent of the protein extract was mixed with the 5X SDS–PAGE sample buffer, boiled for 5 min, and separated using 12% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% milk (w/v) in Tris-buffered saline (TBS) for 1 h and then incubated with primary antibodies overnight at 4°C. The antibodies used at the following dilutions: SP1, Abcam 13370 (1: 4,000); NFκB p65, Abcam 7970 (1: 1,000), IkBα, Abcam 32518 (1: 5,000); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Abcam 9484 (1: 4,000); H3K9-acetylation, Abcam 10812 (1: 500); H3K9-trimethylation, Abcam 8898 (1: 500); and histone H3, Abcam 1791 (1: 5,000). The membrane was washed three times with TBS and then incubated with horseradish peroxidase (HRP)-conjugated goat secondary antibody (1: 20,000) at RT for 1h. After incubation, the membrane was washed with TBS, and the signal was developed with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher, Cat # 34095).

3.2.6 MTT cell viability assays

1 × 10^5 JurkatTat cells or PBMCs were plated in 96-well plates and treated as indicated. The plates were incubated at 37°C, 5% CO₂ for 24 or 48 h, and then, 20 µl of 5 mg/ml MTT reagent [3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, Sigma- Aldrich, Cat # M2128] was added per well and the plates incubated a further 4 h at 37°C. 100 µl of 10% SDS and 0.01 M HCl was then added, the plates incubated overnight at 37°C, and the A595 was determined for each well using a VictorTM X3
Multilabel Plate Reader (PerkinElmer). Results were normalized to an untreated control and presented as survival rate %.

3.2.7 Primary cells and patient samples

Peripheral blood mononuclear cells were isolated from healthy donors by density centrifugation on a Ficoll-Hypaque gradient (Ficoll-Paque™ Plus, GE Healthcare, Cat # 17144002). HIV-infected patient samples were provided through the BC Centre for Excellence in HIV/AIDS at St. Paul’s Hospital, Vancouver. All participants had been on antiretroviral therapy for a minimum of 6 months with plasma HIV-1 RNA levels < 50 copies/ml. All subjects provided written, informed consent prior to their inclusion in the study and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. CD4+ T lymphocytes were isolated using the EasySep™ Human CD4 Positive Selection Kit (STEMCELL Technologies, Cat # 18052), and further purified by negative depletion of cells expressing CD69 (Miltenyi Biotec, Cat # 130-092-355), CD25 (Miltenyi Biotec, Cat # 130-092-983), or HLADR (Miltenyi Biotec, Cat # 130-046-101) using Miltenyi magnetic beads. The purity of isolated T cells was assessed by staining with FITC-conjugated monoclonal antibody against CD4 (BD Pharmingen™, Cat # 561842) and PE-Cy5 conjugated monoclonal antibodies against CD69 (BD Pharmingen™, Cat # 555532) and analysis by flow cytometry.
3.2.8 Measurement of HIV-1 replication from patient samples

HIV-1 RNA was measured from 1 × 10^6 resting CD4+ T lymphocytes treated with 30 µM of each compound. Extracellular RNA was isolated using the ZR Viral RNA Kit (Zymo Research, Cat # D7022) and total cellular mRNA using the RNeasy MinElute cleanup kit (Qiagen, Cat # 74204). HIV-1 RNA concentrations were assayed in one-step reverse transcription- quantitative polymerase chain reactions (RT–qPCRs) using the iTaq™ universal SYBR Green one-step kit (BIO-RAD, Cat # 1725150) with the HIV-1 U3 LTR-specific primers, AGCCGCCTAGCATTTCATC and CAGCGGA AAGTCCCTTGTAG. RNA copy number was calculated using a standard curve generated from titrated infections with Red-Green HIV (RGH)-PGK virus 96. Reproducibility of RT–qPCR analysis was confirmed using a second set of primers within HIV-1 p24 Gag, AGCAGCCATGCAAATGTTA, AGAGAACCAAGGGGAAGTGA. HIV-1 RNA determinations were normalized to GAPDH RNA, measured using the primers CAGCCTCAAGATCATCAGCA and TGTGGTCATGAGTCCTTCCA.

For quantitative viral outgrowth assays (qVOA), serial dilutions of CD4+ T lymphocytes from pooled HIV-infected patients were plated in 6- or 24-well plates (1 × 10^6 cells or <1 × 10^6 cells, respectively). The cells were treated as indicated for 24 h, when the media were removed and replaced with fresh media. Following an additional incubation for 5 h, 4 × 10^6 or 1 × 10^6 MOLT4/CCR5 cells were added to the 6- or 24-well plates, respectively. The plates were incubated for a further 14 days and the media changed every 5 days, when the supernatants were collected and filtered through a 0.45-µm filter unit to remove cell debris. Viral RNA was isolated from the culture supernatants...
and analyzed as described above. The number of latently infected cells was determined from the initial number of CD4+ T cells in the assay, the number of replicates, and the number of positive outcomes, using the IUPMStats V1.0 infection frequency calculator (http://silicianolab.johnshopkins.edu) to produce a “maximum-likelihood estimation (MLE)” 349.

3.2.9 Statistics

For statistical analysis, mean and standard error (SE) for the results were determined from three biological replicates and technical duplicates, unless otherwise stated. Statistical significance analysis involved the ratio paired t-test or one-way Analysis of variance (ANOVA), as indicated in the Figure legends.
3.3 RESULTS

3.3.1 HTS of small molecules identifies compounds capable of reversing HIV-1 latency

A primary high-throughput screen (HTS) was performed with ~180,000 small molecules within three separate libraries CCBN, LCGC, and DIVERSet, representing broad chemical diversity. For this purpose, we used the A1 J-Lat Tat-GFP T-cell line with a latent reporter HIV-1 provirus (Figure 3.1A). In this cell line, expression of the GFP reporter is under control of the HIV-1 LTR promoter such that under basal conditions, in unstimulated cells, GFP expression is not observed (Figure 3.1B, left panel). However, stimulation by signaling agonists, such as PMA, causes induction of GFP expression from the LTR (Figure 3.1B, right panel), as determined by an Arrayscan Imager.

A pilot screen with 4,761 compounds from a bioactive library “KD2” was initially performed to assess reliability of the assay, using a single-point final concentration of 7 µM for each compound in a 348-well plate format; GFP expression was measured following 24-h treatment. Throughout this study, 50 nM PMA was used as a positive control, and results from the pilot screen were normalized as a percentage activation relative to PMA treatment (Figure 3.2A). The calculated Z’ factor was higher than 0.5 for the pilot screen, suggesting that the assay was sufficiently robust to be scaled up for a full HTS of larger compound libraries.

The strategy for the HTS process is shown in Figure 3.2B. Initially, a final concentration of 7 µM, or 1 µM for the LCGC library, of each compound was tested for activity in the A1 J-Lat cell line, where we observed 104 compounds from the CCBN library that produce > 25% activation, 225 compounds that produced > 40% activation
from the LCGC library, and 111 compounds that cause > 40% activation from the DIVERSet, relative to PMA (Figure 3.1C). By these criteria, the Z’ factor for the HTS effort was above 0.5 for all of the libraries. We confirmed activity of compounds that produced significant induction, including 38 from the CCBN library, 26 from the LCGC library, and 42 from the DIVERSet (Figure 3.2C). Compounds with confirmed activity were further tested on parental Jurkat cells lacking the GFP reporter provirus, to eliminate false-positive signals, typically produced by fluorescent properties of the compounds themselves. Compounds that showed activation values for the LTR-GFP reporter, ranging from 30 to 90% relative to PMA in these primary screening assays, were selected for further analysis.
A

5' LTR – Tat – IRES – GFP – 3' LTR

B

50 µm

C

104 Hits > 25%

Z' = 0.70

CCBN library PMA-treated (50nM) Untreated

225 Hits > 40%

Z' = 0.67

LCGC library PMA-treated (50nM) Untreated

111 Hits > 40%

Z' = 0.73

DIVERSet library PMA-treated (50nM) Untreated
**Figure 3.1** High throughput screening of compounds to identify HIV-1 latency reversing activity.

A  Schematic representation of the integrated mini virus (A1 J-Lat Tat-GFP) utilized in the primary screen of small molecules. Expression of the GFP reporter is under control of the HIV-1 LTR promoter in this cell line.

B  Arrayscan images of untreated cells (left panel), or cells treated with 50 nM PMA for 24 hours (right panel: Green-GFP; Blue-Hoechst; Red-PI).

C  Distribution of GFP expression produced by 24h-treatment with compounds from the CCBN, LCGC and DIVERSet libraries (black), or untreated cells (grey). Results are presented as percent GFP expression relative to a positive control reference sample treated with 50 nM PMA (green) and determined from three biological replicates (n=3) (Figure has been provided as collaboration with the CDRD).

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**Diagram Description**

- **Compounds libraries**
  - CCBN (n=25,989)
  - LCGC (n=104,000)
  - DIVERSet (n=50,000)

- **Perform High throughput screen**
  - Single-point confirmation (7/1 µM)
    - A1 J-Lat Tat-GFP cell line

- **Re-examine identified hits**
  - Single-point re-confirmation (7/1 µM)
    - A1 J-Lat Tat-GFP cell line

- **Order in actives to test fresh stock**

- **Select potential compounds for secondary screens**
  - (test on an alternative reporter cell line)
    - Activation values 30-90% relative to PMA

- **Test compounds on parental JurkatTat cells**
  - Elimination of false positive signals

- **Validate activity of the selected compounds**
  - Single-point confirmation (7 µM)
    - JurkatTat LTR-Luciferase cell line

- **Generate concentration response curve**
  - Serial dilutions
    - JurkatTat LTR-Luciferase cell line

- **Select compounds showing latency reversal activity in a dose dependent manner for detailed investigations**
Figure 3.2 Development of a high throughput screening assay for HIV-1 latency reversing activities.

A The bioactive library (KD2) was screened in 384 well format for latency reversing activity using the A1 J-Lat cell line as described in Materials and Methods. The distribution of GFP expression produced by treatment with the compounds is shown as percentage activation relative to control samples treated with 50 nM PMA for 24 hours. The pilot screen produced a Z’ factor higher than 0.5, suggesting that the assay was sufficiently robust for larger screens.

B Overview of the high throughput screening plan and validation with secondary assays. Three compound libraries encompassing 180,000 small molecules were subjected to screens for disruption of HIV-1 latency at a concentration of 7/1 µM. Activity of compounds producing GFP expression upon re-assay at the same single concentration
were reordered from their respective vendors and assayed using the LTR-luciferase cell line to confirm dose response effects.

**C** The active compounds, identified in the first round of screening were retested in the same format described above. In the second round of screening, a smaller number of those active compounds were reconfirmed and selected for further analyses.

Data information: Results are determined from three biological replicates (n=3) (Figure has been provided as collaboration with the CDRD).

### 3.3.2 Five compounds from the primary screens produce concentration-dependent activity

Activity of compounds identified for latency-reversing activity in the primary screens was further validated by examining the effect of a single concentration (7 µM) on an alternative reporter cell line, bearing an integrated HIV-1 reporter virus where luciferase expression is produced from the 5’ LTR, in cells constitutively expressing the viral Tat protein (Figure 3.3A), referred to as Jurkat_{Tat} LTR-luciferase. Importantly, this cell line has the HIV-1 reporter virus integrated at a different chromosomal location than that of the A1 J-Lat cell line and also enables validation of provirus reactivation with a second enzymatic luciferase assay. In these assays, after 24-h treatment we observed 10 compounds that produced significant induction of luciferase activity, consisting of two compounds from the KD2 library, three from the CCBN library and five compounds from the DIVERSet. Compounds that produced significant effects in these secondary assays were re-ordered from their respective suppliers and examined in more detail using the HIV-luciferase reporter cell line. Among the 10 compounds subjected to further analysis, five, designated PH01, PH02, PH03, PH04, and PH05, showed considerable luciferase induction after 24-h treatment in a concentration-dependent manner (Figure 3.3B–F, Table 3.1). The
maximum responses for the compounds ranged from 28 to 60%, relative to PMA treatment, at effective concentration 50s (EC50) between 0.1 and 5.9 µM (Figure 3.3B–F). These five compounds represent diverse chemical structures (Figure 3.3G). In an analysis of time course response, we found that all five compounds produced small effects after only 8-h treatment, and the response continued to increase beyond 48 h (Figure 3.4A and B).
Figure 3.3 Concentration response analysis for five selected PH compounds.

A  Schematic representation of the pTY-LAI-luciferase reporter virus used in secondary screening assays, where luciferase expression is under the control of the 5' LTR, in cloned lines derived from Jurkat{TAT} cells bearing single copy integrants.

B-F  Luciferase activity was measured from reporter cells (Jurkat{TAT} LTR- Luciferase cells) treated for 24 hours with the indicated compound concentrations. Results are presented as percent activation relative to 50 nM PMA treated controls. The EC50 for each compound is indicated. Mean and standard error (SE) for the results are determined from three biological replicates (n=3) and technical duplicates.

G  Illustration of chemical structures of the PH compounds.
Table 3.1 Details of compounds identified from initial HTS

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<th>Name</th>
<th>Library</th>
<th>Vendor</th>
<th>Compound ID</th>
<th>IUPAC Designationa</th>
<th>M.W.b</th>
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<td>C₁₈H₁₈N₄O₂</td>
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</tbody>
</table>

aInternational union of pure and applied chemistry designation; bMolecular weight (grams/mole).
Figure 3.4 Time dependent latency reversing activity of the PH compounds.

A JurkatTαl LTR-luciferase cells were treated with the EC50/4, EC50, and EC50*4 of PH01 - PH05 for the indicated time points. Luciferase activity was measured post-treatment, and the results are presented as percent activation relative to results from PMA treatment. (PH01, EC50: 0.14µM; PH02, EC50: 0.78µM; PH03, EC50: 1.25µM; PH04, EC50: 2.56µM; PH05, EC50: 5.6µM).

B Activity of compounds PH01 - PH05 on JurkatTαl LTR-luciferase cells treated for 24 and 48 h are compared. Concentrations of the compounds are indicated. Data information: Mean and SE for the results are determined from three biological replicates (n=3) and technical duplicates.

3.3.3 PH compounds do not cause global T-cell activation at concentrations that reactivate latent HIV-1

A major obstacle to the clinical use of LRAs is that signaling for activation of HIV-1 transcription overlaps that for T-cell activation, and that global T-cell activation results in a significant production of inflammatory cytokines, which can produce excessive toxicity. Therefore, ideal LRA candidates should be capable of stimulating expression of latent HIV-1 without causing T-cell activation. Two reliable indicators of T-cell activation
are the production of Interleukin-2 (IL-2) and expression of cluster of differentiation 69 (CD69) on the T-cell surface. We examined these parameters using a relatively high concentration of each PH compound (7 µM) in the JurkatTat cell line and found that after 24-h of treatment none of the five compounds significantly increased IL-2 production (Figure 3.5A) or CD69 expression (Figure 3.5B), whereas PMA did.

**Figure 3.5 Effect of PH compounds on global T cell activation.**
JurkatTat cells were treated with 7 µM of each compound, 50 nM PMA or left untreated (No drug control) for 24 h.

**A**  IL-2 expression was measured in culture supernatants using an ELISA.

**B**  Cell surface CD69 expression was measured by flow cytometry.

Data information: For statistical analysis, mean and SE for the results are determined from three biological replicates (n=3). Statistical significance, as determined by ratio paired t test is indicated: ***, P= 0.0008; ns, no significant difference.

### 3.3.4 PH compounds act synergistically with other LRAs

The establishment and maintenance of HIV-1 latency involve a combination of factors and cellular regulatory mechanisms. Therefore, a combination of agonists that
trigger multiple pathways is likely needed to reactivate the full spectrum of provirus in latently infected cells. To examine this potential for the PH compounds, we examined their activity in combination with four previously characterized LRAs using the Jurkat\textsuperscript{Tat} LTR-luciferase cell line. For this purpose, we used LRAs that included the HDAC inhibitor SAHA and the histone methyl transferase inhibitor chaetocin, in addition to ionomycin, and ingenol-3-angelate/PEP005, which activate NFAT and NFκB signaling, respectively. These were examined at concentrations previously shown to induce activation of latent HIV-1 in cell lines or primary cells\textsuperscript{98,134,145,354}. Among these, both SAHA and PEP005 have FDA approval and are currently being used in various cancer treatments, which make them ideal candidates for novel combination treatments. As illustrated in Figure 3.6A–E, all of the PH compounds produced at least a twofold induction in combination with the additional stimuli. Although higher latency-reversal activities were observed for all combinations compared with the single treatments, the highest activity was obtained with 10 nM PEP005 in combination with any of the PH compounds at their EC\textsubscript{50}, which provided between six-fold and 280-fold induction (Figure 3.6A–E). To determine whether these effects met the criteria for drug synergy, we applied the Bliss independence model to test the combined effects. Based on this model, a synergistic effect is defined as an observed result that exceeds the predicted additive effect of the compounds administered separately. As defined by these criteria, each of the PH compounds was capable of producing synergy for reactivation of HIV-1 luciferase expression in combination with 10 nM PEP005 (Figure 3.7A). Furthermore, at lower concentrations of PEP005, which do not cause reversal of latency on their own (between 1 and 4 nM), combination with the PH compounds caused at least a twofold induction of HIV-luciferase activity (Figure 3.6F).
From Bliss independence analysis, the $\Delta F_{axy}$ calculated for each combination treatment shows values higher than 0, demonstrating that there is significant synergy between the PH compounds in combination with PEP005 (Figure 3.7B).
Figure 3.6 Effect of PH compounds in combination with additional treatment on HIV-1 LTR expression.

A–E The JurkatTat LTR-luciferase cell line was treated with the EC50/4, EC50, EC50*4 of PH01 - PH05, alone or in combination with 300 nM SAHA, 100 nM Chaetocin, 1 µM Ionomycin, or 10 nM PEP005 as indicated. Luciferase activity was measured after 24 h, and the results are presented as percent activation relative to results from PMA treatment. Percent activation for the EC50 of each compound are shown here.
The Jurkat T Tat LTR-luciferase cell line was treated with the indicated concentrations of PEP005 alone (left panel; 1nM, 2nM, 4nM) and in combination with the PH compounds (EC50) (right panel). Luciferase activity was measured after 24 h, and the results are presented as percent activation described above.

Data information: Mean and SE for the results are determined from three biological replicates (n=3) and technical duplicates. Statistical significance, derived from one-way ANOVA analysis is indicated as: *, P<0.05; **, P<0.005; ***, P<0.0005; ****, P<0.00005; PH01, EC50: 0.14µM; PH02, EC50: 0.78µM; PH03, EC50: 1.25µM; PH04, EC50: 2.56µM; PH05, EC50: 5.6µM.

**Figure 3.7 Statistical analysis assessing the latency-reversing activity of drug combinations.**

A  Bliss independence determinations were calculated for results from treatment with PH01 - PH05 in combination with 300 nM SAHA, 100 nM chaetocin, 1µM ionomycin, and 10 nM PEP005.

B  The Bliss independence model was applied to calculate $\Delta f_{axy}$ associated with PH compounds (EC50) in combination with the indicated PEP005 concentrations.
Data information. Statistical significance, derived from ratio paired t test is indicated as: *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$; ****. PH01, EC50: 0.14µM; PH02, EC50: 0.78µM; PH03, EC50: 1.25µM; PH04, EC50: 2.56µM; PH05, EC50: 5.6µM.

3.3.5 Compound PH02 reactivates latent HIV-1 integrated at multiple chromosomal locations

HIV-1 tends to integrate into actively transcribed regions of the host genome. Each HIV-1-infected patient carries viral DNA integrated at different chromosomal locations, and as we have previously shown, the site of viral integration influences how latently infected T cells respond to different stimuli. Therefore, an ideal LRA should be capable of disrupting latency in cell lines carrying integrated viral reporters at multiple different regions within the host genome. To assess this capability for the PH compounds, we used two additional previously described cell lines, designated Jurkat\textsuperscript{Tat} LTR-DsRed clones #11, and #131, that carry a mini-dual fluorescent HIV-1 LTR reporter (mdHIV) at different chromosomal locations. The minivirus mdHIV has two fluorescent reporters in which DsRed expression is driven from the 5’ LTR, and eGFP is constitutively expressed from an internal EIF-1α promoter (Figure 3.8A). With this reporter virus, we typically observe expression of eGFP in unstimulated cells, where viral expression is latent, and where stimulation with PMA causes induction of LTR activity and DsRed expression, producing a shift from the DsRed+/eGFP+ population toward double positive cells (DsRed+/eGFP+), representing active infection (Figure 3.8B).

Using the mdHIV minivirus reporter virus, we have observed distinct phenotypes produced by the dual reporters in provirus integrated at different chromosomal locations. For example, in cell line Jurkat\textsuperscript{Tat} LTR-DsRed clone #11, we observe eGFP expression in
most unstimulated cells, and ~40% of these produce dsRed expression upon PMA stimulation (Figure 3.9). In contrast, the Jurkat\textsuperscript{tat} LTR-DsRed clone #131 has reduced expression of EIF-1\(\alpha\)-eGFP in a significant proportion of unstimulated cells, where both reporters appear to have been shut down; however, in these cells, both the LTR and EIF-1\(\alpha\) promoters are induced after overnight treatment with 50 nM PMA (Figure 3.9). We selected the clonal cell lines, which produce these distinct expression phenotypes, for further analysis of the latency reversing activity of the PH compounds. We examined the effect of serial dilutions (28–0.2 µM) of each compound on LTR-DsRed expression after 24h treatment, and results are presented as Δ mean fluorescence intensity (ΔMFI). Among the five compounds, only PH02 was capable of reactivating viral transcription in both of the cell lines at concentrations above 1.75 µM, and without any apparent cellular toxicity (Figure 3.8C and D left panels). These data corroborate previous observations that chromosomal integration site strongly influences response to latency reversing activities and suggest that PH02 might have broad capabilities for reactivation of viral latency. We also tested the effect of PH02 in combination with 10 nM PEP005 in these different clonal cell lines and found concentrations of PH02 higher than 1.75 µM produced increased effects on the HIV-1 LTR transcription with 10 nM PEP005 (Figure 3.8C and D right panels).

![Diagram](A)
Active LTR Transcription

Latency

E1Fα-eGFP

Figure 5
**Figure 3.8 Effect of PH02 on HIV provirus integrated at different chromosomal locations.**

**A**  The effect of PH compounds was analyzed using clonal lines bearing single copy integrations of the mini-dual fluorescent HIV-1 LTR reporter, md-HIV, where GFP is constitutively expressed from the EIF-1α internal promoter, and DsRed from the 5' LTR. Clonal lines are referred to as JurkatTat LTR-DsRed cells, clone 11, and 131.

**B**  Fluorescence-activated cell sorting (FACS) of cells bearing md-HIV provirus indicates expression of EIF-1α-GFP and LTR-dsRed. Stimulation of the md-HIV lines generally causes a shift of the fluorescence profile from GFP+/DsRed- toward GFP+/DsRed+.

**C-D**  Clone 11 or clone 131 were treated with the indicated concentrations of PH02 (left panels) or PH02 in combination with 10 nM PEP005 (right panels) for 24 h and cells analyzed by flow cytometry. Results presented as Δmean fluorescence intensity (ΔMFI) of DsRed expression and fold increase in DsRed expression for solo and combination treatments, respectively.

Data Information: Mean and SE are determined from three biological replicates (n=3) and technical duplicates. Statistical significance, as determined by one-way ANOVA is indicated: ****, P<0.00005.
Figure 3.9 Fluorescence-activated cell sorting (FACS) of clonal lines 11, and 131 bearing md-HIV provirus indicates expression of EIF-1α-GFP and LTR-dsRed.

Stimulation of the md-HIV lines generally causes a shift of the fluorescence profile from GFP+/DsRed- toward GFP+/DsRed+.

A Unstimulated cells.

B 50nM PMA-stimulated cells.

3.3.6 PH02 causes histone modification at the 5’ HIV-1 LTR

To characterize induction of HIV-1 expression caused by PH02 in more detail, we employed chromatin immunoprecipitation (ChIP) assays to examine changes at the LTR promoter using JurkatTat LTR-DsRed clone #11. We observed that H3K9-me3 at the LTR, a chromatin modification typically associated with transcriptional repression, in cells treated with PH02 for 24 h was significantly reduced relative to total histone H3 (Figure 3.10A left panel), and a similar effect was observed in cells treated with PEP005. Despite the observation that the combination of PH02 and PEP005 produced additive or synergistic effects on expression from the LTR, this combination produced an equivalent reduction in H3K9-me3 as either drug treatment separately (Figure 3.10A left panel). This result suggests that reactivation of HIV-1 expression by these compounds must involve multiple parallel mechanisms in addition to demethylation of repressive chromatin modifications at the promoter.

Accompanying the loss of H3K9me3, we observed accumulation of H3K9 acetylation on the LTR in cells treated with PH02 and PEP005 for 24 h (Figure 3.10A right panel). However, the combination of these two compounds caused a significant elevation in H3K9-ac, unlike that observed for loss of H3K9-me3 (Figure 3.10A). This observation is consistent with the well-defined role of histone acetyl transferase complexes as
coactivators of transcription and their known requirement for induction of HIV-1 expression. Although PH02 caused accumulation of histone H3K9 acetylation at the LTR, we did not observe a significant effect of this compound on global acetylation or methylation of lysine 9 on H3 in treated cells (Figure 3.10B, H3K9-ac and H3K9-me3). These observations indicate that the compound likely does not function by inhibiting HDAC or histone methyl transferase activities. Rather, it seems more likely that accumulation of H3K9-ac and loss H3K9 methylation is an indirect consequence of enhanced transcriptional activation by proteins bound to the enhancer region. The HIV-1 LTR is known to be bound by numerous different transcription factors capable of recruiting histone modifying complexes. Because the PH compounds produce an effect only after 24h treatment, we reasoned that they could be altering expression of one or more factors, rather than acting as direct signaling agonists. Consequently, we examined expression of NFκB p65 and SP1, but found their expression to be mostly unaffected by PH02 in the Jurkat Tat LTR-DsRed clone 11 line (Figure 3.10B). We did observe a small increase in expression of SP1 in some cell lines treated with the PH compounds (Figure 3.11), but we do not believe this could account for reactivation of provirus because it is not observed in all of the lines where we observe their effect. Consistent with its known effect on PKC–NFκB signaling, we find that PEP005 causes loss of IκBα after 24-h treatment, alone or in combination with PH02 (Figure 3.10B). A similar effect is observed in cells treated with the PH compounds, but only after 48 h of treatment (Figure 3.11), and consequently, consistent with their synergistic effect with PEP005, we do not believe they could be causing reactivation through upregulation of NFκB activity on the LTR.
Figure 3.10 Effect of PH02 on histone modification at HIV-1 LTR and global histone modification.

A  Chromatin immunoprecipitation assays were performed using Jurkat Tat LTR-DsRed, clone 11. The cells were treated for 24 h with PH02 and/or PEP005, and ChIP was performed using antibodies against histone H3K9-me3 (left panel) and histone H3K9-ac (right panel). Purified DNA was assayed by qPCR primers specific to the conserved region designated RBEI (Ras-responsive binding element-1) which is located within the
LTR core promoter at the LTR U3 sub-region. The results are presented relative to ChIP with anti-histone H3 antibodies.

Western blot analysis was performed using Jurkat<sup>Tat</sup> LTR-DsRed, clone 11, to detect the expression of SP1, NFκB p65, IκBα, total H3K9-me3, and H3K9ac. The cells were treated for 24 h with PH02 and/or PEP005.

Data information: The results are determined from three biological replicates (n=3). Statistical significance, as determined by ratio paired t test, is indicated as follows: ***, P<0.0005; ****, P<0.00005.

Figure 3.11 Effect of PH compounds on SP1, NFκB p65, and IκBα expression.

Jurkat<sup>Tat</sup> LTR-Luciferase cells were treated with a single concentration (3 µM) of each PH compound for the indicated time points. Whole cell protein extract was separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with antibodies against SP1, NFκB p65, and IκBα. GAPDH was used as the loading control.

3.3.7 Structural moieties of PH02 required for latency-reversing activity

To probe chemical structure(s) necessary for PH02 to function as a latency-reversing agent, five analogs representing two-dimensional (2D) 75–90% similarity to the parental structure were obtained and tested on the Jurkat<sup>Tat</sup> LTR-luciferase cell lines and the Jurkat<sup>Tat</sup> LTR-DsRed clone #11 and #131 cell lines (Figure 3.12A, Table 3.2).
Interestingly, among these analogs, none showed higher latency-reversal activity than the parental structure with either of the reporter cell lines tested (Figure 3.12B and C). Furthermore, PH02a and PH02b with the highest 2D similarities (95 and 90%, respectively) to the parent compound showed significant reduction in latency-reversing activity, indicating that omission of the carbonyl group from PH02a or both the carbonyl and methyl groups from PH02b inhibits the ability of PH02 to reverse viral latency. Three additional analogs with 2D similarities of 75–80% did not cause reactivation of viral latency in either of the reporter cell lines (Figure 3.12B and C).
Figure 3.12 Ability of PH02-associated analogs to reactivate HIV-1 LTR expression.

A  Illustration of the chemical structures associated with the PH02 analogs, referred to as PH02a-e, examined on the in vitro reporter cell lines for structural optimization.

B  Jurkat\textsuperscript{tat} LTR-luciferase cell line, (C) Jurkat\textsuperscript{tat} LTR-DsRed clone 11 (left panel) and clone 131 (right panel) were treated with the indicated concentrations of PH02-associated analogs. Luciferase activity or overall DsRed expression were measured 24 hours post-treatment. Results are presented as percent activity relative to the positive control (50 nM PMA-treated cells), or \( \Delta \) mean fluorescence intensity (\( \Delta \)MFI) of DsRed expression, respectively. Mean and SE for the results are determined from three biological replicates (n=3) and technical duplicates.
Table 3.2 Summary of PH02 analog structures

<table>
<thead>
<tr>
<th>PH02 Analogs</th>
<th>Vendor</th>
<th>Compound ID</th>
<th>IUPAC Designation</th>
<th>M.W.</th>
<th>Formula</th>
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<tr>
<td>PH02a</td>
<td>Chembridge</td>
<td>7111897</td>
<td>N-(2-methoxyphenyl)-N-[(3-oxo-1-benzothien-2(3H)-ylidene)methyl]acetamide</td>
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<td>C_{18}H_{15}N_{3}O_{3}S</td>
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<td>6568745</td>
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<tr>
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<td></td>
<td>6854909</td>
<td>methyl 2-(5-[(3-(4-methoxyphenyl)-4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl]-2-furyl)benzoate</td>
<td>452</td>
<td>C_{23}H_{17}N_{2}O_{5}S_{2}</td>
</tr>
</tbody>
</table>

aInternational union of pure and applied chemistry designation; bMolecular weight (grams/mole).

3.3.8 the PH compounds have negligible effects on cell viability

Prior to further analysis of the efficacy for HIV reactivation in primary CD4+ T cells, we measured toxicity of each PH compound on Jurkat-Tat cells and peripheral blood mononuclear cells (PBMCs). In both cases, cells were treated with serial dilutions (28–0.1 µM) of each compound for 24 or 48 h, and examined using an MTT tetrazolium reduction assay, which measures metabolic activity. The results were normalized to untreated controls and are presented as a survival percentage. Survival rates for both Jurkat-Tat cells and primary cells (PBMCs) 24 h post-treatment were above 80% for most compounds at the concentrations used, while reduced viabilities were observed after the 48-h treatment (Figure 3.13A and B, C). We also examined toxicity produced by the combination of PH compounds with 10 nM PEP005 and other stimuli on PBMCs. No major effects were observed on cell viability after 24 h, except for cells treated with chaetocin in combination with the PH compounds (Figure 3.13D).
Figure 3.13 Effect of PH compounds on cell viability.

MTT assay was performed to assess cell viability 24/48 hours post treatment. Results are normalized relative to untreated controls and presented as survival rate percentage. The indicated mean and SE for the results are determined from three biological replicates (n=3) and technical duplicates.
A Jurkat\textsuperscript{Tat} cells were treated with the indicated concentrations of the PH compounds for 24h.

B PBMCs from healthy donors were treated with the indicated concentrations of the PH compounds for 24h.

C Jurkat\textsuperscript{Tat} cells (left panel) and PBMCs from healthy donors (right panel) were treated with the indicated concentrations of the PH compounds for 48 hours.

D PBMCs from healthy donors were treated with the indicated concentrations of the PH compounds in combination with 10 nM PEP005/300 nM SAHA/100 nM Chaetocin for 24h.

3.3.9 The PH compounds activate viral transcription in resting CD4\textsuperscript{+} (rCD4\textsuperscript{+}) T cells from HIV-1-infected patients

We next examined the effect of the PH compounds on T cells purified from HIV-1-infected individuals. At the time of enrollment, all participants in the study from which samples were collected had been on antiretroviral therapy a minimum of 6 months and had viral loads below 50 HIV RNA copies/ml, the clinically accepted limit of detection, and therefore carried only genome-integrated latent provirus.

For this analysis, we purified resting CD4\textsuperscript{+} (rCD4\textsuperscript{+}) T lymphocytes from pools of patient samples by two-step negative selection (Table 3.3). Purity of the isolated cells was assessed by flow cytometry (Figure 3.14), which indicated that 96% expressed the CD4 marker but not the activation marker CD69. Purified rCD4\textsuperscript{+} T cells were treated with 30 µM of each PH compound for 24 h, and RNA from the culture supernatants was analyzed by one-step RT–qPCR, where results are presented as copy numbers of the virion/ml. As shown in Figure 3.15, all of the PH compounds demonstrated moderate viral latency reversing activity compared to the PMA-treated positive controls, but among these, PH02 consistently produced the strongest effect. Based on its capability for
reactivating latent provirus at multiple chromosomal locations in cell lines, its negligible
toxic effect, and that it produces the most robust reactivation of virus from patient
samples, we focused our subsequent analysis on this compound (Table 3.1).

Table 3.3 Pool of aviremic HIV-1 infected patients used in this study

<table>
<thead>
<tr>
<th>No. of rCD4+ T cell poolsa</th>
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</tr>
</thead>
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<td>P.1, P.2, P.3, P.4, P.5, P.6, P.7, P.8, P.9, P.10, P.11, P.12, P.13, P.14, P.15, P.16, P.17, P.18</td>
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</table>

a: cells used in initial evaluations of latency reversing activity of the PH compounds; b: cells used for measuring intracellular HIV-1 mRNAs post compound-treatments; c: cells used in qVOA. Abbreviations: No, number; rCD4+ T cells, resting CD4+ T cells; P, patient; qVOA, quantitative viral outgrowth assay.

Figure 3.14 Assessment of purity for resting T helper cells isolated patient samples.

A  CD4+ T cells isolated from whole blood of patient samples were stained with monoclonal antibodies against CD4+ and were analyzed by flow cytometry;
approximately 98% of the isolated cells stained positive (lower panel) relative to unstained control cells (upper panel).

**B** Isolated CD4+ T cells from patient samples were stained with monoclonal antibodies against both CD4 and CD69 and analyzed by flow cytometry. Approximately 96% of the purified cells express CD4 but not CD69 (lower panel) compared to unstained control cells (upper panel).

Figure 3.15 Effect of PH compounds on expression of HIV provirus in latently infected cells from patients.

Resting CD4+ T cells purified from patient samples (n= 18, Table 3.3) were treated with 30 µM of each PH compound. 24 h post treatment, extracellular HIV-1 RNA was purified from cell culture supernatants. cDNA was synthesized from the purified HIV RNA, and assayed by qPCR using primers specific to a conserved region within the LTR U3 sub-region designated as RBEIII (Ras-responsive binding element III). Results are presented as HIV virions/mL.
3.3.10 Identification of minimal concentrations of PH02 and PEP005 that induce T-cell activation

As discussed earlier, an ideal combination of latency-reversing compounds would reverse HIV-1 latency in a broad spectrum of infected cells, without causing global T-cell activation. Accordingly, we examined the effects of PH02 and PEP005 on T-cell activation and toxicity on primary cells (PBMCs) isolated from healthy donors. Previous results have indicated that 10 nM PEP005 does not cause IL-2 production but does induce upregulation of CD69, in primary cells. Consequently, we examined the effect of lower concentrations of PEP005, alone and in combination with PH02 on these primary cells. As illustrated in Figure 3.16, 3 µM PH02 and 1 nM PEP005 alone or in combination did not cause significant CD69 induction, whereas PEP005 concentrations higher than 1 nM alone and in combination with PH02 caused expression of CD69 at similar levels as observed for cells treated with PMA (Figure 3.16). Based on these observations, we expected that 1 nM PEP005 in combination with PH02 should produce negligible toxicity, and have minimal effects on T-cell activation, while being capable of causing significant reversal of HIV latency (Figure 3.6F and Figure 3.7B).

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![Graph showing CD69 expression levels](image-url)
Figure 3.16 Effect of PH02 in combination with PEP005 on T cell activation. Expression of CD69 on PBMCs from three healthy donors (A, B, C- n=3) was measured by flow cytometry following 24 h treatment with 3 µM PH02 alone, or in combination with the indicated concentration of PEP005. Positive control samples were treated with 50 nM PMA.

Data information: Statistical significance, as determined by one-way ANOVA is indicated: *, P<0.05; **, P<0.005; ***, P<0.0005; ****, P<0.00005; ns, no significant difference.

3.3.11 A combination of PH02 and PEP005 produces HIV latency reversing activity on CD4+ T cells from HIV-infected patients

Consistent with the above results, we found that 1 nM PEP005 alone did not cause induction of viral RNA after 24-h treatment of CD4+ T lymphocytes from patients (Figure 3.17, Table 3.3). However, 1 nM PEP005 in combination with 1 µM PH02 produced a significant response that was double that produced by 1 µM PH02 on its own (Figure 3.17). We confirmed this effect using a quantitative viral outgrowth assay (qVOA) with pools of CD4+ T cells isolated from HIV 1-infected patient samples (Figure 3.18A, Table 3.3). Consistent with the results shown above, 1 nM PEP005 alone did not produce reactivation of virus, whereas 1 µM PH02 in combination with 1 nM PEP005 produced significant virus replication (Figure 3.18B). We conclude that this combination of treatments may represent a promising candidate therapy to target persistent latent viral infection.
Figure 3.17 Effect of PH02 and PEP005 on expression of HIV provirus in latently infected cells from patients.

Expression of intracellular HIV-1 mRNA was determined from pools of CD4+ T cells from aviremic HIV-infected patients (n= 3, Table 3.2) following 24 h treatment as indicated. Purified intracellular mRNA was assayed by RT-qPCR using primers specific for the conserved region within the HIV-1 LTR U3 sub-region (Ras-responsive binding element III (RBEIII)). Results have been shown as fold induction relative to untreated controls.
Figure 3.18 Assay for reactivation of HIV-1 from latently infected cells using a quantitative viral outgrowth assay.

A  Quantitative viral outgrowth assays (qVOA) were used to measure reactivation of HIV-1 provirus from latency in pools of cells from patients. As indicated in the Table 3.3C, five different CD4+ T cells pools (n=5) were used for this analysis. Resting CD4+ T from patient samples were serially diluted in 6 or 24 well plates in duplicate (top), and treated...
as indicated (left) (dots represent the treated wells). After 24 h MOLT4/CCR5 cells were added to the wells, and the cultures incubated a further 14 days. HIV-1 RNA was detected in filtered culture supernatants using RT-qPCR, and wells producing the specific PCR product are indicated in green.

B The frequency of latently infected cells where viral replication was reactivated by treatment was estimated using the IUPMStats V1.0 infection frequency calculator (http://silicianolab.johnshopkins.edu) which generates a maximum likelihood estimation (MLE) for latently infected cells.

Data information: Statistical significance was determined by one-way ANOVA analysis and indicated as *, P<0.05; **, P<0.005; ***, P<0.0005; ****, P<0.00005.

3.4 DISCUSSION

According to recent statistics released by the United Nations Programme on HIV/AIDS (UNAIDS), the number of HIV-infected individuals receiving antiretroviral treatment has increased considerably within the last decade, particularly in African countries, which have the highest number of infected individuals. Effective antiretroviral treatment can control the virus, allowing patients to enjoy healthier lives and reducing their likelihood to transmit the virus. Despite the many benefits of this treatment, a cure cannot be achieved through HAART and will require a clearer understanding of HIV-1 pathogenesis and the mechanisms contributing to provirus latency. Cells that are latently infected with HIV-1, carrying the stable viral genome, are considered a major obstacle to a cure.

Various technologies have been proposed for eliminating latently infected cells. In particular, the gene-editing technology CRISPR/Cas9, which is aimed at disrupting genes associated with HIV-1, has recently attracted considerable attention in the field 357-360. To date, this approach has been met with limited success because of the incomplete
silencing of genes of interest or interference with the expression profiles of other unintended genes, known as “off-target effects”, thus requiring a more in-depth investigation into the safety and feasibility of such applications. Challenges in implementing direct gene manipulation strategies for therapy highlights the fact that the proposed shock-and-kill strategy, which is based on pharmaceutically overcoming mechanisms involved in viral latency, to expose latently infected cells to boosted immune responses or apoptosis, may currently be considered a relatively safer and, more clinically accessible strategy for treating the HIV epidemic. Persistent HIV infection is known to be fueled by the presence of latent HIV-1 reservoirs, but additionally, Lorenzo Redondo et al. (2016) have demonstrated active viral replication in sanctuaries of lymphoid tissues in patients on antiretroviral therapies, where the concentration of drugs may not be sufficient to destroy the replicating virus. Thus, clearance of viral reservoirs through the proposed shock-and-kill strategy, along with optimized applications capable of delivering effective drug concentrations more efficiently to sites of active infections, will be necessary for achieving a permanent HIV cure. To this end, the identification of new latency-reversing agents or regulatory factors involved in establishing HIV latency will contribute to ideal therapeutic targets toward the shock-and-kill approach.

Consistent with this aim, this study was initiated to identify new potent latency-reversing agents, with a particular focus on a requirement for global reactivation of latent provirus with synergistic effects in combination with other LRAs. For this purpose, high throughput screening of small molecules was performed using the A1 J Lat reporter cell line (Figure 3.1A). Based on the initial screens, 10 compounds that showed significant latency-reversal activity, compared to the effect of PMA, were selected for follow-up
analyses. Five of these, designated PH01, PH02, PH03, PH04, and PH05, reproducibly reactivated viral transcription in a dose-dependent manner when examined using an alternative reporter cell line with an LTR-luciferase reporter (Figure 3.3B–F). In general, we found that all of the PH compounds from the screen produced synergistic responses for reactivation of HIV in combination with additional latency-reversing compounds, including HDAC and HMT inhibitors and the PKC–NFκB agonist ingenol-3-angelate/PEP005 (Figures 3.6A–E). Among these, PEP005 was found to be particularly effective for viral reactivation in combination with our compounds after 24-h treatment (Figures 3.6 and 3.7B).

Since minimal latency-reversing activity was observed after 8-h treatment with each of the PH compounds in the luciferase reporter cell line and significant induction of viral transcription was only observed at 24 h (Figures 3.4A and B), we believe it is unlikely that they act as signaling agonists for pathways directly downstream of the T-cell receptor (TCR), including those involving protein kinase C (PKC)–NFκB, MAPK–AP1, or calcineurin–NFAT, which typically produce immediate transcriptional responses. For instance, it has been previously demonstrated that the latency-reversal activity of PEP005, which was used throughout this study as a second stimulus along with the PH compounds to augment the induction of HIV transcription, is mediated through the PKC–NFκB signaling pathway, where the phosphorylation of PKCδ/θ and IκBα/IκBε is induced by 12 nM of PEP005 as early as 0.5–2 h post-treatment\textsuperscript{134}. However, this compound does not cause modulation of NFκB protein expression, making it clinically more favorable compared to other ingenol esters\textsuperscript{135,361}. In contrast, the delayed responses demonstrated here by the PH compounds suggest that they may cause upregulation of gene expression
or recruitment of one of the many additional transcription factors that bind the HIV-1 LTR but are not the direct targets of T-cell signaling\textsuperscript{57}. Accordingly, we have examined effects of the compounds on expression of Sp1, which has three binding sites (GC boxes) within HIV-1 LTR, and is known to be required for HIV enhancer activation\textsuperscript{362}, but we do not observe alterations that would be consistent with a role in causing reactivation (Figure 3.10B). However, it is possible that altered expression of one or more other factors with binding sites on the LTR could result in the delayed transcription activation observed with the PH compounds. Similarly, because of the many additional mechanisms proposed to be involved in HIV latency, including non-coding RNAs and recruitment of repressive complexes by additional sequence specific binding factors, and DNA methylation, it is also possible that alterations of these more poorly characterized mechanisms might also contribute to delayed responses produced by the compounds.

Several additional observations indicate that the PH compounds are not likely inducing HIV expression by upregulation of NFκB activity. First, we observe minimal toxicity of the compounds in both the Jurkat\textsuperscript{Tat} cell line and PBMCs, even at relatively high concentrations (Figures 3.13A and B), whereas upregulation of NFκB protein typically results in excessive cytotoxicity\textsuperscript{363}. Secondly, none of the PH compounds caused IL-2 expression (Figure 3.5A), which is normally induced in response to T-cell receptor engagement. Similarly, none of the compounds caused CD69 upregulation in either Jurkat\textsuperscript{Tat} cells or PBMCs (Figures 3.5B and 3.16), implying that these agents do not directly trigger NFκB nuclear translocation, as the CD69 promoter was shown to contain multiple NFκB binding sites\textsuperscript{364}. 
Other important observations from this study were the effects of both PH02 and PEP005 treatments on chromatin modifications in the HIV-1 promoter region, which were examined in the JurkatTat LTR-DsRed clone #11 (Figure 3.10A). Interestingly, cells treated with either PH02 or PEP005 showed a reduction in H3K9 methylation as well as an accumulation of H3K9 acetylation on the HIV-1 LTR relative to total histone H3 (Figure 3.10A). PEP005, which is known as a PKC agonist involved in the T-cell signaling pathway, likely causes indirect accumulation of H3K9 acetylation as a consequence of enhanced histone acetyltransferase (HAT) recruitment from transcriptional activation. Consistent with this assumption, it is unlikely that these modifications, accompanied by the PH02 treatment, resulted from a direct inhibition of either HDACs or HMTs by this compound. Indeed, we do not observe alterations in global histone H3K9 acetylation or methylation in cells treated for 24 h (Figure 3.10B), which supports the possibility that it may promote histone modification at the LTR through mechanisms involving enhanced recruitment of HATs.

We have previously shown, using in vitro analyses of clonal cell lines carrying the mini-dual HIV reporter virus at different chromosomal locations, that the epigenetic landscape at the site of integration has a significant effect on the efficacy of various LRAs in disrupting latency. Consistent with these findings, we observed different responses to the PH compounds using four latently infected reporter cell lines bearing the virus at different genomic locations. Importantly, however, we demonstrated that PH02 is capable of reactivating viral transcription in all of the reporter cell lines we examined. These observations support the contention that a combinational regimen targeting multiple distinct pathways involved in the establishment of viral latency would be required for
global stimulation of latent proviruses integrated at various sites within the genome, as different provirus species exhibit a unique sensitivity to signaling agonists and chromatin-remodeling agents.

The highest latency-reversing activity associated with PH02, presumably independent of the proviral integration site, which was observed alone and in combination with PEP005 through our intensive in vitro analyses, led us to further investigate the effect of these two compounds on CD4+ T lymphocytes purified from HIV-1- infected individuals on HAART. The ability of PH02 to significantly reactivate HIV-1 transcription in combination with only 1 nM of PEP005 was initially demonstrated by measuring HIV-1 intracellular RNA 24 h post-treatment (Figure 3.17). This finding was further confirmed by performing a viral outgrowth assay quantifying only the replication-competent provirus (Figure 3.18B).

In conclusion, in this chapter we describe a potentially novel combination of treatments that may be used to uniformly induce HIV transcription in a broad range of latently infected cells. We suggest that this combination may be a candidate for application with the shock-and-kill strategy to expose reactivated cells for the purging of latent infection.
CHAPTER 4 CONCLUSIONS AND FUTURE DIRECTIONS

4.1 TARGETING TRANSCRIPTIONALLY REPRESSED PROVIRUSES WITH PH COMPOUNDS

HIV-1 infection is clinically characterized with three phases. During the initial acute phase, rapid viral replication occurs in productively infected cells that results in an initial high level of plasma viral load and a rapid decline in the CD4+ T lymphocytes. This is followed by the second phase, referred to as the chronic or asymptomatic period, during which viral replication continues at a slower pace and CD4+ T cells are further depleted over a span of years to a decade. Eventually, the gradual decline of CD4+ T lymphocytes renders infected patients susceptible to opportunistic infections and development of AIDS, which represents the last phase of the disease. Although antiretroviral therapies have been remarkably effective in suppressing the plasma viral load, consequently increasing life expectancy of infected individuals and reducing the likelihood of transmission, viral infection persists due to the presence of the latent viral reservoirs. Upon interruption of therapy, virus can again be detected in the plasma as early as two to eight weeks. Consequently, patients infected with HIV are destined to remain on current therapies for the remainder of their lives.

The frequency of cells carrying HIV provirus has been estimated to be less than $10^{-5}$ in the peripheral T cell population of patients on antiretroviral therapies. However, the majority of these cells carry defective provirus genomes, and only a minute subset is replication-competent. Although an accurate estimation of the absolute number of cells bearing latent proviruses is technically challenging, current estimates indicate a total of...
10⁸ latently infected cells, but where the frequency of latent but replication-competent provirus-infected resting memory CD4+ T cells of less than 10⁶. 

There is consensus that development of strategies to efficiently purge this population of cells latently infected with provirus will be required for long-term management of the HIV-1 pandemic. One proposed strategy would involve reactivating virus transcription in the latently infected cellular population, which mark these cells for elimination via the consequent production of viral proteins. Towards this purpose, in Chapter 3 I have described the identification of five novel small molecule LRAs capable of efficiently reactivating transcription of repressed provirus. One of these compounds, PH02, demonstrated capacity to induce HIV provirus in multiple different cell lines, as well as T cells from patients on antiretroviral therapy. Additionally, PH02 was shown to produce significant synergism for induction of viral transcription in combination with the PKC agonist PEP005. Notably, the ability of the PH02-PEP005 combination was assessed using a quantitative viral outgrowth assay, which provides a sensitive and reliable measure for reactivation of replication-competent provirus.

Since the PH compounds described here represent novel chemical structures with previously undefined biological targets, the mechanism of action through which these small molecules stimulate viral transcription needs to be elucidated. Accordingly, clues as to mechanism of action may be derived from further experiments as performed in Chapter 2, to examine effects produced by treatment of PH compounds in combination with LRAs with previously known mechanisms of actions, with the rationale that compounds affecting parallel mechanisms would be expected to produce synergistic
responses, whereas compounds that affect the same pathway are likely to produce less than additive effects (Figure 2.1).

Additional investigations to gain insight into these compounds’ biological targets may involve strategies such as RNA sequencing and quantitative MS proteomics, where alterations in RNA or protein abundance might be useful for predicting biochemical targets. Additionally, biochemical techniques may also be employed to directly identify proteins capable of interaction with the compounds by purification. However, this typically first requires identification of derivatives of the compound that can be coupled to a solid support, or alternatively a tag such as a fluorescent dye, that can be used to identify bound proteins. For PH02, five related analogs were evaluated for latency reversing activity, where both the carbonyl and methyl groups of the parental structure were found to be particularly important for activity. Additional structure-activity relationship studies with this compound may indicate modifications that could enable covalent coupling to supports or tags, without impairing effectiveness for reactivation, and possibly other functional groups that may trigger parallel pathways that could improve the latency-reversing activity. Accordingly, carbons at the C2-C5 positions of the benzene ring fused to a thiolane in the PH02 parental structure, might serve as proper sites at which this compound could be modified. Proteomic strategies such as thermal proteome profiling (TPP), can sometimes also be used to identify biochemical targets for small molecules. TPP is a modified version of the cellular thermal shift assay (CETSA) that enables detection of direct compound/drug-protein interactions within the proteome. This approach takes advantage of thermostability changes that can occur upon
compound-target protein interaction and may be useful to identify targets of the PH compound latency reversing agents.

The effectiveness of novel latency reversing compounds to reactivate viral transcription should ideally be assessed in vivo, using an animal model. Prior to the generation of humanized mice, SIV-infected macaques served as the only animal model for HIV pathogenesis. Although these studies involving SIV have been highly informative, their implementation is prohibitive for routine analysis, considering cost and ethics considerations. The use of humanized mice circumvents these issues, as much as possible, to provide an animal model for HIV pathogenesis. The bone marrow, liver, and thymus (BLT) humanized mice are produced by fragment transplantation of human fetal liver and thymus tissues into immuno-deficient mice, followed by intravenous administration of CD34+ hematopoietic stem cells (HSCs). Thymus transplantation is needed for human immune reconstitution, while HSCs provide progenitor cells that differentiate into lymphoid and myeloid cells. Although this model provides a robust representation of a human immune system, BLT mice typically develop graft versus host disease (GVHD), which may limit the use for certain HIV-1-related studies, including viral latency. However, recently developed modifications may have overcome such limitations, and it would be informative to examine the effect of PH compounds for reactivation of provirus in latently infected cells in vivo using this humanized system.

4.2 DIVERSITY IN MECHANISMS OF ACTION FOR DIFFERENT LRAS

Establishment of HIV-1 latency occurs in vivo because HIV transcription is coupled to T cell signaling and consequently is naturally programmed to occur during development.
of immunological memory\textsuperscript{377}. During the initial acute phase of infection, HIV-1 predominately infects CD4\textsuperscript{+} effector T cells\textsuperscript{378}. Although the majority of these infected cells die rapidly due to vial cytopathic effect and/or host antiviral immune defenses, a significant number survive as the result of activated CD4\textsuperscript{+} T cells reverting to a G\textsubscript{0} resting memory state where transcription of genome-integrated virus is repressed\textsuperscript{378}.

One aspect of current research towards applying the shock and kill therapy focuses on developing potent LRA combinations capable of producing broad reactivation of viral transcription. Theoretically, targeting any of the molecular mechanisms contributing to the establishment and maintenance of viral silencing could modulate the HIV-1 transcription. LRAs identified to date can be classified into different groups based on the predominate molecular pathway which they affect. In chapter 2 of this thesis I discuss different groups of LRAs, including epigenetic modifiers, chromatin modulators, signalling effectors/modulators, and transcriptional elongation modulators, that have emerged from low and high throughput screens of small molecules over the past decade (Table 2.1-2.2). Results from these efforts suggest that development of compounds targeting pathways that are unique to regulation of HIV, including possibly less-explored or uncharacterised mechanisms for modulating viral transcription may represent an important focus for future studies. Accordingly, Figure 2.1 illustrates an overview of the diverse mechanisms through which different groups of LRAs, discovered in small molecule screens are thought to cause activation of HIV expression. Many novel compounds discovered with this effort function to stimulate HIV transcription through less well characterized or previously unrecognised mechanisms. These include the BET and BAF inhibitors, benzotriazoles, and SMAPP1.
The BET family member Brd4 has been shown to enhance NFκB transcriptional activation function by interaction with the p65 acetylated at lysine 310. BET inhibitors were shown to disrupt this interaction, consequently causing a reduction in the NFκB-stimulated inflammatory responses\textsuperscript{379}; BET inhibitors are currently in clinical trials as anti-inflammatory agent and cancer therapy. Considering the central role that NFκB plays for activating HIV transcription in response to T cell signaling, it is curious that BRD4 inhibitors should act as latency reversing agents. This effect revealed the inhibitory role of the BET protein Brd4 for transcriptional elongation from the HIV promoter, and that BET inhibitors inhibit binding of Brd4 to TAT and promote release of pTEFb from 7SK snRNP thus stimulating TAT-activated transcription. Therefore, BET inhibitors can alleviate adverse effects of immune signaling while inducing transcription from the HIV-1 LTR, which indicates that these may provide safe therapies for implementation of the shock phase of the shock and kill strategy\textsuperscript{190}.

Several studies analyzing the responsiveness of the latently infected cellular reservoirs to various LRAs have shown that the broad integration landscape of the proviruses produces both inducible and more poorly inducible infected cells\textsuperscript{100,380,381}. A recent study utilizing a dual reporter virus demonstrated that only a small fraction of the newly established latent population was responsive to various LRAs, and that these effects heterogeneity of the chromatin landscape at various sites of HIV-1 integration \textsuperscript{381}. Such observations raise concerns about the feasibility of the shock and kill strategy for effective clearance of the latent proviruses, and there is recognition that no single LRA is likely capable of inducing a sufficient proportion of this population to achieve a cure\textsuperscript{382,383}. Recently, more attention has shifted toward other possible curative approaches,
particularly a mechanistically opposing strategy from shock and kill, generally known as “block and lock.” In this proposed strategy, latency promoting agents (LPAs) are employed with the purpose of driving the provirus into a deep transcriptionally repressed state\textsuperscript{382,383}. One rationale for this strategy includes recent recognition that HIV provirus in latently infected cells must produce sporadic transcripts that may maintain low levels of viral proteins, particularly TAT. Fewer LPAs have been characterized to date, and may affect either virus-specific or host-dependent mechanisms. One notable example includes di-dehydro Cortistatin A (dCA), which selectively interferes with the Tat-TAR interaction. Additionally, ruxolitinib, and tofacitinib inhibit the Jak-STAT5 signalling pathway\textsuperscript{280,384-386}. Initial results with these agents appear promising, but identification of additional and more effective LPAs is clearly an important goal for future studies.

The increasing understanding regarding complexity of the latent HIV-1 population has led to realization that a combination of therapeutic strategies may be necessary to produce a cure. The shock and kill strategy, as initially proposed, may not be effective on its own, but various observations suggest that both the shock and kill parameters of this approach can be enhanced to improve elimination of latently infected cells. Additionally, a successful therapeutic approach may depend upon the HIV integration profile of individual patients; the proviral population in some patients may be more suited to treatment with an enhanced shock and kill approach, while other patients may be more effectively treated with a block and lock strategy. In view of this possibility it will be important for further development of both strategies.
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